HEI HEALTH EFFECTS INSTITUTE

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

Part XI: Integrative Summary

The Collaborative Ozone Project Group (in Alphabetical Order): Paul J. Catalano, Ling-Yi L. Chang, Jack R. Harkema, Debra A. Kaden, Jerold A. Last, Paul W. Mellick, William C. Parks, Kent E. Pinkerton, Bhandaru Radhakrishnamurthy, Louise M. Ryan, and John L. Szarek

Includes the Commentary of the Institute's Health Review Committee

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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 XI

The Consequences of Prolonged Inhalation of Ozone on Rats: An Integrative Summary of the Results of Eight Collaborative Studies

BACKGROUND

Ozone is a highly reactive gas that is formed when components of emissions from mobile and industrial sources react in the presence of sunlight. Ozone is a major component of urban smog and a public health concern. It has been well documented that short-term exposure to ozone can cause transient health effects in some people. Symptoms include cough, pulmonary function changes, and the appearance of inflammatory cells in the lungs. What is not known is whether repeated exposures to ozone over a period of many years cause damage to the respiratory tract and lead to the development or worsening of chronic lung diseases such as chronic obstructive pulmonary disease, asthma, or diffuse pulmonary fibrosis. This is of serious concern because chronic lung diseases are major contributors to morbidity and mortality in developed countries, where over half of the population is exposed to elevated levels of ozone, especially during the summer months. In order to protect public health, the U.S. Environmental Protection Agency sets National Ambient Air Quality Standards for ozone. The current ozone standard is 0.12 parts per million (ppm), a level that is not to be exceeded for more than one hour once per year.

Recently, the National Toxicology Program (NTP) conducted an animal bioassay designed to evaluate whether prolonged exposure to ozone causes cancer in rodents. The NTP bioassay presented a unique opportunity to examine noncancer endpoints as well. Therefore, the NTP and HEI set up a collaboration that allowed HEI-funded investigators access to rats that were exposed to ozone using the same rigorous exposure protocol and quality assurance procedures as the NTP bioassay animals. The HEI studies addressed respiratory diseases other than cancer. The results of the individual studies have already been published. The Integrative Summary that is discussed in this Statement highlights the major findings, integrates the results, and discusses their implications for human health.

APPROACH

Healthy male and female F344/N rats were exposed to filtered air or to one of three concentrations of ozone for six hours per day, five days per week, for twenty months: 1.0 ppm ozone (the highest level the animals would tolerate), 0.12 ppm ozone (the National Ambient Air Quality Standard), or 0.5 ppm ozone (an intermediate concentration). (It should be noted that the cumulative annual ozone exposure, even for the low-dose group, was much higher than that received by individuals who live in polluted areas of the United States.) The HEI funded eight independent research studies, including investigations of lung biochemical constituents, structural and cellular changes, lung function, and nasal structure and function. In addition, a Biostatistical Advisory Group developed an animal allocation scheme that allowed several investigators to measure endpoints on the same set of animals, assisted the individual investigators with data analyses, and developed a statistical approach for analyzing multiple endpoints across the individual studies.

RESULTS

Twenty months of exposure to ozone, even at the highest concentration, had no effect on animal survival and only minimal effect on weight gain. Some of the most striking effects of prolonged ozone exposure were observed in the nose. They included structural alterations of the epithelium that lines the nasal cavity and impairment in the flow of mucus; the severity of both of these changes depended on ozone concentration. These effects were seen when rats exposed to 0.5 or 1.0 ppm ozone were compared with rats that had breathed clean air. Exposure to 0.12 ppm ozone had no effect on nasal structure or function.

Prolonged exposure to ozone had mild to moderate effects on other regions of the respiratory tract. The most notable changes occurred in the centriacinar region of the lung, which is the anatomical site that is the junction of the conducting airways and gas exchange region. In rats exposed to 0.5 or 1.0 ppm ozone, the thin epithelial cells that normally line the alveolar ducts were replaced by thicker cells that are more characteristic of the small bronchioles, and the volume of the underlying interstitium increased. These changes were specific to the centriacinar region and were not seen in randomly chosen alveolar ducts. Some alterations were reported in the epithelial cells of the centriacinar region of rats exposed to 0.12 ppm ozone; however, these are difficult to interpret because the changes

were small, they were found in some lung regions but not in others, and some of them occurred only in male rats. Thus, whether prolonged exposure to 0.12 ppm ozone causes alterations in the respiratory tract requires further study.

Biochemical studies indicated small ozone-induced changes in some components of connective tissue (collagen and glycosaminoglycans), substances that provide structural support to the lung. Analyses by light microscopy showed an increase in collagen fibrils in the centriacinar region of animals exposed to 0.5 or 1.0 ppm ozone compared with rats that breathed clean air. Other analyses indicated that the increase in collagen most likely occurred early in the exposure period.

The results of extensive pulmonary function tests indicated that ozone exposure had little or no measurable impact on lung function. A small decrease in residual volume (the amount of air remaining in the lungs at the end of an expiration) was the only change that was found; however, when compared with control animals, the change was statistically significant only in the group exposed to 0.5 ppm ozone. Thus, one can conclude that the minor alterations in lung structure and biochemistry did not affect overall lung function.

Because the 20-month exposure to ozone resulted in some biochemical and structural changes in the rat lungs that were protective in nature, and because the effects on overall pulmonary function were minimal or not measurable, the authors suggest that the animals had become tolerant to the injurious effects of ozone. Although this is a reasonable hypothesis, the study design, in which the animals were examined at only one time point, did not permit a true test of whether tolerance had, indeed, developed. Examining the animals at multiple time points would be required to determine conclusively if the rats developed tolerance as a result of repeated exposures to ozone.

The investigators used three analytical approaches to integrate the results of the multiple studies. One innovative technique involved combining data for a set of related parameters that were measured in different animals. Using a statistical technique called median polish analysis, composite scores for the combined data were developed. This analytical technique, when applied to three disease surrogates (defined by the investigators as a collection of endpoints "potentially related to different pathogenic processes"), revealed new relationships among the structural, biochemical, and functional endpoints investigated in the individual studies. When ozone exposure was clearly related to a disease process, as it was with rhinitis, the composite scores showed highly significant differences between the control rats and those exposed to 0.5 or 1.0 ppm ozone, but no differences between rats exposed to 0 or 0.12 ppm ozone. However, for the other disease surrogates, centriacinar fibrosis and airway disease, the analyses were heavily influenced by the structural changes in the centriacinar region, or were sensitive to the inclusion or exclusion of some endpoints.

IMPLICATIONS FOR HUMAN HEALTH

The results of this project provide insight into the question of whether prolonged exposure to ozone leads to permanent injury of the respiratory tract or to the development of chronic diseases. The nasal changes in rats exposed to 0.5 or 1.0 ppm ozone are very similar to the lesions that have been observed in humans living in areas with high levels of ozone in the ambient atmosphere, and suggest that rhinitis may be a consequence of exposure to high ambient concentrations of ozone. However, because of marked differences in the structures of the nasal cavities of rats and humans, it is difficult to draw conclusions about this aspect of the human response, and to extrapolate exposure-response data from rats to humans. Although rats exposed to 0.5 or 1.0 ppm ozone developed mild to moderate fibrotic lesions in the centriacinar region of the lungs, they did not develop diffuse pulmonary fibrosis analogous to the human condition. In humans, pulmonary fibrosis of either known or unknown origin has characteristic clinical, physiologic, and pathologic features, including a diffuse inflammatory response and a reduction in some pulmonary function measurements. The rats exposed to ozone for 20 months, even at the highest ozone concentrations, did not show any of the characteristic features of human diffuse pulmonary fibrosis, including the inflammatory changes that might lead to fibrotic lesions. The lesions observed in the centriacinar region of the rat lungs were very similar to respiratory bronchiolitis in humans, which is asymptomatic and is associated with either no measurable functional abnormalities or only minimal functional abnormalities.

Some limitations must be considered when extrapolating the results of the NTP/HEI Collaborative Ozone Project to humans. First, the protocol for exposing the rats to ozone did not simulate the ambient exposure pattern of humans, which is intermittent and includes extended periods during which peak ozone levels are less than 0.12 ppm. Second, despite the fact that both the exposure and experimental components of the study were conducted rigorously and thoroughly, the results are directly applicable only to healthy animals exposed to a single pollutant. Prolonged ozone exposure may have a more severe effect in animals with lungs already compromised by previous injury or disease, or when the exposure includes other air pollutants.

This Statement, prepared by the Health Effects Institute and approved by its Board of Directors, is a summary of research on the health effects of prolonged ozone exposure that was sponsored by HEI from 1991 to 1994. The inhalation component of the NTP/HEI Collaborative Ozone Project was supported by the National Toxicology Program as part of its studies on the toxicologic and carcinogenic effects of ozone. The Integrative Summary was developed and prepared jointly by the Collaborative Ozone Project Group. The following Research Report contains both the Integrative Summary and a Commentary on the Integrative Summary prepared by the Institute's Health Review Committee.

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INTRODUCTION

The National Toxicology Program/Health Effects Institute Collaborative Ozone Project

INTRODUCTION

In 1987, the Health Effects Institute entered into a partnership with the National Toxicology Program (NTP) 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. By developing a partnership, the HEI and NTP were able to optimize use of their funds to develop a comprehensive research program that extended beyond the NTP bioassay's focus on carcinogenicity to include other health effects in addition to cancer.

The potential adverse health effects of ozone exposure have been extensively studied using a variety of research approaches. Most of our information concerning these effects on humans is based on multihour, controlled, laboratory exposures, and on epidemiological studies of air pollution episodes of a few days' duration (reviewed by Lippmann 1989; U.S. Environmental Protection Agency 1994). These studies provide evidence that short-term exposure of healthy young adults and children to low concentrations of ozone can, produce a transient decrease in lung function and an increase in markers of inflammation in the respiratory tract. Whether prolonged ozone exposure causes or exacerbates chronic lung diseases is not known. In addition, ozone's quality of being highly reactive has stimulated much speculation about its role as a possible cause or promoter of lung cancer (reviewed by Witschi 1988). Understanding what role ozone and other pollutants have in the development of these diseases is an important public health issue because they affect millions of individuals in the United States and are prominent causes of morbidity and mortality.

Because of the widespread exposure to ozone and concerns about its potential health effects, the 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 diseases of concern, included additional animals for HEI-supported studies of the pathologic and physiologic consequences of prolonged ozone exposure. The HEI animals were housed in cages that would otherwise have been empty. The HEI-sponsored research focused on the relation between prolonged ozone exposure and the pathogenesis of chronic lung diseases, such as asthma, emphysema, and fibrosis. The HEI 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.

The HEI component of the Collaborative Project had three phases. During the initial phase, HEI supported six pilot studies to encourage innovative experimental approaches and to allow potential investigators to test the feasibility of their proposed methods before applying them to aged and potentially fragile animals.*

During the second phase, eight investigator-initiated projects 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." Proposals were first evaluated by an ad hoc review panel and then selected by the HEI Research Committee on the basis of their scientific merit and their likely contribution to a coherent research program. Because of the complexity of a multi-investigator, multi-endpoint project, the Research Committee also funded a biostatistical team to provide assistance with experimental design, animal allocation, and data analyses. Figure 1 in the Integrative Summary presents the studies in the NTP/HEI Collaborative Ozone Project. It includes those studies that were part of the NTP bioassay and the eight studies and the biostatistical component funded by HEI. In addition, HEI engaged Battelle Pacific Northwest Laboratories to provide support services for the HEI-sponsored investigators.

The overall design and protocols of the NTP/HEI Collaborative Ozone Project and the details regarding the studies funded by HEI can be found in Part VI of Research Report Number 65 (Boorman et al. 1995). Briefly, 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 for six hours per day, five days per week. These concentrations were selected to include the maximum dose 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 HEI animals were housed in

* The pilot studies were chosen through a competitive process in response to HEI's Request for Applications 89-2, "Health Effects of Chronic Ozone Inhalation: Collaborative NTP/HEI Studies. Phase I. Pilot Studies and Preproposals." The pilot study results, together with comments from the Institute's Health Review Committee, have been published in the HEI Communications Number 1, New Methods in Ozone Toxicology: Abstracts of Six Pilot Studies (1992).

cages located in the rat and mouse chambers used for the three NTP studies. The ozone exposures were initiated between October 1989 and January 1990 and, for the HEI animals, ended after 20 months to avoid confounding the results with effects from leukemia and other naturally occurring degenerative diseases in aged rats.

Throughout the course of the NTP/HEI Collaborative Ozone Project, HEI conducted workshops and meetings with the project investigators, the staffs of the participating institutions, and other scientists to encourage investigators' interactions and to develop plans for individual and integrated data analyses. The Biostatistical Advisory Group developed a sample allocation scheme that allowed several researchers to obtain measurements or tissue samples from the same subset of study animals; this plan provided the maximum overlap of animals and tissues among the eight studies and ensured balance with respect to dose, gender, and time of death. When the ozone exposures for the HEI animals ended (summer and fall of 1991), 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.

During the final phase of the Project (1992 to 1994), the HEI Review Committee began its evaluation of the Investigators' Reports. Upon completion of their study, each investigator team submitted a draft final report on their work, which underwent a detailed peer review by three or four outside reviewers, including a biostatistician, and then by the Institute's Health Review Committee. The Review Committee's objective was to help ensure that the reports were as complete, accurate, and understandable as possible. After the investigators had revised their reports, the Committee, in accordance with standard HEI practices, prepared a Commentary on each study. Parts I through X of HEI Research Report Number 65 contain the Investigators' Reports, together with the Review Committee's Commentaries, for each of the studies funded by HEI in the NTP/HEI Collaborative Ozone Project; Part VI contains details on the NTP exposure protocol and Study design.

Each study in the NTP/HEI Collaborative Ozone Project was conducted independently, and many conclusions can be drawn on the basis of the results from the individual studies. However, the overall project was designed to allow correlation and synthesis of the multiple outcomes. In this Integrative Summary, the investigators present the key findings of the individual studies and integrate the biochemical, functional, and structural data. During the review of the Integrative Summary, the HEI Review Committee and the investigators had an opportunity to exchange comments and to clarify issues in the Integrative Summary and the Health Review Committee's Commentary. The Commentary is intended to serve as an aid to the Institute's sponsors and the public by highlighting the strengths and limitations of the results of this Project and by discussing their public health implications.

The importance of the NTP/HEI Collaborative Ozone Project is the unparalleled opportunity it provided to examine the effects of 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 provide new information about the threshold effects of ozone exposure on nasal and lung injury in rats 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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REFERENCES

Boorman GA, Catalano PJ, Jacobson B, Kaden DA, Mellick PW, Nauss KM, Ryan LM. 1995. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part VI, Background and Study Design. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Lippmann M. 1989. Health effects of ozone: A critical review. J Air Pollut Control Assoc 39:672–695.

U.S. Environmental Protection Agency. 1994. Air Quality Criteria for Ozone and Related Photochemical Oxidants: External Review Draft, 1993. EPA/600/AP-93/004a. Office of Research and Development, Washington, DC.

Witschi H. 1988. Ozone, nitrogen dioxide and lung cancer: A review of some recent issues and problems. Toxicology 48:1–20.

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PREFACE

Ozone, one of the six air pollutants that are regulated by the U.S. National Ambient Air Quality Standards (NAAQS)*, is a major constituent of photochemical smog. The current NAAQS for ozone is 0.12 parts per million (ppm) averaged over one hour and not to be exceeded more than once per year. The U.S. Environmental Protection Agency (EPA) estimates that 67 million people in the United States, or slightly more than one quarter of the population, live in areas that were out of compliance with this standard in 1989 (U.S. Environmental Protection Agency 1991). Due to the difficulties faced in meeting the ozone standard, and given the large cost of compliance, it is important to understand the relation between ozone exposure levels and adverse health effects.

Short-term exposure (1 to 90 days) to levels of ozone ranging from 0.1 to 1 ppm cause changes in several important biochemical (Last et al. 1979, 1984; Pickrell et al. 1987), structural (Stephens et al. 1974; Evans et al. 1976; Schwartz et al. 1976; Plopper et al. 1978; Boorman et al. 1980; Castleman et al. 1980; Harkema et al. 1987a,b), and functional (Costa et al. 1983, 1995) parameters in animals. Ultrastructural changes induced by ozone are evident in rats (Boorman et al. 1980; Barry et al. 1985, 1988; Barr et al. 1988) and monkeys (Eustis et al. 1981; Hyde et al. 1992; Harkema et al. 1993), particularly in the nasal cavity, trachea, and the centriacinar region of the lung (Plopper et al. 1994b).

Several studies using rodents have been conducted to address the questions regarding repeated exposure to ozone over long time periods. In one study (Gross and White 1987), male F344/N rats were exposed by inhalation to 0.5 ppm ozone for 20 hours per day, 7 days per week, for one year. Slight increases were observed in residual volume and functional residual capacity of the lungs, and decreased carbon monoxide diffusing capacity was seen. These changes returned to normal after three months of breathing clean air. No changes related to ozone were observed in the total collagen content of the lung (Wright et al. 1988). In another set of studies (Grose et al. 1989), F344/N rats were exposed to a regimen designed to mimic the daily urban profile of ozone exposure for 12 or 18 months. This included a baseline ozone concentration of 0.06 ppm for 13 hours per day, 7 days per week, upon which was superimposed a 0.25 ppm ozone exposure for 9 hours per day, 5 days per week (a 9-hour time-weighted average of 0.19 ppm for 5 days per week). In these studies, reductions were seen in lung residual volume and total lung capacity; these changes also returned to normal after a 1.6-month period of breathing clean air. No evidence of airflow obstruction was observed in these animals (Costa et al. 1995). Increased antioxidant enzyme activity (glutathione peroxidase, glutathione reductase, and reduced nicotinamide adenine dinucleotide phosphate–cytochrome *C* reductase) was observed after 12, but not 18, months of exposure. Glutathione peroxidase activity was increased, but superoxide dismutase activity was unchanged at 18 months (Grose et al. 1989). In another experiment, a similar group of animals showed decreased breathing frequency and increased expiratory resistance upon challenge with carbon dioxide (Tepper et al. 1991); however, minimal changes were noted in the centriacinar region of companion animals exposed to ozone under the same exposure pattern (Chang et al. 1992). Although the exposure regimen differed among these studies, some of them show

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

This Investigators' Report is one section of Part XI 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 Integrative Summary. Authors are listed alphabetically because many people had varied roles in this integrative process. Correspondence concerning the Investigators' Report may be addressed to Dr. Debra A. Kaden at the Health Effects Institute, 141 Portland Street, Suite 7300, Cambridge, MA 02139.

The studies discussed in this report were 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.

trends toward obstructive changes and others show trends toward restrictive changes. Thus, it is not known whether repetitive daily or intermittent exposure to ozone over the course of an animal's lifetime produces cumulative damage or lasting deficits in lung function, or if it alters airway structure; furthermore, no data on a concentration-response relation are available.

Studies of lung structure, biochemistry, function, and clearance suggest that the response to ozone may diminish as exposure is repeated or increases in duration; this process of diminished response is termed tolerance (Penha and Werthamer 1974; Nikula et al. 1988; Schlesinger et al. 1992). Tolerance complicates extrapolating the results from studies of short-term exposure to estimate the results of prolonged ozone exposure. Studies of moderate ozone exposure have shown persistent inflammation, but of reduced severity, and other cellular responses (Boorman et al. 1980; Eustis et al. 1981; Moore and Schwartz 1981). The potential adverse health effects of prolonged exposure to ozone include short-term respiratory effects, chronic nonneoplastic lung disease, and cancer. Although some of these changes do not threaten life, they may affect a large segment of the population. In addition, subpopulations (such as people with asthma) may exist that are more sensitive to the adverse effects of ozone.

Because of the need for more reliable information on the effects of prolonged ozone exposure, HEI and the California Department of Health and Human Services recommended that ozone be evaluated by the National Toxicology Program (NTP). In 1987, the NTP planned an inhalation bioassay to evaluate the carcinogenic potential of ozone. These studies were conducted at Battelle Pacific Northwest Laboratories in male and female F344/N rats and $B6C3F_1$ mice. For the NTP studies, animals were exposed for 24 or 30 months to 0, 0.12, 0.5, or 1.0 ppm ozone for 6 hours per day, 5 days per week. Because it is possible that ozone may promote but not initiate cancer, a study of rats also was done with concomitant exposure to a known nasal and pulmonary carcinogen, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

In conjunction with the studies planned by the National Toxicology Program, the Health Effects Institute organized the NTP/HEI Collaborative Ozone Project. As part of its carcinogenicity studies of inhaled ozone, the NTP included in its exposure chambers 164 rats for HEI-funded studies designed to evaluate noncancerous respiratory effects of ozone. These animals were exposed for 20 months to 0, 0.12, 0.5, or 1.0 ppm ozone for 6 hours per day, 5 days per week. The results of these studies are reported in HEI Research Report Number 65, Parts I through X. This Part (XI) summarizes and integrates the results of the set of HEI-funded studies.

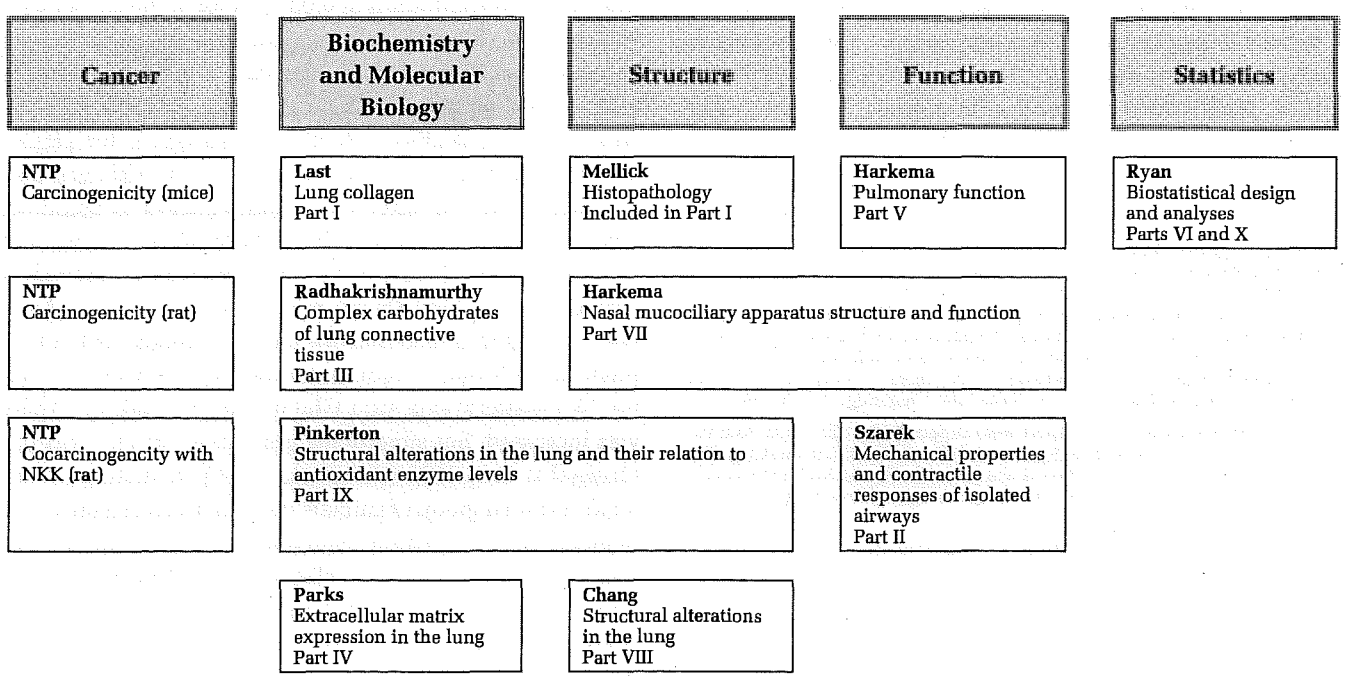

Figure 1. The NTP/HEI Collaborative Ozone Project: Individual Studies. The Part of Research Report Number 65 that presents the findings of each study is given at the end of each description.

INTRODUCTION

This group of studies was designed to address the question of whether repeated episodic exposure to ozone under ambient conditions would cause permanent deleterious effects in rats. Animal studies involving prolonged exposure to ozone are few. However, studies of effects of short-term human exposure suggest that chronic disease states are possible. Therefore, this Project was carefully designed to examine three levels of ozone exposure and several different endpoints.

Eight investigators who conducted nine studies were selected as a result of a competitive, peer-reviewed process to participate in the collaborative effort (Figure 1). Two studies evaluated respiratory and airway function; two studies involved lung and airway morphometry; one study involved nasal structure and function; three studies evaluated connective tissue biochemistry; one study provided statistical support and statistical methods for integration of the studies. In addition, Battelle Pacific Northwest Laboratories provided technical support when the animals were killed and the tissues were prepared.

Figure 2 is a diagram of the rat respiratory system. The endpoints examined in this set of studies (structure, function, and biochemistry) are indicated in this drawing at the approximate location within the respiratory system. Mucociliary structure and function were explored in the nasal apparatus; basic pulmonary function was assessed in the live animals; stress and tension in the airways were measured; structural features of the acinus were investigated morphometrically; and biochemical characteristics were examined both on the level of the whole lung and in focal sites within the airways.

Two photographs compose Figure 3: one of a silicone corrosion cast, and one of a microscopic portion of a left lung lobe from an F344/N rat. The labeled regions include (A) a small-diameter short-path airway with a small number of generations of branches, but a large cumulative branching angle (termed the cranial bronchus); (B) a large-diameter, short-path airway with the same number of generations of branches as the cranial bronchus, but with a very small cumulative branching angle (termed the central bronchus); and (C) a small-diameter, long-path airway with twice the number of generations of branches (termed the caudal bronchus).

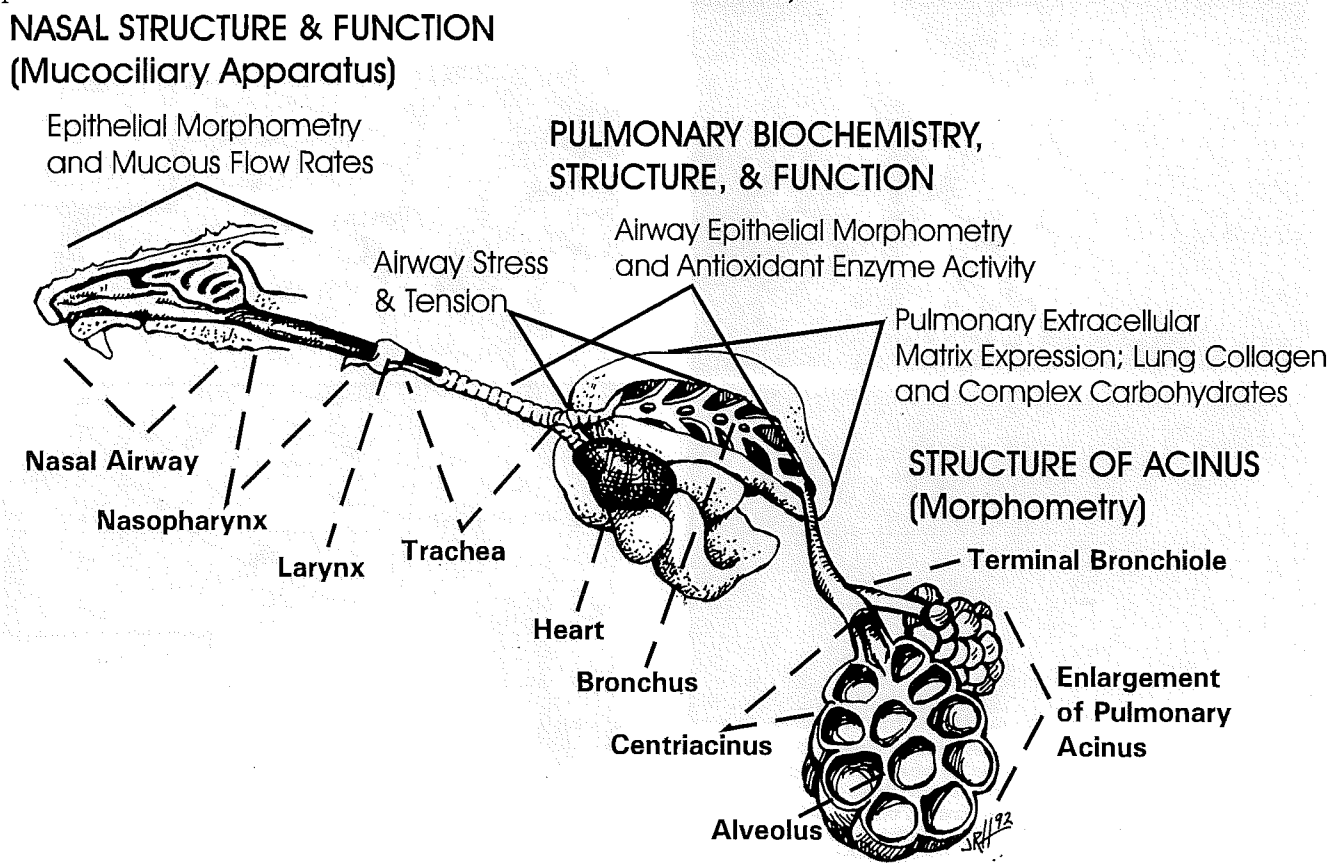

Figure 2. Schematic drawing of the rat respiratory system showing locations where various structural, functional, and biochemical parameters were measured.

Finally, Figure 4 shows examples of sites within the acinus that were sampled in the morphometric studies. These sites included the terminal bronchiole and alveolar duct paths, and especially the proximal and distal alveolar duct regions.

Broad categories and individual endpoints examined in the studies are listed in Table 1; individual endpoints are further described in Appendix A. The large number (more than 244) of individual endpoints that were examined by the entire group of investigators give rise to potential issues of multiplicity in performing and interpreting statistical comparisons. In particular, the prominent danger is focusing on those particular endpoints that happen to be the most significant statistically. To minimize this problem, this summary concentrates on a set of specific questions about the relations among the results of different studies. Subsets of endpoints that are pertinent to answering these questions were selected for statistical analysis on the basis of their scientific relevance, and not on the basis of the effects found and their statistical significance. These parameters were then analyzed using a variety of statistical procedures designed to allow an overall assessment of effects revealed by the aggregation of individual endpoints.

The results of individual studies are summarized in the following section, and then integrated to respond to four basic questions using three types of analysis (Integration Across Studies section). (Full details of individual studies can be found in HEI Research Report Number 65, Parts I through X.)

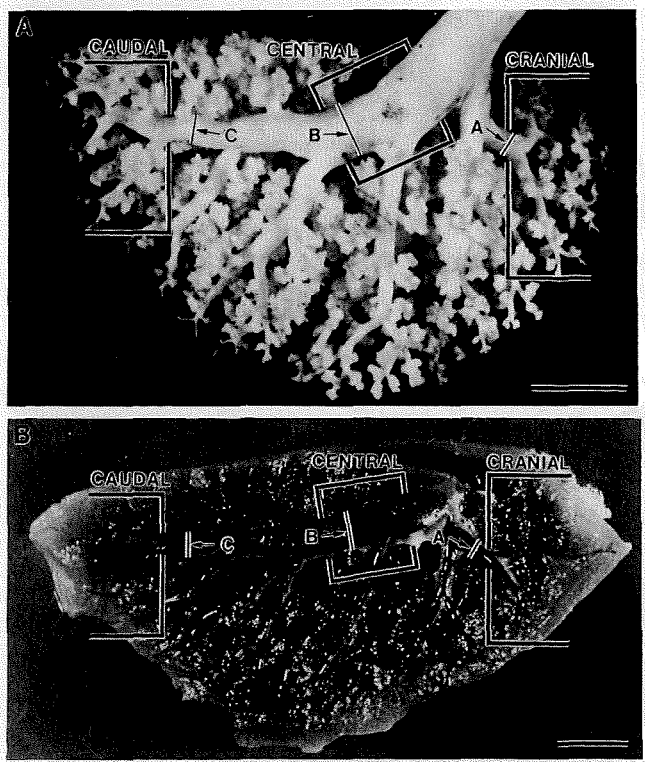

Figure 3. Location of tissue samples taken from rat lung. A: Silicone cast of the tracheobronchial airway tree from the left lobe of a rat. Lung casts served to standardize sampling. Samples of terminal bronchiole–alveolar duct junctions were taken from the three regions (cranial, central, and caudal) indicated. The letters and arrows within the figure indicate the precise locations from which samples of intrapulmonary conducting airways were taken. (Bar = 4 mm.) B. Mediastinal half of a fixed, microdissected rat lung. Note how closely the pathways and sampling regions match the silicone cast shown above. (Bar = 4 mm.)

RESULTS OF INDIVIDUAL STUDIES

RESPIRATORY TRACT STRUCTURE

The mammalian respiratory system is the primary target for a variety of inhaled oxidant gases. However, the response of various regions of the respiratory system to ozone is not uniform. Some sites are either sensitive or resistant to the effects of short-term exposure to ozone (Eustis et al. 1981; Nikula et al. 1988). Therefore, three studies used morphometric techniques to examine a number of anatomical sites along the nasal cavity, tracheobronchial tree, and pulmonary acini. The sensitive sites evaluated included the

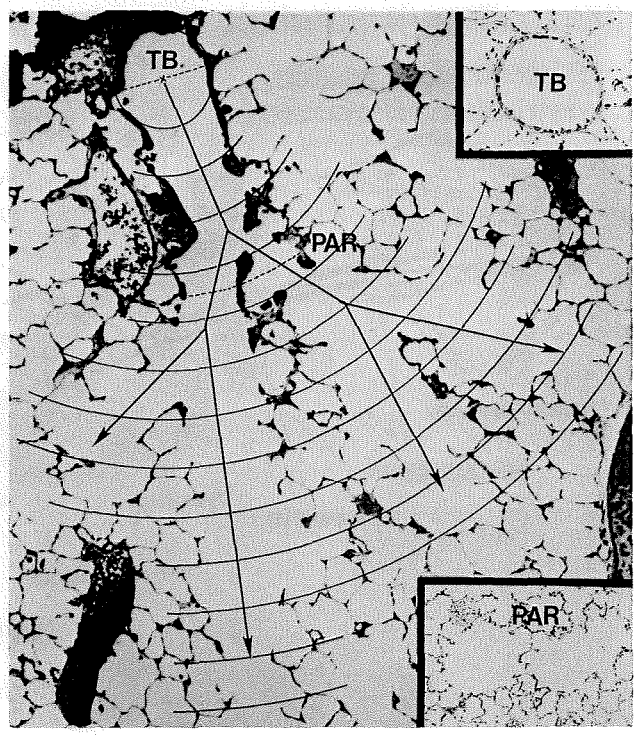

Figure 4. Photomicrograph of a single isolated ventilatory unit overlaid with concentric arcs at 100-µm intervals. Morphometric analysis of the terminal bronchiole (TB) and complete alveolar duct paths forming the pulmonary acinus (arrows) were analyzed in longitudinal profile by light microscopy. The TB and proximal alveolar region (PAR; see inset) were also analyzed in cross-sectional profile using transmission electron microscopy.

nose, trachea, lobar bronchus, and distal conducting airways (terminal bronchioles and proximal alveolar ducts) of the central pulmonary acinus. Areas examined that are considered to be less susceptible to acute injury and inflammation included the airways located between the trachea and the terminal bronchiole, and the lung parenchyma beyond the centriacinar region (see Figure 2).

The different investigators who examined structural features of the respiratory system shared tissues from the same animals. Because these studies required that whole lungs be fixed by perfusion, these samples were unavailable for some other studies.

Histopathology

A complete necropsy and histologic analysis was performed on the remains of all animals at Battelle Pacific Northwest Laboratories after tissues had been distributed to HEI investigators. Results of these analyses are presented

Table 1. Summary of Endpoints Examined by HEI Investigators[a]

General Category and Site	Principal Investigator	Individual Endpoints	Total Endpoints
Structure			
Nasal epithelium	Harkema	6 Measurements at 1 site	6
Tracheobronchial epithelium	Pinkerton	3 Measurements at 10 sites	30
Pulmonary acinus	Pinkerton	5 Measurements at 2 sites at 8 distances from the bronchiole–alveolar duct junction	80
Mucus stored in the airways	Pinkerton, Harkema	3 Nasal regions and 4 airway locations	7
Alterations in terminal bronchioles, and proximal and distal alveolar regions of the lung	Chang	15 Primary measurements	15
Biochemistry			
Collagen and measures of collagen cross-links in lung tissue	Last	1 Measurement of total collagen and 5 measurements of cross-linked collagen	6
Individual and total glycosaminoglycans in lung tissue	Radhakrishnamurthy	6 Measurements of individual glycosaminoglycans and 1 measurement of total glycosaminoglycans	7
Messenger RNA for extracellular matrix components	Parks	5 Measurements of individual matrix components	5
Antioxidant enzyme activity as a function of airway location	Pinkerton	4 Enzymes at 9 locations	36
Prostaglandin and leukotriene release from airways	Szarek	2 Eicosanoids at 2 locations under 2 airway conditions	4
Function			
Nasal mucous flow rates (in vitro)	Harkema	Flow rates in 13 regions	13
Pulmonary function (in vivo)	Harkema	19 Primary measurements and multiple derived values	19
Contractile responses in airways (in vitro)	Szarek	4 Agonists at 2 locations under 2 airway conditions	16

[a] Appendix A gives a detailed list of endpoints.

in Appendix B of this Integrative Summary, the Supplement in Last and associates (1994) and in Boorman and colleagues (1995). Histologic lesions attributable to ozone exposure consisted of subtle fibrotic changes in the centriacinar region of the lung, an extension of the bronchiolar epithelial lining into the alveolar duct region, and alveolar macrophage accumulation in alveolar ducts and proximal alveoli in rats exposed to 0.5 or 1.0 ppm ozone. The severity and extent of these changes were greater in animals exposed to 1.0 ppm ozone. Mononuclear cell leukemia was observed in 37 of the 164 rats, but was not related to ozone exposure. Five rats had primary lung tumors (alveolar or bronchiolar adenomas or carcinomas). Although all five primary lung tumors occurred in animals exposed to ozone, the incidence of these lesions was within the range of historical data for F344/N control rats. The lung tumors had the typical histologic characteristics of those commonly seen in this strain of rat. Information on the carcinogenicity results for the NTP animals is presented elsewhere (Appendix B of this report; National Toxicology Program 1995). Both the necropsy results on HEI animals and the serum testing for antibodies to rodent pathogens in sentinel animals (rats housed in the exposure chambers but not used for experiments) indicated that the Project animals had no infections.

Nasal Morphometry

After nasal mucociliary function was assessed (see the Nasal Mucociliary Tract section under Respiratory Tract Function),

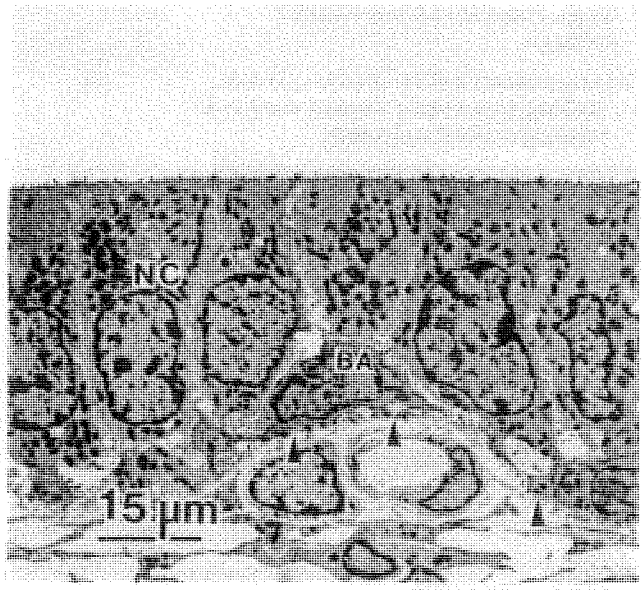

Figure 5A. Transmission electron photomicrographs of nasal transitional epithelium lining the lateral walls of rats exposed to 0.0 ppm ozone for 20 months. BA = basal cell; NC = nonciliated cuboidal cell; arrowheads = basal lamina.

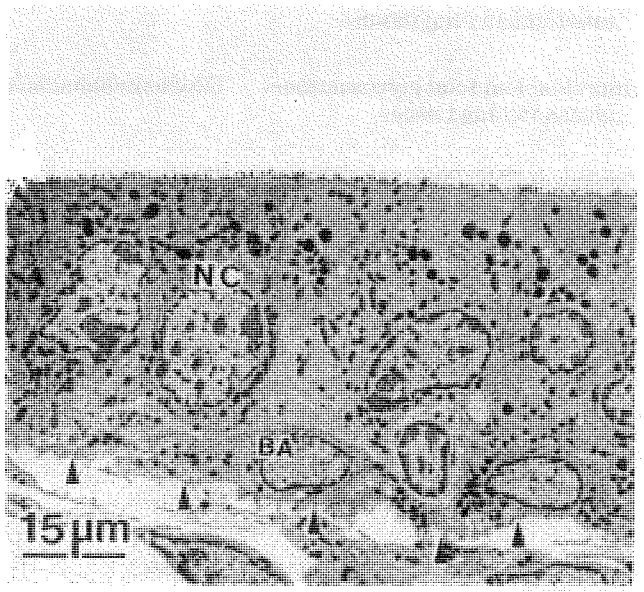

Figure 5B. Transmission electron photomicrographs of nasal transitional epithelium lining the lateral walls of rats exposed to 0.12 ppm ozone for 20 months. BA = basal cell; NC = nonciliated cuboidal cell; arrowheads = basal lamina.

nasal tissues were processed for light and electron microscopy and stained so the intraepithelial mucus could be detected (Harkema et al. 1994). Image analysis was used to quantify the amount of mucus within the nasal transitional epithelium located in the proximal nasal airway. This study focused on changes in the cellular populations that compose the surface epithelial lining of the nasal lateral wall and on the intracellular mucus within these cells. Structural changes were correlated with functional alterations in the mucociliary apparatus, as described later in this Integrative Summary (the Nasal Mucociliary Tract section under Respiratory Tract Function).

The lateral wall and turbinates of the proximal third of the nasal airway in animals exposed to 1.0 ppm ozone contained marked mucous cell metaplasia (the appearance of mucous secretory cells in the transitional epithelium that is normally devoid of them; Figure 5 and Table 2) and greater amounts of mucus in nasal transitional epithelium (Table 3). Mucous cell metaplasia associated with an increase in intraepithelial mucus also was evident in the nasal transitional epithelium of rats exposed to 0.5 ppm ozone, but to a lesser degree than in rats exposed to 1.0 ppm ozone (Tables 2 and 3).

In addition, male and female rats exposed to 0.5 or 1.0 ppm ozone had moderate mucosal inflammation, atrophy of turbinates, and histologic alterations in the olfactory epithelium, and showed evidence of bony atrophy. No significant morphologic alterations were evident in the rats exposed to 0.12 ppm ozone.

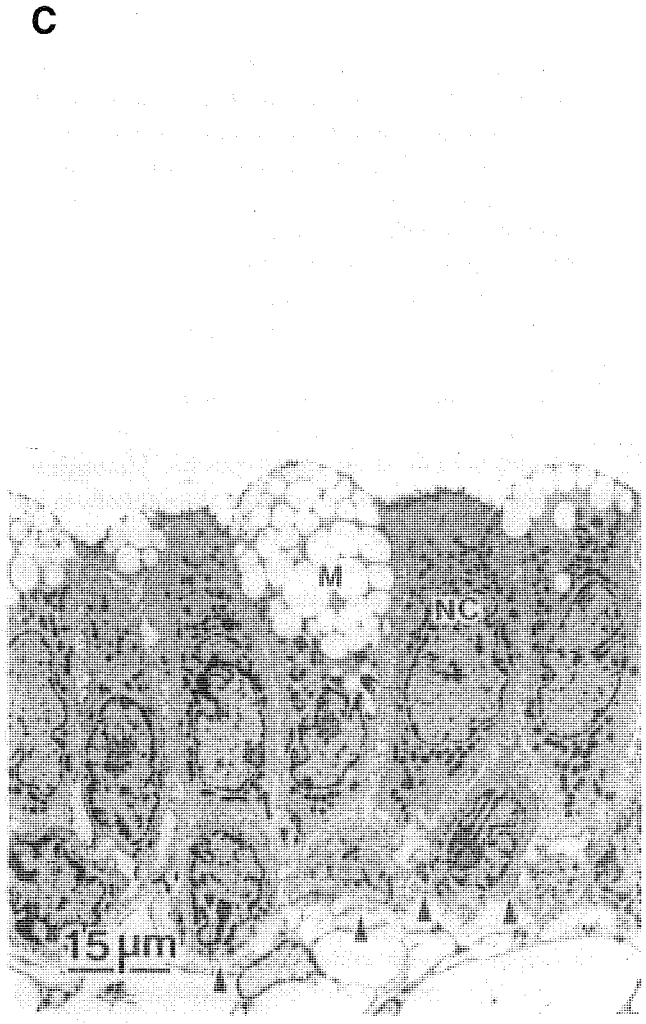

Figure 5C. Transmission electron photomicrographs of nasal transitional epithelium lining the lateral walls of rats exposed to 0.5 ppm ozone for 20 months. M = mucous cell; NC = nonciliated cuboidal cell; arrowheads = basal lamina.

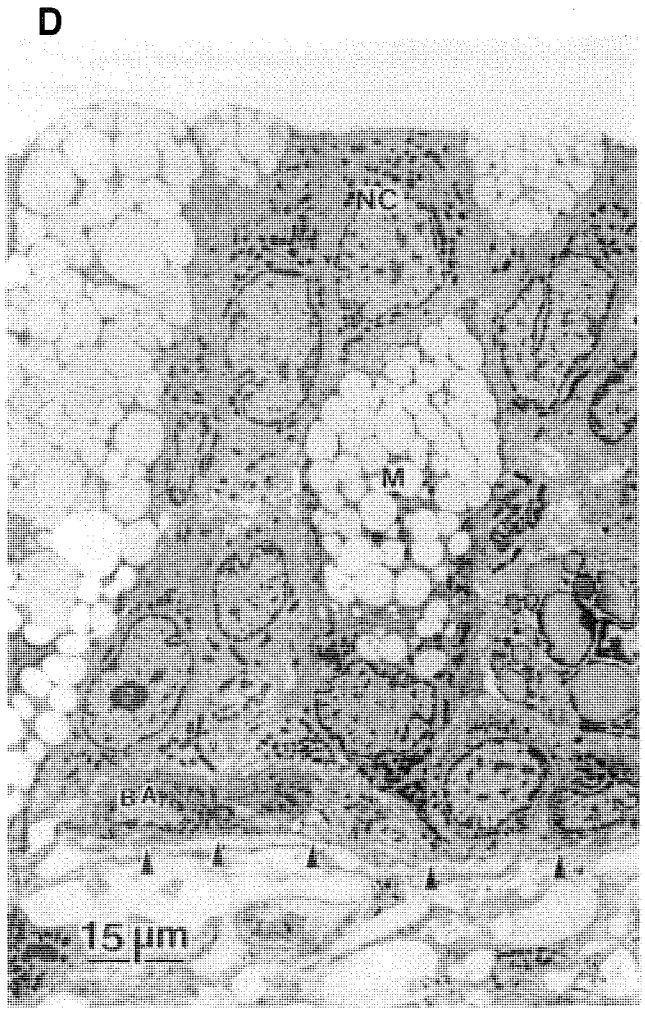

Figure 5D. Transmission electron photomicrographs of nasal transitional epithelium lining the lateral walls of rats exposed to 1.0 ppm ozone for 20 months. M = mucous cell; BA = basal cell; NC = nonciliated cuboidal cell; arrowheads = basal lamina.

Table 2. Abundance of Cells in the Nasal Transitional Epithelium Lining the Lateral Walls of the Proximal Nasal Airway[a]

Epithelial Cell Type	Ozone Concentration (ppm) 0.0 ($n = 4$)	0.12 ($n = 4$)	0.5 ($n = 4$)	1.0 ($n = 5$)
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 number of cells per millimeter of basal lamina.

[b] Significantly different ($p < 0.05$) from control group.

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

Ozone Concentration (ppm)	n (males/females)	Volume/Surface Area (nL/mm^2 of basal lamina $\times 10^{-2}$)[a]
Lateral Aspect of Nasoturbinate		
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 Nasoturbinate		
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 (7/8)	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]
Maxilloturbinate		
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]

[a] Values are given as means ± SEM.

[b] Significantly different ($p < 0.05$) from control group.

Tracheobronchial and Centriacinar Morphometry

Ozone is toxic to the epithelial cells of the respiratory system, and the responses are highly focal and specific to site. Furthermore, the majority of injury to the cell and accompanying inflammation occurs during the initial phases of exposure (Eustis et al. 1981; Dungworth 1989; Chang et al. 1992; Harkema et al. 1993). Repeated exposures to ozone, even at concentrations that *initially* produce epithelial necrosis and acute inflammation, have been shown to cause minimal inflammation and cell death, and the remodeling and emergence of the epithelial cell population (Boorman et al. 1980; Barr et al. 1988, 1990; Chang et al. 1992; Pinkerton et al. 1993). The two lung morphometry studies from this Project (Chang et al. 1995; Pinkerton et al. 1995) demonstrate the importance of looking for changes potentially induced by ozone at specific regions believed to be the most sensitive and at multiple time points after exposure. Although it is known that the changes that occur in the epithelium progress over time, the NTP exposure protocol was designed in such a way that animals could be studied at only one time point. This precluded examining the temporal changes in epithelial cells during the exposure that might account for the regional sensitivity to ozone that apparently had been altered.

The two pulmonary morphometry studies in this Project used different techniques to examine changes in tracheobronchial and centriacinar regions. In one study (Pinkerton et al. 1995), cellular and enzymatic changes in the respiratory system were investigated at discrete locations throughout the airways. This study involved morphometric and three-dimensional analyses of longitudinal sections of the conducting airways and pulmonary acini to identify changes in cell numbers and types of cells, measurements of the amount of secretory product in airway epithelial cells, and evaluation of changes in the cellular composition and organization (architectural remodeling) of the acinar region. The ventilatory unit was examined at a series of locations,

starting at the bronchiole–alveolar duct junction and extending distally by 100-μm intervals to at least 800 μm further along the alveolar duct. In addition, biochemical changes in precisely defined lung regions were examined in a second set of animals, and correlated with any structural alterations observed in the first set of animals. The other study (Chang et al. 1995) used electron microscopy to examine ultrastructural features in two specific cross-sectional regions within the centriacinar region: the terminal bronchiole, and the proximal and distal alveolar regions. The proximal alveolar region is located approximately 600 μm from the bronchiole–alveolar duct junction at the first alveolar duct bifurcation after the terminal bronchiole (see Figure 4). In this study, changes in cell populations and characteristics of the interstitial matrix were examined. Volume-to-surface ratios were examined in alveolar regions selected at random.

Mathematical dosimetry models (Overton et al. 1987; Mercer et al. 1991) predicted that the dose of ozone to lung tissues would be distributed heterogeneously throughout the airways; that is, some tissues would receive high and some low doses. On the basis of these models, airways for analysis in the Pinkerton morphometry studies included tissues that (1) would receive a range of low to high dose levels, and (2) could be identified reproducibly in all animals. As predicted, the changes in the endpoints examined in this study varied with location (Plopper et al. 1994a), as is consistent with findings previously reported (Plopper et al. 1991). Both the amount of stored secretory product and the epithelial organization were examined. A decrease in stored secretory product that was dependent on ozone concentration was noted in the trachea. However, an increase in stored secretory product was noted in intrapulmonary conducting airways of differing size and branching history; this increase was consistent with the differences described in models of ozone exposure concentration in these regions (Overton et al. 1987; Mercer et al. 1991). The regions examined included the cranial bronchus (a small-diameter short-path airway with a small number of generations of branching but a large cumulative branching angle), the central bronchus (a large-diameter, short-path airway with the same number of generations of branching as the cranial bronchus but with a very small cumulative branching angle), and the caudal bronchus (a small-diameter, long-path airway with twice the number of generations of branching). Locations of the caudal, central, and cranial bronchi are shown in Figure 3.

No statistically significant cellular reorganization was evident after ozone exposure in the epithelium of the trachea or distal conducting airways (cranial, caudal, or central bronchi) both in terms of total epithelial mass (determined as thickness), and nonciliated cell volume (Table 4, Figure 6).

As would be expected from previous studies (Plopper et al. 1978, 1979; Boorman et al. 1980; Barr et al. 1988, 1990), the Pinkerton study found that the centriacinar region was most responsive to ozone. In this region, the epithelial composition of ventilatory units arising from two bronchi-

Table 4. Statistical Analysis of Tracheobronchial Airway Data

	Multivariate p Values[a]			Univariate p Values[b]		
Primary Variables	Ozone Concentration	Gender	Concentration × Gender	Ozone Concentration	Gender	Concentration × Gender
Stored secretory product	< 0.01[c]	0.02[c]	0.47			
Trachea				0.03[d]	0.43	
Cranial bronchus				< 0.01[d]	0.19	
Central bronchus				0.23	< 0.01[d]	
Caudal bronchus				0.01[d]	0.24	
Nonciliated cell volume	0.11	0.26	0.04[c]			
Trachea				0.91	0.35	0.12
Cranial bronchus				0.08	0.36	0.59
Central bronchus				0.03[d]	0.02[d]	0.23
Caudal bronchus				0.95	0.18	0.79
Total epithelial thickness	0.15	0.47	0.40			

[a] Multivariate significance was tested by the Hotelling-Lawley trace.

[b] Univariate significance was tested by ANOVA factor F tests. A blank column indicates that the multivariate tests did not permit subtests of that factor at the univariate level.

[c] Statistically significant effect.

[d] Statistically significant effect that was subtested if needed.

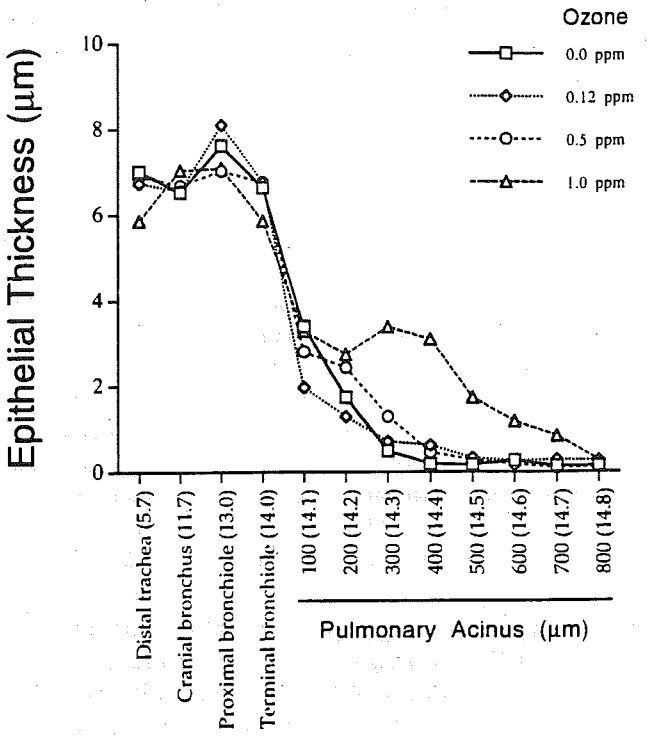

Figure 6. Epithelial thickness (in micrometers) **of the cranial pathway of the lower respiratory tract after 20 months of exposure to 0.0, 0.12, 0.5, or 1.0 ppm ozone.** The estimated distance (in millimeters) of each anatomical site from the larynx is given in parentheses. Values presented are the average for both male and females.

oles was examined: one arising from the cranial region and one arising from the caudal region of the left lung. The most sensitive area in each of these two regions was found to be just beyond (100 to 200 µm) the bronchiole–alveolar duct junction of the terminal bronchiole (Figure 6). Within the centriacinar region, the organization of the epithelium changed most dramatically, evidenced primarily by changes in the composition of the epithelial cell types (an increase in nonciliated cells) and an increase in the total cell mass. These changes were dependent on the dose delivered to the site. The cranial region of male rats showed significant changes even at the lowest ozone exposure concentration of 0.12 ppm.

In the alveolar duct, examined up to 800 µm distal from the junction with the terminal bronchiole, a general reorganization of the epithelium was noted in the form of bronchial epithelial metaplasia; this could be due to migration of bronchial epithelial cells into the alveolar duct regions, or reversion of epithelial cells to a more primitive form that subsequently redifferentiated into bronchial cells. Where such metaplasia occurred, the nature of the epithelium was similar to that found in terminal bronchioles in control animals. Bronchial epithelial metaplasia extended a significant distance into the alveolar duct in animals exposed to ozone (as far as 600 µm in animals exposed to 1.0 ppm ozone), with the extent of bronchiolarization being dependent on the ozone concentration (Figures 7 and 8). In the caudal region, the extent of bronchial epithelial metaplasia was greater in females than in males, whereas in the cranial region, it was greater in males. The epithelium was characterized by a greater proportion of nonciliated cells compared with the terminal bronchioles of control animals. This was true only in the cranial region for females exposed to 1.0 ppm ozone, and in all regions in males exposed to 0.5 or 1.0 ppm ozone. The Pinkerton study demonstrates that ozone exposure primarily affects the structure of the centriacinar region, causing a greater proportion of nonciliated epithelial cells to form, and bronchial epithelial cells to

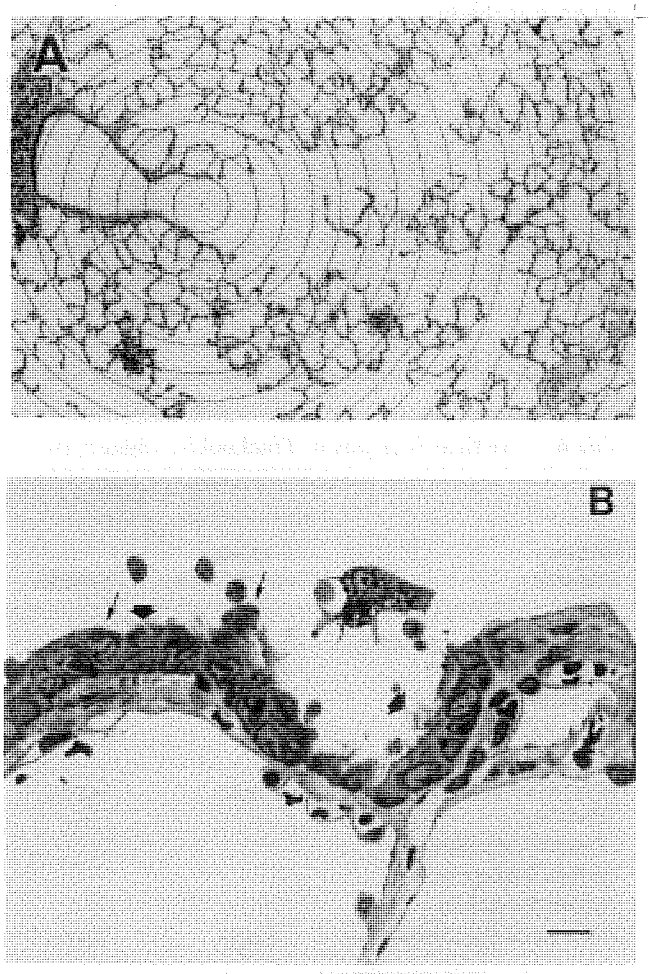

Figure 7. A: Bronchiole-alveolar duct junction and centriacinar sliced longitudinally from the lung of a rat exposed to 1.0 ppm ozone for 20 months. The concentric arc pattern (at 100-µm intervals) radiates from the first alveolar outpocketing (arrow indicates the proximal position). (Bar = 100 µm.) B: A higher magnification of the area in panel A marked by the arrowhead. It shows the presence of ciliated cells (thick arrows) and nonciliated cells (thin arrows) on septal tips and walls of the alveolar duct. (Bar = 100 µm.)

extend into normally nonepithelial regions of the alveolar ducts. The severity of the lesions differed by gender, with males showing more effects than females. Earlier research has shown that body weight, lung surface area, and ventilatory rates in rats differ by gender. Using the results of the Pinkerton study in a dosimetry model, the data suggest that, for animals with similar body weights (as were the animals in this Project), the delivered dose to both the cranial and caudal sites will be higher in male rats than in female rats. This would help to explain why effects were seen in male rats at lower ozone exposure concentrations than in female rats.

In the second morphometry study, by Chang and associates (1995), no significant differences were observed between animals exposed to ozone and control animals when volume-to-surface ratios in randomly selected alveolar regions were compared. Both male and female animals exposed to 0.5 or 1.0 ppm ozone demonstrated 1.6- to 1.8-fold increases in the volume of interstitial matrix as indicated in Figure 9 and in Table 5 by MANOVA, and for individual interstitial variables (acellular space, basement membrane, collagen, elastin, and interstitial cells) by ANOVA. As in the Pinkerton study, increases in the thickness of the epithelium in the proximal alveolar regions of these animals were noted. The thickening of the epithelium was caused by bronchial epithelial metaplasia, in which the normal squamous epithelium was modified to a cuboidal epithelium, similar, but not identical, to that found in terminal bronchioles. Bronchial epithelial metaplasia was composed of differentiated ciliated cells and nonciliated cells similar to those found in terminal bronchioles, and undifferentiated cuboidal cells unique to the animals exposed to 0.5 or 1.0 ppm ozone. The amount of metaplasia increased with an increase in ozone concentration. The bronchial epithelial metaplasia observed in the proximal alveolar ducts may indicate that a protective mechanism against the effects of

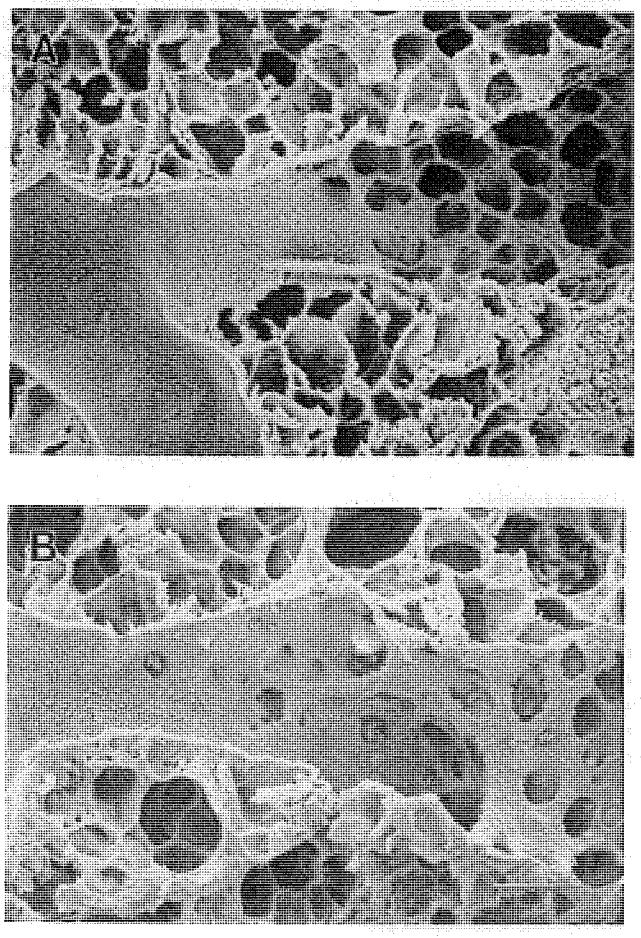

Figure 8. Scanning electron micrographs of the bronchiole-alveolar duct junction from the lungs of a control rat (top panel) and a rat exposed to 1.0 ppm ozone (bottom panel) for 20 months. In the control animal, the transition from the terminal bronchiole to the alveolar duct is abrupt; in the animal

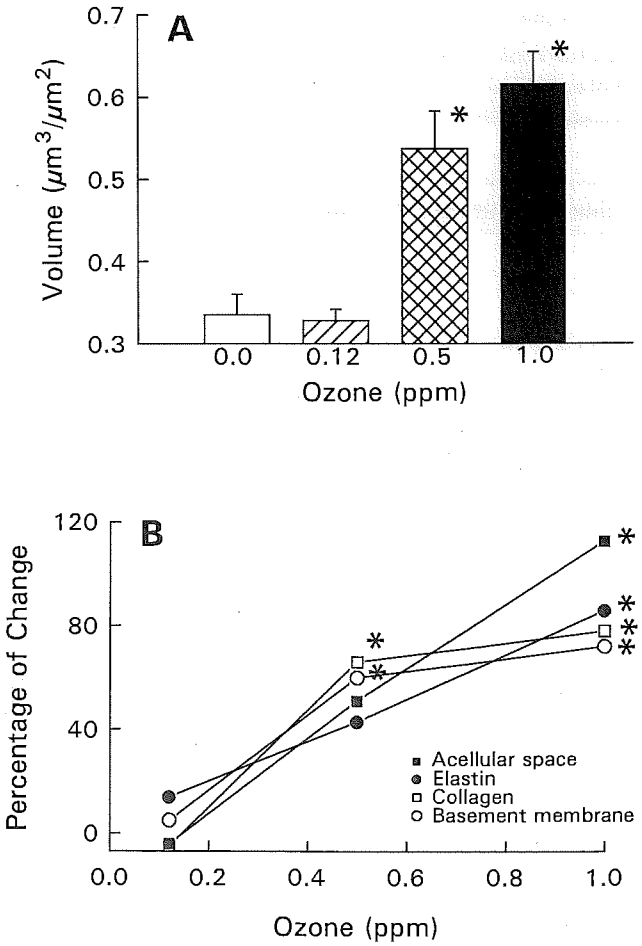

Figure 9. Changes of the interstitial matrix in proximal alveolar regions induced by prolonged exposure to ozone. (A) total volume of interstitial matrix increased after 20 months of exposure to 0.5 and 1.0 ppm ozone. Error bars represent the SEM.(B) After ozone exposure, the percentage of change from the control value for each component of interstitial matrix.

prolonged exposure to ozone (tolerance) may form by more resistant cell types being placed in the areas most susceptible to ozone injury.

A mild fibrotic response was seen in the animals exposed to 1.0 ppm ozone, with increases in both noncellular and cellular components in the interstitium. The individual components of the matrix, including collagen, elastin, basement membrane, and acellular spaces, all increased after exposure to 1.0 ppm ozone (Figure 9). The increase in cellular interstitium was due to an increase in the volume of interstitial fibroblasts. A slight inflammatory response, including an increase in alveolar macrophages, was observed in animals exposed to 1.0 ppm ozone. The terminal bronchioles were less affected than the proximal alveolar regions by the ozone exposure, which may indicate a resistance of these cells to ozone damage. Changes in the terminal bronchioles consisted mainly of a shift in cell type from ciliated to nonciliated cells in the animals exposed to 1.0 ppm ozone.

In the Pinkerton and Chang studies, two indices of bronchial epithelial metaplasia, or "bronchiolarization," were used: an assessment of the distance the metaplasia extended, and an assessment of the percentage of cells that were affected. Because of the different techniques used, the two studies examined different locations within the alveolar duct (see Figure 4). The Pinkerton study examined 100-μm arcs radiating from the bronchiole–alveolar duct junction for at least 800 μm down the alveolar duct. The Chang study examined cross-sections of the terminal bronchiole and proximal alveolar region from random alveolar regions, either at the bronchiole–alveolar duct junction or approximately 600 μm from it. Thus, the changes that Pinkerton observed in regions 100 to 400 μm from the bronchiole–alveolar duct junction in animals exposed to 0.12 ppm ozone would not have been detected by the techniques used in the Chang study.

LUNG BIOCHEMISTRY AND MOLECULAR BIOLOGY

Four studies evaluated biochemical parameters in the lungs. On the basis of earlier short-term studies (for instance, Last 1988), three of these studies were designed to examine changes in the rate of synthesis and deposition of components in the lungs of animals exposed to ozone. The studies in biochemistry and molecular biology examined total

Table 5. Summary of Statistical Analyses for Effects of Ozone Concentration in the Proximal Alveolar Region

Class of Injury	Significance of Ozone Concentration by MANOVA for Class of Injury[a]	Key Variable	Significance of Ozone Concentration by ANOVA for Key Variable[a]
Bronchiolarization	*	Volume of Clara cells	*
		Volume of ciliated cells	*
		Volume of other epithelial cells	*
Epithelial		Volume of type I cells	
		Volume of type II cells	
		Number of type I cells	
		Number of type II cells	
Interstitial	*	Volume of acellular space	*
		Volume of basement membrane	*
		Volume of collagen	*
		Volume of elastin	*
		Volume of interstitial cells	*
Vascular	*	Volume of plasma	
		Volume of red blood cells	*
Inflammation	*	Volume of alveolar macrophages	*
		Volume of interstitial macrophages	

[a] An asterisk (*) in this column indicates a statistically significant result ($p < 0.05$).

and cross-linked collagen content, complex carbohydrates of pulmonary connective tissue, and connective tissue protein gene expression. In addition, one of the lung morphometry studies had a component that examined antioxidant enzyme activity and its distribution within specific airway generations of the tracheobronchial tree.

Expression of Extracellular Matrix Genes

In specific lung regions, levels of expression of genes coding for connective tissue proteins were evaluated using in situ hybridization techniques (Parks and Roby 1994). Due to the possible lability of messenger RNA (mRNA), this study required that tissues be sampled on the day immediately after the ozone exposure ended; lung tissue samples then were stored. Sections of the accessory lung lobes were hybridized with ^{35}S-labeled probes for various matrix proteins, including procollagen types I and III, elastin, and fibronectin, and for interstitial collagenase, a matrix metalloproteinase. Fetal rat lung was used as a positive control tissue for in situ hybridization.

No signal for any mRNA related to matrix was detected in terminal airway stromal cells of lungs from control animals or animals exposed to ozone. Only very weak signals were seen in occasional cells within the interstitial spaces around the airways and blood vessels. In contrast, a strong signal for matrix mRNA was detected in fetal lung tissue. These findings indicate that active or enhanced matrix production is turned off in the adult animals, and suggest that any increase in matrix deposition results from a transient and early fibrotic response. Indeed, in a separate study, signal for type I procollagen and tropoelastin mRNAs was seen in alveolar septal cells of lungs from a limited number of rats exposed to ozone for two months (Parks 1992). No signal was seen in alveolar cells of age-matched controls. These findings suggest that ozone mediates a transient fibrotic response that results in a sustained increase in lung extracellular matrix (see the next section).

Content and Cross-Linking of Lung Collagen

Measurements of pulmonary collagen were made using high performance liquid chromatography and radiometric techniques (Last et al. 1994). Three different indices of lung collagen content were studied: total collagen was assessed as 4-hydroxyproline, an amino acid found predominantly in collagen; mature collagen was assessed as hydroxypyridinium cross-link, a biomarker that occurs in relatively higher concentrations in fibrotic lung collagen than in normal lung collagen; and recently synthesized collagen was assessed as the ratio of dihydroxylysinonorleucine:hydroxylysinonorleucine (DHLNL:HLNL) cross-links.

Data for 4-hydroxyproline and hydroxypyridinium cross-links were expressed per lung lobe, or were normalized to tissue weight or to protein content, and the DHLNL:HLNL cross-link results were expressed as a ratio. When data were expressed per lung lobe, significant changes related to exposure concentration were observed in female, but not male, animals for the 4-hydroxyproline and hydroxypyridinium content of the lungs after exposure to 0.5 or 1.0 ppm ozone. 4-Hydroxyproline content of the lung after ozone exposure is presented in Figure 10. When data were normalized to lung weight, no statistically significant differences in the biochemical assays for lung collagen content were noted between any of the exposure groups.

A statistically significant change (on the basis of the slope of the concentration-response line) in the ratio of DHLNL:HLNL cross-links was recorded for the rats exposed to ozone. The small change in the ratio of DHLNL:HLNL cross-links may be viewed as inconsistent with the negative findings using in situ hybridization for detecting alpha1(I) and alpha1(III) procollagen mRNAs (see the Expression of Extracellular Matrix Genes section). Both methods provided information regarding newly synthesized and deposited collagen in the lung. However, the relative sensitivity of the two methods is not known, and the two determinations measure very different parameters. Furthermore, it is only an assumption that the collagen contributing to the increased ratio of DHLNL:HLNL cross-links is fibrotic collagen.

One possible interpretation of these observations is that the alterations in whole lung collagen content and cross-linking observed in the female rats reflected growth and

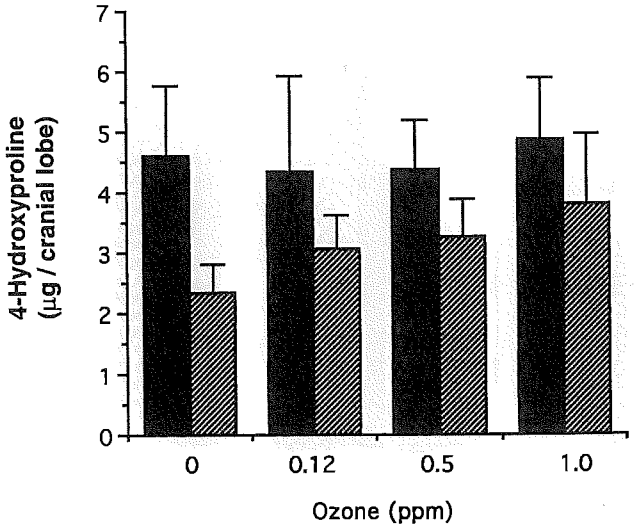

Figure 10. Collagen content of cranial lung lobes, expressed as the content of 4-hydroxyproline in the whole lobe. Data are presented as mean values, with error bars representing one SD. Filled bars = males; hatched bars = females.

repair processes, such as the extension of bronchial epithelium into alveolar regions (as described in the Tracheobronchial and Centriacinar Morphometry section), rather than increased deposition of fibrotic lung collagen. If this interpretation is true, then female animals may have been less susceptible to fibrotic changes induced by ozone than male animals. An alternative interpretation might be that differences are related to the large differences in body size between the male and female rats in this Project. We could speculate that these gender or size differences altered the actual ozone dose to the lungs (see Tracheobronchial and Centriacinar Morphometry section), intrinsic resistance of the lungs to ozone, adaptation of the lungs to ozone, or other factors that affect the ability of the lungs to respond to ozone throughout the duration of the exposure period.

Complex Carbohydrates of Lung Connective Tissue

Most glycosaminoglycans are constituents of proteoglycans, which are integral components of lung connective tissue. Glycosaminoglycans not only provide structural support to the organ system, but also have several biologic properties including influencing extracellular matrix assembly, cell adhesion, and cell proliferation.

Analyses of pulmonary complex carbohydrates focused on the relation between ozone exposure concentration and the composition of glycosaminoglycans (Radhakrishnamurthy 1994). A statistically significant decrease ($p < 0.05$ or $p < 0.01$, two-tailed) in total glycosaminoglycan concentration was observed in animals exposed to 0.5 ppm ozone (a decrease to 81% of the control value; $p < 0.05$, two-tailed) or 1.0 ppm ozone (a decrease to 78% of the control value; $p < 0.01$, two-tailed) when compared with control animals. Analyzing individual glycosaminoglycans showed statistically significant decreases in the concentrations of hyaluronan ($p < 0.001$, two-tailed), chondroitin 4-sulfate ($p < 0.05$, two-tailed), and chondroitin 6-sulfate ($p < 0.01$, two-tailed) when compared with control animals (Figure 11). Heparan sulfate concentration exhibited a statistically significant trend ($p < 0.05$, two-tailed) toward increasing concentrations with increasing concentrations of ozone. Gel filtration studies of hyaluronan

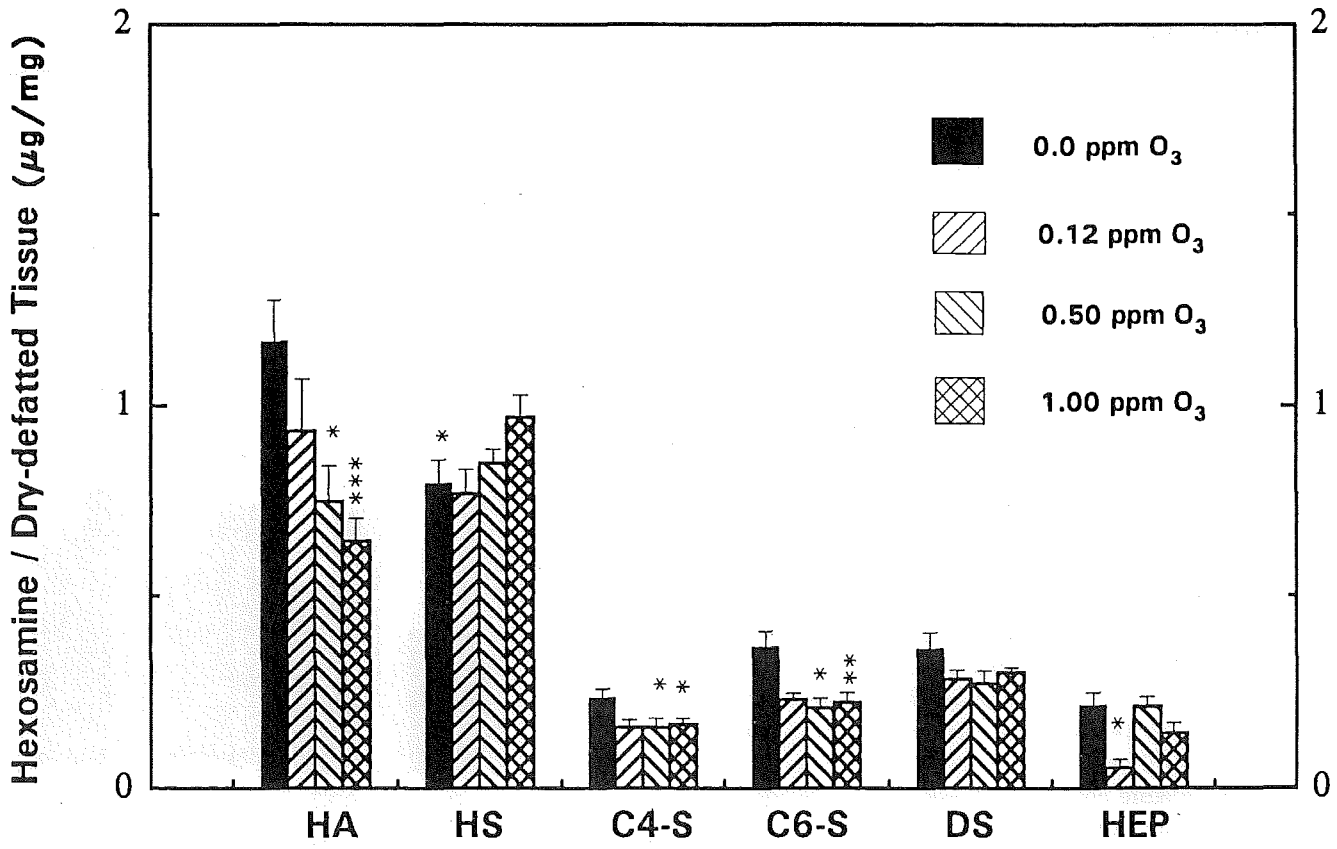

Figure 11. Concentration of individual GAGs from lung tissues of rats exposed to ozone. Fractionation of GAGs was done by HPLC as described in the Methods section. The results are expressed as micrograms of hexosamine per milligram of dry-defatted tissue. Concentrations of individual GAGs were calculated from the amount of hexosamine in pooled fractions under each peak and from the amount of uronate that was fractionated. The p values ($^{*} = p < 0.05$; $^{**} = p < 0.01$; $^{***} = p < 0.0001$; all two tailed) shown on ozone groups correspond to pairwise t tests that compare each ozone contration group to the control group. Control group, $n = 10$; exposure to 0.12 ppm ozone; $n = 4$; exposure to 0.5 ppm ozone, $n = 7$; exposure to 1.0 ppm ozone, $n = 10$.

samples indicated that the molecular size of hyaluronan was smaller in animals exposed to ozone (K_{av} = 0.24, where K_{av} is the fraction of gel volume available for diffusion of solute, and is proportional to log molecular weight) when compared with control animals (K_{av} = 0.18). When the chemical properties of heparan sulfate were examined, differences between animals exposed to ozone and control animals also were noted. These differences included *N*-sulfate content, iduronic acid content, and antithrombin III binding affinity. The concentrations of dermatan sulfate and heparin showed no significant differences between exposure groups. Overall, the observed decrease in total glycosaminoglycans at 0.5 and 1.0 ppm ozone can be accounted for by the decreases observed in individual glycosaminoglycans. In addition, structural changes were noted for hyaluronan, and changes in the biochemical properties were noted for heparan sulfate.

Antioxidant Enzyme Activity

A second component of the Pinkerton morphometric study (described in the Tracheobronchial and Centriacinar Morphometry section) evaluated three antioxidant, or phase II, enzymes: superoxide dismutase, glutathione peroxidase, and glutathione S–transferase in comparable locations within the respiratory tract (Pinkerton et al. 1995). The levels of activity of these enzymes was highly heterogeneous when comparing various locations in the respiratory system. Superoxide dismutase activity was elevated in the trachea, distal bronchiole, and centriacinar regions (Figure 12). Glutathione peroxidase activity was reduced in the major daughter pathway located along the axial airway path of the left lung lobe, but was increased in the portion of the airway tree that included the minor daughter bronchi. Glutathione S–transferase activity was unaffected by ozone exposure in this Project. As expected from previous studies, the airways of the centriacinar region were the most responsive to changes that resulted from continual oxidant stress, showing ozone-related elevations in the activities of superoxide dismutase and glutathione peroxidase.

RESPIRATORY TRACT FUNCTION

Short-term ozone exposure at concentrations of 0.08 to 0.6 ppm causes changes in pulmonary function and forced expiratory volume in one second in people (McDonnell et al. 1985; Seltzer et al. 1986; Drechsler-Parks et al. 1987; Hazucha 1987; Linn et al. 1988; Hazucha et al. 1989; Aris et al. 1993; Fox et al. 1993). Whether prolonged ozone exposure elicits similar effects in people is unknown. These findings from studies of short-term exposures provided the impetus for the functional studies conducted as part of the NTP/HEI Collaborative Ozone Project. In vivo and in vitro evalu-

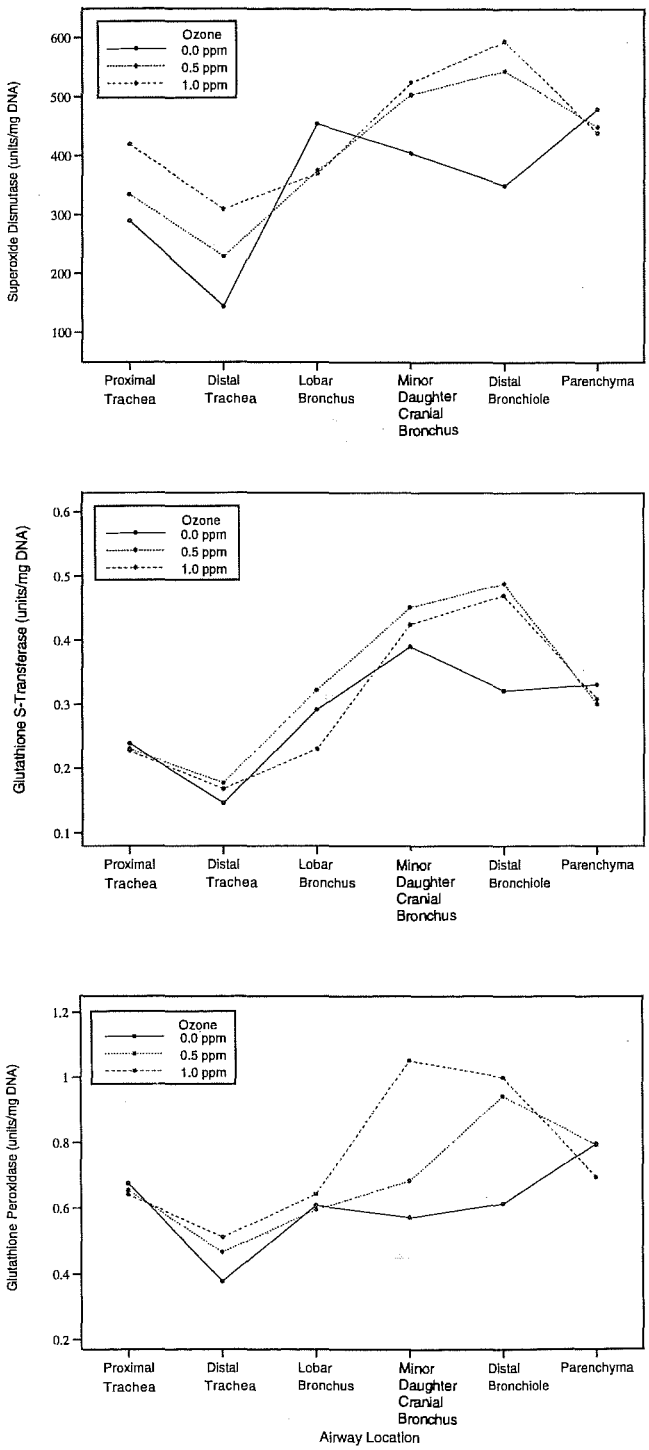

Figure 12. Antioxidant enzyme activity levels of the respiratory tract following 20 months of exposure to 0.0, 0.5, or 1.0 ppm ozone. Antioxidant enzyme activity is expressed in units per milligrams of DNA for SOD, GST, and GPx. Activity levels are shown for the proximal and distal trachea, lobar bronchus, minor daughter (cranial) bronchus, distal bronchiole, and lung parenchyma.

ations of lung and airway function were conducted in two parallel studies. The indices were chosen to determine the functional implications, if any, of the structural changes caused by ozone. In addition, the effects of prolonged ozone exposure on the nasal mucociliary apparatus were examined.

Nasal Mucociliary Apparatus

The functional component of this study involved identifying alterations in nasal mucous flow rates and patterns using video motion analysis and examining the luminal surface of the nasal airway using in vitro video microscopy (Harkema et al. 1994). All animals were killed one week after the last exposure; within 20 minutes of death, mucous flow rates throughout the nasal passages were determined on one half of the nasal apparatus. Immediately after video motion analysis, tissues were prepared for microscopy.

Rats exposed to 0.5 or 1.0 ppm ozone had markedly slower mucous flow rates over the lateral wall and turbinates of the proximal third of the nasal airway when compared with rats exposed to 0 or 0.12 ppm ozone (Figure 13). These areas were selected a priori as sites believed to be sensitive to ozone. No significant decreases in mucous flow rates were measured in rats exposed to 0.12 ppm ozone when compared with controls. However, mild increases in mucous flow rates were noted in some areas of the nasal airways in rats exposed to 0.12 ppm ozone when compared with controls. Together with structural findings, these results indicate that the structure and function of the nasal mucociliary apparatus is significantly altered in rats exposed for a prolonged time to 0.5 or 1.0 ppm ozone.

Lung

Pulmonary function parameters were evaluated in vivo one to six days after the end of exposure, using plethysmographic techniques (Harkema and Mauderly 1994). Measurements were made on live, anesthetized animals fitted with oral endotracheal and esophageal catheters. These measurements were made during the one-week period between the end of ozone exposure and when the animals were killed. More than 30 parameters were measured and calculated, including breathing pattern, dynamic lung mechanics, lung pressure and volume characteristics, intrapulmonary gas distribution, expiratory flow limitation, and alveolar-capillary gas transfer. Tissues from the same animals also were examined by other investigators for functional, structural, and biochemical endpoints.

The mean values and intersubject variability of measured endpoints were similar to those reported previously for similarly aged rats (Mauderly and Gillett 1992). The only consistent effect related to ozone was a small reduction of residual volume measured during slow lung deflation. This trend was observed in most exposed groups, but was only statistically significant (p = 0.03, two-tailed) in females exposed to 0.5 ppm ozone, where a 38% decrease was observed. Overall, ozone had little impact on pulmonary function in vivo.

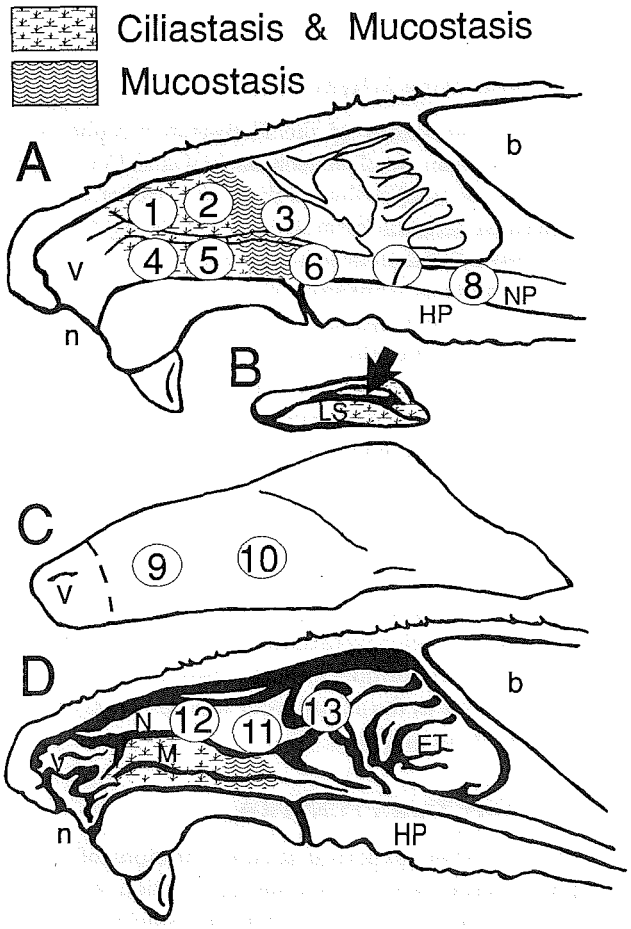

Figure 13. Diagram of rat nasal passages opened near the midline with the septum removed to reveal the turbinates. Areas of ciliastasis and mucostasis and areas of mucostasis only in the nasal mucosa after exposure to 0.5 or 1.0 ppm ozone are indicated. Numbers represent the areas that were analyzed 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. In all panels, V = vestibule; b = brain; HP = hard palate; n = naris; N = nasoturbinate; M = maxilloturbinate; ET = ethmoid turbinates; NP = nasopharynx.

Mechanical Properties of Airways and Contractile Responses

The pulmonary function studies described above did not include an assessment of the responsiveness of the airways to bronchoactive stimuli because of the possibility that exposure to bronchoconstrictive drugs would compromise the data in studies by other investigators. Instead, contractile properties of large (fourth generation) and small (eighth generation) bronchi were examined in vitro with several bronchoconstrictor stimuli (Szarek 1994). For comparative purposes, these airway generations correspond, respectively, to the central bronchus and the cranial and caudal bronchi described in the morphometric studies. Because ozone exposure may be involved in the remodeling of airways, smooth muscle areas were measured in each segment and tension responses normalized to these measurements.

Responsiveness of the airways to the contractile stimuli was described by the maximal response and the effective concentration or frequency that elicited a half-maximal contraction (EC_{50}). The effects of prolonged ozone exposure on structure or function of isolated large bronchi were unremarkable. Before the tension data were normalized to smooth muscle area, neither the EC_{50} nor the maximal response to the contractile stimuli obtained in the small bronchi was altered after prolonged ozone exposure. Smooth muscle areas were significantly larger in airways isolated from animals that had been exposed to 0.5 ppm ozone ($p = 0.004$, two-tailed), but not in those exposed to 0.12 or 1.0 ppm ozone (Figure 14). After accounting for smooth muscle area, the maximal stress (force/smooth muscle area) measured in the small bronchi isolated from male rats was reduced by up to 50% after exposure to 0.5 ppm ozone ($p \leq 0.001$, two-tailed). A comparable decrease in maximal stress was observed in airway segments after exposure to 0.12 ppm ozone as well. However, statistical significance ($p = 0.012$, two-tailed) was attained for only the contractile stimulus bethanecol. A similar trend was observed in airways isolated from female rats in these exposure groups, but none of the stimuli produced statistically significant results. Although some interesting trends were observed in these stress and tension measurements, no consistent concentration-response pattern was noted. Further research is needed to understand the changes observed in the animals exposed to 0.5 ppm ozone and whether, after near-lifetime exposure to ozone, smooth muscle cell contractile function is compromised.

Eicosanoids are a class of metabolites derived from arachidonic acid, formed through the cyclooxygenase pathway, that are important mediators of inflammation. Eicosanoids also seem to be important in affecting airway contractile responses to bronchoactive stimuli, and are likely candidates for causing both the airway alterations and the inflammatory response that can occur after inhaling oxidants such as ozone (Barnes 1986; Drazen and Austen 1987; Shore et al. 1989; Holtzman 1991). Therefore, one component of this study examined the release of two specific eicosanoids, prostaglandin E_2 and leukotriene C_4, from the caudal airway segments isolated from rats exposed to ozone.

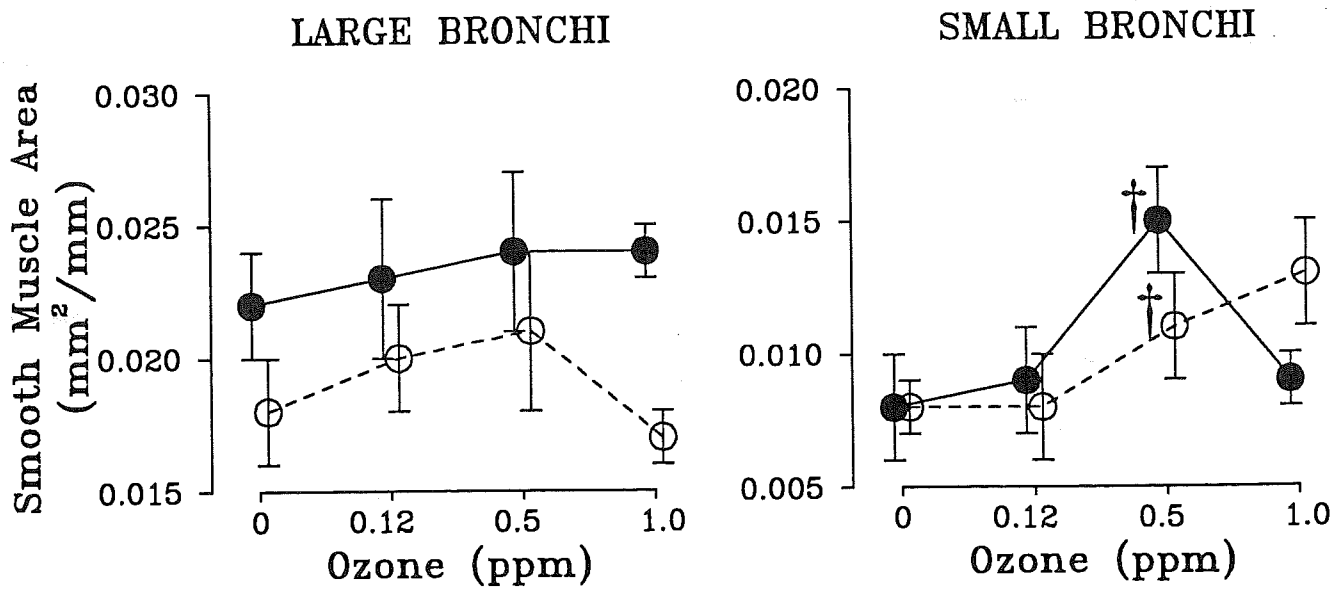

Figure 14. Smooth muscle area determined in large and small bronchi isolated from male (●) and female (○) rats after 20 months of exposure to ozone. Ozone had no effect in the large bronchi, but smooth muscle area was significantly increased after exposure to 0.5 ppm ozone ($p = 0.004$, two-tailed, comparing pooled data from male and female animals).

Basal and stimulated release of eicosanoids was measured in media surrounding airway segments using enzymoimmunoassays. Basal release of prostaglandin E_2 was unaffected by ozone exposure (Table 6). Incubation of the segments with the calcium ionophore A23187 increased the release of the prostaglandin; the percentage of increase in the release of prostaglandin E_2 over basal levels induced by A23187 was approximately two times greater in airway segments isolated from rats exposed to 1.0 ppm ozone than in those from control animals ($p = 0.005$, two-tailed); this was not true in the animals exposed to 0.12 or 0.5 ppm ozone. Basal release of leukotriene C_4 was below the limits of detection. Measurable amounts of the leukotriene were released during incubation with A23187; however, ozone was without effect on these levels. The results suggest that the cyclooxygenase pathway of the arachidonic acid cascade may be affected by prolonged ozone exposure. Which of the processes of prostaglandin production and release are affected remains to be determined.

INTEGRATION ACROSS STUDIES

This Project was designed to investigate the structural, functional, and biochemical effects of prolonged ozone exposure. Groups of investigators met throughout this Project to discuss how results from individual studies that measured diverse endpoints could be integrated to provide information about the overall effects of ozone on the respiratory system. As discussions progressed, it was agreed that the proposed analyses could be organized around a framework of four basic questions:

- What changes in nasal function, if any, are correlated with the structural changes in the nose?
- How are the structural, functional, and biochemical changes in the lung interrelated?
- How are biochemical changes correlated with structural and functional indices of lung stiffness?
- What is the relation between changes in the nose and changes in the rest of the respiratory tract?

These questions were addressed through three analytical approaches, as described in the next section.

ANALYTICAL APPROACHES

Because studies in this Project were conducted in the same set of animals after a common exposure protocol, this Project provided the opportunity for statistical integration of results beyond that which is ordinarily available when attempting to draw conclusions from a collection of interdisciplinary studies. For instance, it is possible in this Project to assess whether changes in a particular functional parameter occurred in the same animals that showed structural alterations. This is not possible in comparing most data sets because the relationships between exposure concentration and response are usually observed in different sets of animals treated with different protocols by different laboratories, which makes direct comparison of data impossible. In some of the analyses, we have exploited the unique features of this Project's design to gather and interpret the most information possible about a wide range of parameters. The sections that follow describe the results of analyses undertaken to address the four scientific questions outlined above. In the rest of this section, we provide details regarding the three statistical methods used to perform these integrative analyses.

Table 6. Release of Prostaglandin E_2 from Airway Segments[a]

	Ozone Concentration (ppm)							
	0.0		0.12		0.5		1.0	
	Male	Female	Male	Female	Male	Female	Male	Female
Basal[b]	3.5 ± 2.5	2.6 ± 0.9	3.9 ± 1.0	3.6 ± 0.5	2.7 ± 1.0	1.9 ± 0.5	2.7 ± 0.3	2.5 ± 1.2
A23187[c]	6.7 ± 4.3	6.4 ± 2.3	8.4 ± 2.4	8.8 ± 1.4	5.8 ± 2.4	5.5 ± 1.8	9.7 ± 2.0	7.3 ± 2.9
Percentage of Increase	112.5 ± 27.5	114.0 ± 27.6	114.3 ± 29.5	142.5 ± 3.5	88.0 ± 36.9	166.0 ± 42.7	244.2 ± 52.2[d]	222.0 ± 23.8[d]

[a] Values are given as means ± SEM in either nanograms of prostaglandin E_2 per milligram of protein or in percentages; $n = 2$ to 6 airway segments.

[b] The absolute amount of PGE_2 accumulated in 10 minutes in the medium surrounding the airway segments.

[c] The absolute amount of PGE_2 accumulated after 15 minutes of incubation with 5 μM A23187.

[d] Significantly different from 0.0 ppm ozone as determined by contrasts in the ANOVA.

Consistency Among Concentration-Response Relations

Although many endpoints were examined in each animal in this Project (Boorman et al. 1995), some endpoints that one would want to correlate could not be measured or compared in the same animals. This was because individual studies required measurement procedures that conflicted with procedures in other studies. For these sets of endpoints, concentration-response curves were compared qualitatively to determine consistency across the studies. Thus, the results from one study were used to help explain or corroborate findings in another study. This aspect of data analysis was strengthened by the fact that the animals used in these studies were exposed together and treated according to the same protocols.

Comparing dose-response curves is an effective way to evaluate any number of endpoints, but it has several limitations. Because it does not require an overlap in samples, it does not quantitatively measure the association between the endpoints being compared. Detailed knowledge of the biological relations and several mechanistic assumptions are required in order to draw conclusions from these comparisons. Furthermore, because no statistical power is gained from such a qualitative comparison, its sensitivity is limited by the sample sizes of the individual endpoints compared. Finally, it is difficult to interpret such comparisons when many endpoints are involved. Nevertheless, examining consistencies among sets of concentration-response relations is a common and useful method for comparing the major endpoints from different studies, and is, thus, a familiar technique for toxicologists.

Correlations Between Endpoints

In the studies in which endpoints of interest were measured on the same animals, direct pairwise correlation between the data measured in both studies for the same animal was used to evaluate the degree to which concurrent changes occurred in the animal. Pairwise correlation quantitatively assesses the interrelations between any two of the biochemical, structural, and functional measurements in the same animal, whereas inferences drawn solely from examining the concentration-response curves for a group of animals may not reflect changes occurring in the same animals. For the pairwise correlations, standard correlation coefficients were computed (Snedecor and Cochran 1989) on rankits of the data (Mosteller and Tukey 1977). One-tailed or two-tailed significance tests were performed depending upon whether the direction of change for an endpoint in response to ozone exposure had been established a priori. One-tailed tests were used for comparisons in which the direction of change for both endpoints had been assumed; two-tailed tests were used for comparisons in which the direction of change for one of the endpoints was unknown. Significance levels of $p = 0.025$ for one-tailed tests and $p = 0.05$ for two-tailed tests were used to assess all correlations. Although these are somewhat conservative guidelines, some caution still must be used when interpreting individual correlation coefficients.

Although it may not be as intuitive as examining the consistencies among concentration-response relations, pairwise correlation between endpoints is a standard statistical technique that directly compares the behavior of endpoints measured in the same animals. Because of the large number of measurements involved in this Project, the process requires that endpoints to be analyzed be selected carefully. Furthermore, this method provides insight beyond that of the usual concentration-response analysis because, for any pair of endpoints, information is obtained on whether changes in one endpoint are observed concurrently with changes in another endpoint. This level of analysis is usually unobtainable when comparing the effects observed in a series of studies because the measurements are not taken on the same animals. Thus, statistical strength is gained by examining responses using the pairwise correlation approach. On the other hand, this technique requires that both correlated endpoints be evaluated in the same animals, and is, therefore, not possible for all sets of endpoints.

Combined Analyses

A third approach was taken to integrate many endpoints related to a question and to evaluate the overall effects of ozone exposure. This form of analysis involved constructing composite scores, which allowed data to be combined across studies in which not all relevant endpoints had been measured on the same animals. An important feature of this Project was the opportunity to synthesize information across related outcomes to assess the concentration-response relation for ozone. Extensive discussions among investigators led to the plan for evaluating sets of endpoints that are potentially related to different pathogenic processes, and the measurements of the endpoints in response to different ozone exposure concentrations. The effects of ozone on the endpoints related to the various processes does not document that a change is clinically detectable, but instead may indicate that stages of the process are occurring. As mentioned previously, to avoid selection bias, the relation between changes in individual parameters and ozone exposure concentrations was not a factor in deciding which parameters to include in these analyses.

Composite scores, a statistical tool for analyzing information for multiple endpoints, were constructed for three

groups of endpoints clustered by their potential association with three pathogenic processes (chronic rhinitis, chronic airway disease, and centriacinar fibrosis). The scores were computed using a technique based on median polish analysis (Hoaglin et al. 1983) and further developed to address some of the specific features of the Collaborative Ozone Project data (e.g., missing data patterns and variability across endpoints). A separate report (Catalano et al. 1995) describes the analyses of the composite scores and compares the analysis method with other techniques used to analyze multiple outcomes.

Of the three methods of analysis that were used, the combined analysis approach offers the greatest statistical strength. Animal allocation in this Project purposely provided a high degree of overlap between each study endpoint and as many other study endpoints as possible (Boorman et al. 1995), allowing a more powerful analysis of combined endpoints. Although significant overlap across many endpoints was achieved, a large number of data were missing because not all animals could be evaluated for all endpoints. Thus, an integrative concentration-response analysis must be able to accommodate the pattern of missing data. In our approach to the combined analysis, any given pair of endpoints need not have been measured in the same animals, and any number of endpoints could be evaluated. Simulations performed by adding or removing specific endpoints show that the method is, indeed, a composite, or summary, of the different endpoints evaluated, and is not driven by inclusion or exclusion of any single variable (Catalano et al. 1995). Although applying this technique is a novel approach, it is robust, and can deal with the limited sample sizes for individual endpoints in this Project. Choice of endpoints for analysis was not straightforward; however, several careful and thoughtful discussions of endpoints among investigators and several outside experts led to a consensus on the parameters to be included.

Conclusions

Table 7 summarizes the features of these three analytical approaches, each of which provides different types and

Table 7. Comparison of Integrative Methods

Method	Strengths	Limitations
Consistency between concentration-response relations	Can be used to compare any two sets of animals Conventional method for comparing different endpoints from individual studies	Trends not necessarily in the same endpoints Qualitative rather than quantitative Sensitive to sample size for individual endpoints; less accurate with a small sample No measure of degree of association Inferences difficult to substantiate (require many scientific assumptions) Difficult to interpret across many endpoints
Correlation between endpoints	Quantitative rather than qualitative Simple interpretation Allows direct comparison of data within each animal Conventional method for examining correlations between pairs of endpoints	Requires that measurements to be compared are obtained from the same animal Compares pairs of endpoints, but not larger groupings Sensitive to the association between individual endpoints
Composite scores for combined analysis	Allows data to be integrated across many studies Quantifies overall assessment of effects Allows for missing data Does not require animal overlap Can accomodate an arbitrary number of endpoints Provides simple summaries for each animal Robust to individual endpoint variability	Choice of endpoints can be difficult Obscures specific endpoint detail Scores difficult to interpret Unconventional method

degrees of information, and allows different interpretations of the composite set of data. For instance, the correlation analyses and the assessment of concentration-response patterns compare particular endpoints and, therefore, specifically address individual measurements. The composite scores analysis, on the other hand, addresses the larger picture and evaluates the generalized effects, but obscures the details. In the following sections, these three analytical approaches are used to address the four scientific questions of interest.

WHAT CHANGES IN NASAL FUNCTION, IF ANY, ARE CORRELATED WITH THE STRUCTURAL CHANGES IN THE NOSE?

The nasal cavity is the first part of the respiratory system exposed to inhaled irritants and other pollutants. It has been described as an efficient "scrubbing tower" that removes inhaled chemicals that may harm the lower respiratory airways and gas-exchange regions of the lungs (Brain 1970). Although the nose is an efficient protector of the lower airways, its proximal location in the respiratory tract makes its mucous membranes vulnerable to injury from inhaled toxicants. Ozone has been shown to be irritating to the mucous membranes that line the noses of laboratory animals and humans. Inflammation (rhinitis) and other alterations to epithelial cells lining the nasal airways have been documented in rats (Harkema et al. 1989) and monkeys (Harkema et al. 1987a,b) after exposure to ozone. It has been reported that people who move into the southwest metropolitan region of Mexico City, where ambient concentrations of ozone often exceed the U.S. NAAQS, develop marked alterations of their nasal mucosa compared with residents of the nonpolluted port cities in Mexico (Calderon-Garcidueñas et al. 1992).

Consistency Among Concentration-Response Relations

Mucous flow rate is one index of mucociliary function, and the amount of mucosubstances within the nasal epithelium is a morphometric indicator of structural alteration. In this Project, these measurements were performed in three specific regions in the proximal nasal airway: the lateral wall, the septum, and the nasoturbinate. These sites were chosen on the basis of the results of previous studies of short-term ozone exposure with rats (Harkema et al. 1989; Hotchkiss et al. 1991).

In this Project, rats exposed to 0.5 or 1.0 ppm ozone showed significantly greater volume densities of intraepithelial mucosubstances and concomitantly slower mucous flow rates when compared with rats exposed to 0 or 0.12 ppm ozone. Thus, the effects of ozone on structure and function were related, and are consistent with the findings of an earlier study (Harkema et al. 1989). The transitional epithelium lining the proximal lateral wall is normally a nonciliated, simple, cuboidal epithelium with few or no mucous cells. After exposure to 0.5 or 1.0 ppm ozone, the transitional epithelium contained a marked mucous cell metaplasia characterized by numerous mucous cells with copious amounts of mucosubstances. Interestingly, the proximal septum and nasoturbinate had no structural or functional alterations induced by any of the ozone exposures. These regions are normally lined by a respiratory epithelium that contains numerous mucous cells. The reason for the insensitivity of these epithelial regions to ozone is unknown. Because airway mucus is known to be an effective antioxidant (Cross et al. 1984), the presence of mucosubstances in these regions may partially explain the absence of ozone-induced injury.

Correlations Between Endpoints

Table 8 presents the results of the analysis of correlations among structural and functional endpoints in the nose on the basis of what was measured in individual animals. As with the concentration-response results discussed above, these analyses indicate a relation between structure and function in the nose. As predicted, a statistically significant

Table 8. Correlation of Structural and Functional Parameters in the Nasal Apparatus

Functional Parameter	Structural Parameter	n	Correlation Coefficient	p[a]
Mucous flow rate in lateral wall	Amount of mucosubstances in lateral wall	40	–0.68	< 0.0001[b]
Mucous flow rate in septum	Amount of mucosubstances in septum	15	0.49	0.97
Mucous flow rate in medial aspect of nasoturbinate	Amount of mucosubstances in medial aspect of nasoturbinate	38	0.23	0.92

[a] Based on a one-tailed test of significance assuming a negative correlation between two parameters.

[b] Statistically significant correlation ($p \leq 0.025$).

($p < 0.0001$, one-tailed) negative correlation between the structural and functional parameters was observed in the lateral walls of the nose (which can be seen in Figure 13). No statistically significant correlation between mucous flow rate and the amount of mucosubstances in the septum or medial aspect of the nasoturbinate was revealed. Comparisons between mucous flow rate and cell types within the same animals were not possible because these parameters were measured on different sets of animals.

Combined Analysis: Endpoints Related to Chronic Rhinitis

Chronic rhinitis refers to a set of pathologic alterations in the nose including mucosal inflammation and proliferation of mucous cells, which results in an increase in intraepithelial and luminal mucus. Table 9 lists the endpoints used in this analysis, along with the reasons for their selection.

The observed changes that could be related to both ozone concentration and chronic rhinitis included mucosal inflammation accompanied by marked alterations of the surface epithelium that lines the nasal and paranasal cavities (for example, epithelial hyperplasia, and mucous cell metaplasia). In addition to these structural alterations in the nose, functional alterations in the nasal mucociliary apparatus (for example, decreased flow rate of the mucus overlying the mucosal surfaces) also were observed.

Figure 15 shows that the trends in analysis for endpoints related to chronic rhinitis are quite strong ($p < 0.0001$, two-tailed). The patterns are solidly maintained in both the male and female subsets ($p < 0.0001$, two-tailed, for each subset); the evidence is clear that the groups exposed to 0.5 ppm or 1.0 ppm ozone are significantly different ($p < 0.0001$, two-tailed) from control animals, but no significant difference was found between the control group and the group exposed to 0.12 ppm ozone.

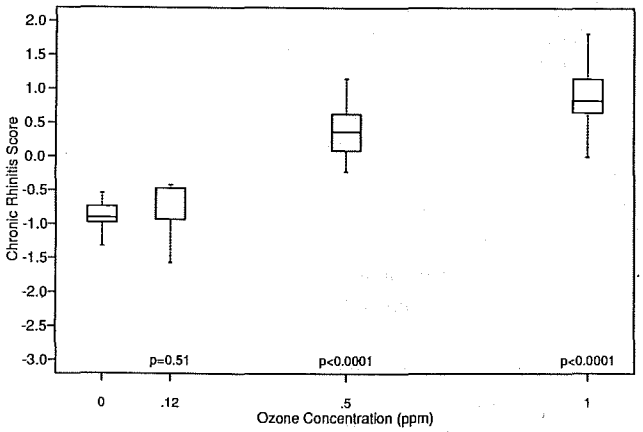

Figure 15. Combined analysis of chronic rhinitis in all animals. Significant concentration-response trend ($p < 0.0001$, two-tailed). p values given in the figure are for pairwise comparisons between the mean for each ozone concentration and the control value.

Table 9. Selected Endpoints Used in the Composite Score for Chronic Rhinitis

Endpoint (Investigator)	Region	Rationale and Comments	Predicted Change After Exposure
Structural			
Stored mucus (Harkema-Nasal Morphometry)	Lateral wall; proposed a priori as an important region	Key histologic feature of chronic rhinitis; correlated with mucous flow	Increase
Nasal secretory cell density (Harkema-Nasal Morphometry)	Lateral wall; proposed a priori as an important region	Cell types change from nonsecretory to secretory in chronic disease; measured as secretory:nonsecretory ratio	Increase
Functional			
Mucous flow rate (Harkema-Nasal Video Microscopy)	Lateral wall; proposed a priori as an important region	Endpoints to assess chronic rhinitis show impaired mucociliary clearance and increased mucous production; this may decrease clearance rates; mucostasis assigned the value of 0 for analysis	Decrease

The mucous cell metaplasia, along with the increase in intraepithelial mucosubstances and their antioxidant properties (Cross et al. 1984), is probably an adaptive mechanism of the rat designed to protect the underlying tissue from further oxidant injury. Therefore, more mucous cells that contribute increased amounts of mucus to the luminal surface could significantly reduce the direct or indirect toxic effects of ozone on airway tissue. However, rats exposed to 0.5 or 1.0 ppm ozone had decreased mucous flow rates, in spite of extensive mucous cell metaplasia in proximal nasal airways. Thus, the concomitant mucous cell metaplasia could not protect the rat from the functional alterations, and may have even contributed to this specific nasal dysfunction.

Marked decrease in mucous flow, as demonstrated in this Project, could lead to significant alterations in nasal mucociliary clearance, an important upper respiratory mechanism that defends the lung from excessive burdens of harmful agents. The nasal damage induced by prolonged ozone exposure also could be a factor in increasing the susceptibility of the upper respiratory tract to acute infections.

Conclusions

Based on these integrative analyses, rats exposed to 0.5 or 1.0 ppm ozone had the structural and functional hallmarks of chronic rhinitis, including chronic inflammation, mucous cell proliferation, increases in intraepithelial mucosubstances, and decreased levels of mucous flow. These alterations were focal, occurring largely in regions proposed a priori as the most sensitive areas. The occurrence of bony atrophy may indicate that some changes induced by ozone may be irreversible. No alterations were evident in rats exposed to 0.12 ppm ozone.

HOW ARE THE STRUCTURAL, FUNCTIONAL, AND BIOCHEMICAL CHANGES IN THE LUNG INTERRELATED?

Consistency Among Concentration-Response Relations

No significant structural changes were noted in the central airways of animals exposed to 0.12, 0.5, or 1.0 ppm ozone. Similarly, the functioning of large bronchi (corresponding to the central airway), assessed by contractile responses in vitro, was unaffected by exposure to ozone. Some statistically significant changes related to ozone exposure were noted in the smaller bronchi in the tracheobronchial airway. These changes included increased amounts of stored secretory product in the cranial and caudal bronchi of animals exposed to 1.0 ppm ozone, as well as trends in the amounts of stored secretory product in the trachea and caudal bronchus that were related to ozone concentration. Antioxidant enzyme activities for superoxide dismutase and glutathione peroxidase were elevated in the trachea (airways) after exposure to ozone. These activities of enzymes also were elevated in the centriacinar region. In addition, smooth muscle area was increased in small bronchi isolated from rats after exposure to 0.5 ppm ozone, but not from those exposed to 0.12 or 1.0 ppm ozone. Functional effects in isolated small bronchi (corresponding to the caudal and cranial airways) also were noted in the reduction in maximal stress by up to 50% after exposure to 0.5 ppm ozone, but not after exposure to 0.12 or 1.0 ppm ozone.

Hypertrophy and hyperplasia have been described in respiratory bronchioles of Bonnet monkeys after a 90-day exposure to 0.4 or 0.64 ppm ozone (Moffatt et al. 1987). Increased amounts of smooth muscle are a feature characteristic of asthmatic airways as well, yet information is insufficient concerning the regulation of airway smooth muscle growth under normal and pathophysiological conditions. Whether the increase in smooth muscle area demonstrated in the present Project represents hypertrophy or hyperplasia remains to be determined. It is probable, however, that growth factors released from epithelial cells or from resident lung cells mediated the increase in smooth muscle area after prolonged exposure to ozone. Although in the small bronchi the increase in the smooth muscle area could have resulted in greater tension in the muscle in response to a contractile stimulus, this was not observed.

In the proximal alveolar region, increases were seen in the volumes of the interstitium and epithelium in animals exposed to 0.5 or 1.0 ppm ozone. Epithelial thickening was characterized by a shift in the cell population from the normal squamous epithelium to a cuboidal epithelium that was similar, but not identical, to that found in terminal bronchioles. The epithelial cells were differentiated ciliated and nonciliated cells, and undifferentiated cuboidal cells found only in the animals exposed to 0.5 or 1.0 ppm ozone. A mild fibrotic response, with increases in both noncellular and cellular components in the interstitium, was seen in the animals exposed to 1.0 ppm ozone. Individual components of the interstitial matrix, including collagen, elastin, basement membrane, and acellular spaces, all were increased after exposure to 1.0 ppm ozone (see the Tracheobronchial and Centriacinar Morphometry section under Respiratory Tract Structure). The increase in cellular interstitium was due to an increase in the volume of interstitial fibroblasts. A slight inflammatory response, including an increase in alveolar macrophages, was observed in

the animals exposed to 1.0 ppm ozone. The terminal bronchioles were less affected than the proximal alveolar regions by the ozone exposure, which may indicate that this tissue either is resistant to ozone damage or absorbed a lower dose of ozone. Changes in the terminal bronchioles consisted mainly of a shift in the type of cell from ciliated to nonciliated cells in the animals exposed to 1.0 ppm ozone. The bronchial epithelial metaplasia observed in the proximal alveolar ducts may indicate that a protective mechanism (tolerance) developed in response to the prolonged exposure to 1.0 ppm ozone.

The pulmonary function parameters showed little or no change in response to ozone exposure (see the Lung section under Respiratory Tract Function). The only exception to this was a small reduction observed in residual lung volume measured during slow lung deflation in animals exposed to 0.5 ppm ozone. Thus, it appears that the structural and biochemical changes in the small bronchi and the proximal alveolar region were not extensive enough to affect basic pulmonary function.

Correlations Between Endpoints

Intraepithelial mucosubstances present within the epithelial cells of the tracheobronchial tree are one source of the mucous layer that lines the conducting airways. Alterations in the amount of stored product, or shifts in the distribution or numbers of cells making these materials could reflect significant or marked biological changes occurring in the respiratory tract in response to prolonged exposure to ozone. To determine if such changes did occur, studies were done to examine the amounts of stored intraepithelial secretory product and the distribution of cell populations in bronchi of different sizes, locations, and generation number within the left lung lobe. Table 10 examines the relation between the percentage of nonciliated cells and stored mucus in the trachea, and the central, caudal, and cranial bronchi. Statistically significant associations were observed in the caudal and cranial regions where a positive relation was observed ($p = 0.020$, one-tailed, and $p = 0.023$, one tailed, respectively). No relation was found, however, between the percentage of nonciliated cells and the stored mucus in the trachea or central bronchi. Note that the cranial and caudal regions were the two regions that also showed significant changes in the amount of stored secretory product in response to exposure to 1.0 ppm ozone. These findings indicate that the distal airways are the site of impact from ozone exposure. Other changes seen include an increase in the nonciliated cell population and an increase in stored mucus; these changes were very specific to small, distal airways.

Parameters assessing the airway caliber in the lungs also were examined. In general, the level of resistance to airflow is inversely proportional to the fourth or fifth power of the radius of the airway lumen; a decrease in the radius of the lumen by one-half results in a minimum of a 16-fold increase in resistance. A decrease in radius of the lumen could occur through several mechanisms including mucous plugging, increased wall thickness, which could result from edema or an increase in deposition of connective tissue components (such as collagen and elastin), or contraction of airway smooth muscle. Pulmonary function parameters useful in assessing the resistance to airflow include total pulmonary resistance and indices extrapolated from the maximal expiratory flow–volume curve. Although neither of these in vivo parameters was significantly affected by ozone exposure, animal-by-animal correlations between parameters of airway caliber and airway wall structure were calculated to see if the sensitivity of this analysis could be

Table 10. Correlation of Functional and Structural Parameters in the Lower Respiratory Tract: Cell Type and Stored Mucus

Structural Parameter	Functional Parameter	n	Correlation Coefficient	p Value[a]
Percentage of nonciliated cells in the trachea	Mucus stored in the trachea	31	−0.03	0.56
Percentage of nonciliated cells in the central bronchus	Mucus stored in the central bronchus	31	−0.10	0.70
Percentage of nonciliated cells in the caudal bronchus	Mucus stored in the caudal bronchus	31	0.37	0.020[b]
Percentage of nonciliated cells in the cranial bronchus	Mucus stored in the cranial bronchus	32	0.35	0.023[b]

[a] Based on a one-tailed test of significance assuming a positive correlation.

[b] Statistically significant correlation ($p \leq 0.025$).

increased. Wall area measurements were obtained in airway segments representative of the conducting airways. Thus, correlation between total pulmonary resistance and wall area was evaluated.

Table 11 shows the results of the correlation analyses between total pulmonary resistance with airway wall area and airway radius. None of these correlations was statistically significant.

Combined Analysis: Endpoints Related to Chronic Airway Disease

Airway disease refers broadly to a set of conditions that could impede airflow to and from the lungs by one or several mechanisms, alone or in combination. These conditions include smooth muscle cell contraction, narrowing of the lumen caused by thickening of the airway wall, obstruction of the airways caused by secretions or fibrous obliteration, mechanistic uncoupling of airways from parenchyma due to peribronchial inflammation, altered capacity of the airway to collapse, and an increased surface tension of the liquid lining the airways. Several structural and functional parameters were included in this analysis that relate to these various pathogenic mechanisms. Some of these parameters were measured in the centriacinar region, and others in the conducting airways. The endpoints used to construct the composite score for chronic airway disease are presented in Table 12 (see Catalano et al. 1995 for further details).

Figures 16 and 17 show the analysis for the airway disease composite score. A moderate overall trend is apparent (p = 0.014, two-tailed), which remains in the analysis of the male (p = 0.033, two-tailed), but not the female (p = 0.16, two-tailed), subset. Further subset analyses are presented by Catalano and associates (1995).

Conclusions

On the basis of these integrative analyses, the rats involved in this Project exhibited some of the structural hallmarks of chronic airway disease. However, other features of chronic airway disease, such as chronic inflammation and changes in the in vivo functional measurements, were not observed. Most alterations were limited to the small airways and gas-exchange region. In the small airways, increased amounts of stored secretory product and secretory cells were evident in rats exposed to 0.5 or 1.0 ppm ozone, but not in rats exposed to 0.12 ppm ozone. These increases in secretory product and cells were correlated to each other when examined within each animal. Also in the small airways, maximal stress was reduced and wall area was increased in male rats, but only at the exposure level of 0.5 ppm ozone. Activities of the antioxidant enzymes superoxide dismutase and glutathione peroxidase were elevated in the bronchus and trachea. In the gas exchange region, epithelial thickening was seen in rats exposed to 0.5 or 1.0 ppm ozone. The amount of interstitial matrix also was increased at these ozone exposure concentrations, with changes evident in collagen, elastin, basement membrane, and acellular space. A slight inflammatory response was noted in rats exposed to 1.0 ppm ozone. Bronchiolarization occurred in the centriacinar region, and the extent of bronchiolarization correlated with the concentration of ozone exposure. Antioxidant enzyme activities also were elevated in the centriacinar region. When all parameters that could be associated with chronic airway disease were examined through a combined statistical analysis, a mild trend (p = 0.014, two-tailed) toward increasing chronic airway disease was found with increasing ozone exposure concentration.

Thus, it appears that prolonged exposure to increasing concentrations of ozone may stimulate some possible manifestations of chronic airway disease; this conclusion is probably driven by the changes that occurred in male rats. Some structural changes could have resulted in encroachment upon the lumina, especially those of the small bronchi and the centriacinar region of the lung. Although observed trends are suggestive of chronic airway disease, no changes were seen in the in vivo parameters of airway patency after prolonged ozone exposure. Thus, the structural changes that were observed in the small airways and centriacinar

Table 11. Correlation of Functional and Structural Parameters in the Lung: Airway Caliber

Functional Parameter	Structural Parameter	n	Correlation Coefficient	p Value [a]
Total pulmonary resistance	Wall area of small airway	20	0.31	0.09
	Wall area of large airway	20	0.13	0.29
	Small airway (radius)$^{-4}$	19	0.09	0.65
	Large airway (radius)$^{-4}$	20	0.08	0.64

[a] Based on a one-tailed test of significance ($p \leq 0.025$) assuming a positive correlation.

Table 12. Selected Endpoints Used in the Composite Score for Chronic Airway Disease

Endpoint (Investigator)	Region	Rationale and Comments	Predicted Change After Exposure
Structure			
Airway cell populations (Chang)	Centriacinar	Ciliated cells changing to nonciliated (Clara) cells is characteristic of airway disease; expressed as ratio of Clara cell density to ciliated cell density	Increase
Airway epithelial cells in the bronchi (Pinkerton)	Three bronchi: cranial (small diameter, short path), central (large diameter, short path), cranial (small diameter, long path)	Airway epithelial cell populations reflect chronic disease process; ciliated cells change to nonciliated cells	Increase
Percentage of bronchiolarization (Chang)	Proximal alveolar	Histologic indicator of chronic airway disease; bronchiolarization is expected to alter airflow rate	Increase
Distance of bronchiolarization (Pinkerton)	Centriacinar	Histologic indicator of chronic airway disease; bronchiolarization is expected to alter airflow rate	Increase
Epithelial volume (Pinkerton)	Data for both cranial and caudal regions in each animal were averaged	Potential alteration due to airflow changes; expressed as estimated initial epithelial volume, or estimated distance down bronchiolar path at which the epithelial volume tended to become asymptotic	Increase
Airway smooth muscle (Szarek)	Distal (both cranial and caudal), and proximal (large) conducting airways	Thickening of the airway wall encroaches on the lumen and obstructs airflow; can increase with asthma, have no change with emphysema, and either increase or have no change with chronic bronchitis	Increase
Mucus stored in the lung (Pinkerton)	Caudal and cranial conducting airways; two fifth-generation bronchi; one eleventh-generation bronchus	Important constituent of epithelial cells; key histologic feature of chronic airway disease; mucus in the lung is thought to contribute to decreased airway function	Increase
Biochemistry			
Basal release of prostaglandin E_2 (Szarek)	Distal conducting airways	Implicated in a number of airway diseases (asthma, chronic bronchitis); reflects inflammation in airways; inhibits smooth muscle cell proliferation and contraction	Increase

(Table continues next page.)

Table 12. Selected Endpoints Used in the Composite Score for Chronic Airway Disease *(continued)*

Endpoint (Investigator)	Region	Rationale and Comments	Predicted Change After Exposure
Function			
Mean midexpiratory air flow (Harkema-Pulmonary Function)	Centriacinar region, or conducting airways	In vivo functional measurement of small airways; reflects how well animal can expel air from the lungs; obstruction of the airways would result in a decrease in the mean midexpiratory flow; can increase, decrease, or have no change with increasing airway disease, depending on the specific disease	Decrease
Airway responsiveness (Szarek)	Large (intrapulmonary bronchus) and small (branch) conducting airways	Changes in airway responsiveness are associated with some airway diseases (in asthma, "twitchy airways"); averaged values across four agonists; evaluated change in maximal tension values and EC_{50}	Increase in maximal tension; decrease in EC_{50}

region are most likely responsible for the observed trend towards chronic airway disease found in the combined analysis. The finding that no changes were apparent in the basic pulmonary function parameters, as assessed in anethesized rats, does not preclude the possibility that functional changes might have been observed had other techniques been used that do not require anesthesia. Furthermore, although basic pulmonary function was unaffected under the conditions of this Project, effects might have been observed if the animals had been physically stressed; this could have occurred during infection or with exercising conditions, which also would have increased the dose of ozone.

HOW ARE BIOCHEMICAL CHANGES CORRELATED WITH STRUCTURAL AND FUNCTIONAL INDICES OF LUNG STIFFNESS?

Consistency Among Concentration-Response Relations

The centriacinar region was particularly sensitive to the effects of ozone, demonstrated by a statistically significant change observed in the percentage of bronchiolarization of the centriacinar region of animals exposed to 0.12, 0.5, or 1.0 ppm ozone. Bronchiolarization was most evident in regions closest to the bronchiole–alveolar duct junction, and the ability to observe subtle changes at the exposure level of 0.12 ppm ozone depended on the location from which samples were taken (see Chang et al. 1995; Pinkerton et al. 1995). Evidence of mild centriacinar fibrosis was observed in the terminal bronchiole–alveolar duct region. These fibrotic changes presumably resulted from a net increase in total collagen, and perhaps from a shift in the balance of collagen types. Residual lung volume was reduced after ozone exposure, reaching statistical significance only in the group exposed to 0.5 ppm ozone.

The residual lung volume is the volume of air that is retained within the lung after maximal expiration. In anesthetized rats, residual volume is thought to be affected primarily by airways collapsing; thus, the gas remaining in the lung at that point would be gas trapped distally to the location of the airway closure (Leith 1976; Gillespie 1983). Because cartilage and wall thickness can prevent airway collapse in the large conducting airways, collapse is assumed to occur in regions where cartilage is absent and the airway wall is very thin, such as the terminal bronchiole–alveolar duct region. These regions have smaller diameters and, thus, can be more easily occluded. It could be hypothesized that structural changes occurring in these regions would stiffen the terminal airway walls and delay collapse during deflation. Centriacinar fibrosis was observed in the terminal bronchiole–alveolar duct region, which would act to stiffen the wall. The fact that a suggestion of decrease in residual volume was observed after ozone exposure supports the hypothesis that an exposure-related stiffening of the terminal bronchiole–alveolar duct region allowed the lungs to deflate to the slightly greater degree of deflation observed in this study. The correlation between these endpoints in the same animals was not possible because very few animals ($n = 7$ for all exposure groups combined) were exam-

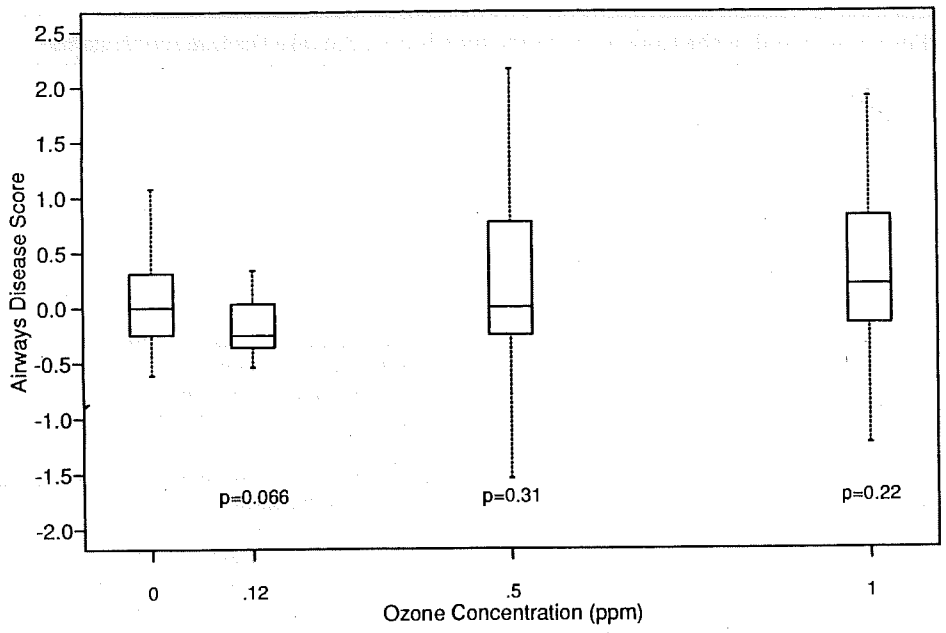

Figure 16. Combined analysis of airway disease in all animals. Significant concentration-response trend ($p = 0.014$, two-tailed). p values given in the figure are for pairwise comparisons between the mean for each ozone concentration and the control value.

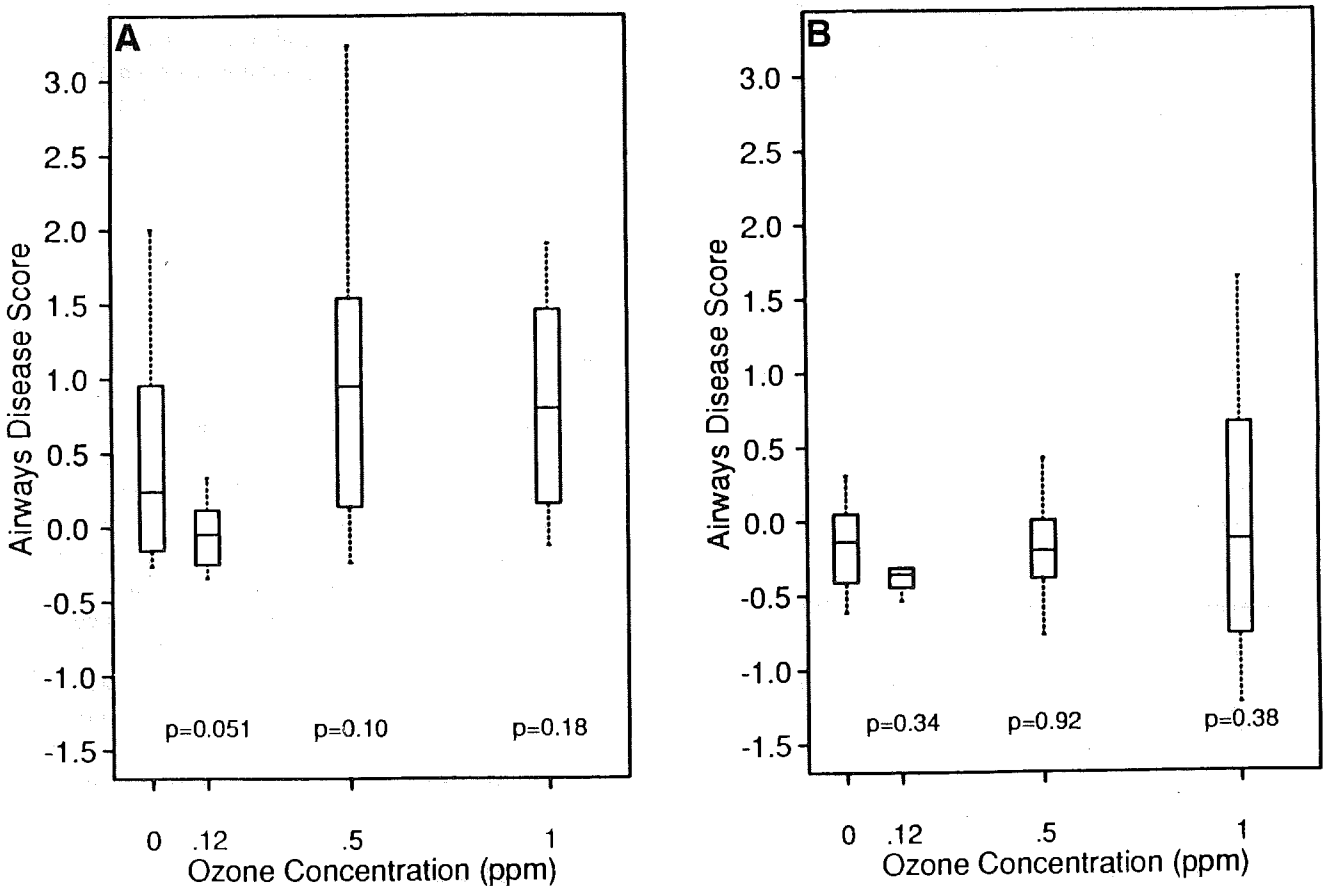

Figure 17. Combined analysis of airway disease by gender. A: Male data, significant concentration-response trend ($p = 0.033$, two-tailed). B: Female data, concentration-response trend ($p = 0.16$, two-tailed). p values given in the figures are for pairwise comparisons between the mean for each ozone concentration and the control value.

ined for both pulmonary function and morphometric features.

Correlations Between Endpoints

To explore the relation between reduced functional parameters and fibrosis in the centriacinar region, correlations were examined between functional measurements (residual volume, dynamic compliance, and quasistatic chord compliance) and the level of 4-hydroxyproline (lung collagen content) (Table 13). Dynamic compliance and quasistatic chord compliance were examined as indices of lung elasticity, because fibrotic changes in the lung decrease the lung elasticity and result in a decrease in compliance. Furthermore, because dermatan sulfate is associated with collagen, and chondroitin 6-sulfate and heparan sulfate are found in extracellular matrix, these measurements were correlated with collagen data as well. A significant association was found between quasistatic chord compliance and 4-hydroxyproline ($p = 0.006$, one-tailed). No significant correlations were found among the other parameters mentioned above, including the glycosaminoglycans and collagen data. However, because sample sizes are small for these comparisons, statistical power was somewhat limited for assessing these direct animal-to-animal correlations.

Structural and biochemical results show that a number of differences occurred in the response of the respiratory epithelium to prolonged oxidant stress. The amount of stored secretory product and the antioxidant enzymes that might provide protection appeared to be altered. Epithelial reorganization in favor of less sensitive cell types (nonciliated cells) was the predominant response of the epithelium throughout the conducting airways. This response was specific to the site within the airway, and included reorganization in conducting airways as well as in the bronchiolarized epithelium of alveolar ducts. The primary target site, the central acinus, where modeling studies have indicated that some of the highest local concentrations of ozone would be expected (Overton et al. 1987; Mercer et al. 1991), had the greatest degree of epithelial change and exhibited the most elevated response in terms of increased antioxidant enzymes.

Correlations of mucosubstances, epithelial thickening, and indices of cell restructuring in the lung were examined in the same animal, whereas biochemical and morphometric endpoints could not be correlated because they were measured in different animals. Table 13 also presents the results of correlation analyses in which the percentage of bronchiolarization and the extent of bronchiolarization are compared with epithelial volume in the cranial and caudal regions. Although the correlation of the percentage of bronchiolarization with epithelial volume was not statistically significant, the correlation of the extent of bronchiolariza-

Table 13. Correlation of Biochemical, Functional, and Structural Parameters in the Lung

Parameter 1	Parameter 2	n	Correlation Coefficient	p Value[a]
4-Hydroxyproline (per lung lobe)	Residual volume	29	0.02	0.47
	Dynamic lung compliance	29	0.17	0.19
	Quasistatic chord compliance	29	0.46	0.006[b]
	Dermatan sulfate	12	−0.26	0.41[c]
	Chondroitin 6-sulfate	12	−0.38	0.23[c]
	Heparan sulfate	12	0.06	0.86[c]
Percentage of bronchiolarization	Epithelial volume in the cranial bronchi	25	0.29	0.08
	Epithelial volume in the caudal bronchi	24	0.35	0.05
Extent of bronchiolarization	Epithelial volume in the cranial bronchi	27	0.45	0.022[b]
	Epithelial volume in the caudal bronchi	27	0.61	0.0014[b]

[a] Based on a one-tailed test of significance assuming a positive correlation, unless otherwise indicated.

[b] Statistically significant correlation ($p \leq 0.025$ for one-tailed test, $p \leq 0.05$ for two-tailed test).

[c] Based on a two-tailed test of significance.

tion and epithelial volume in the cranial and caudal regions was (p = 0.022, one-tailed, and p = 0.0014, one tailed, respectively).

Combined Analysis: Endpoints Related to Centriacinar Fibrosis

Fibrosis is a general condition in which connective tissue becomes thickened and stiffer by excessive accumulation of fibrous material. Because this Project included measurements of a variety of outcomes related to fibrotic development in the centriacinar region of the lung, a composite score representing "centriacinar fibrosis" was constructed.

Centriacinar fibrosis is defined as an increase in some of the interstitial components of the alveoli of the most proximal portions of the pulmonary acinus. Changes typically include a diffuse thickening of the alveolar walls and remodeling of the bronchiole–alveolar duct junction. Because the bronchiole–alveolar duct junction is critical as the opening from the conducting airways to the lung parenchymal region, any changes here tend to be more meaningful. All air must pass through this orifice before gas exchange can occur. Therefore, alterations in this region may impact both air flow and gas exchange in the lungs.

The parameters used in this analysis and the rationale for their selection are presented in Table 14. Although other endpoints such as glycosaminoglycans also were considered to be potentially related to centriacinar fibrosis, they were ultimately excluded because sufficient justification for their inclusion in the analysis could not be found. A potential association of glycosaminoglycans with collagen was considered to be insufficient rationale when no significant correlation between glycosaminoglycans and hydroxyproline could be found in the within-animal correlation analysis (see the Correlations Between Endpoints section). An alternative analysis, including glycosaminoglycans, and an analysis of a subset that excluded leukemic animals can be found in Catalano and colleagues (1995).

Figure 18 is a plot of the results of the combined analysis of endpoints potentially related to centriacinar fibrosis. Using a standard t test, pairwise comparisons of exposure to 0.5 and 1.0 ppm ozone suggest that control and exposed animals were significantly different. Using linear regression analysis, the concentration-response results for the composite score for centriacinar fibrosis show a significant trend with increasing ozone exposure (p = 0.006, two-tailed). This analysis is quite sensitive to the inclusion or exclusion of the glycosaminoglycan endpoints; if two such endpoints are included, the trend is no longer statistically significant (p = 0.091, two-tailed). The main reason for this sensitivity is that several individual glycosaminoglycans decreased in concentration with increasing ozone exposure. This response is contrary to the predicted increase in the concentration of glycosaminoglycans. A subset analysis by gender (Figure 19) shows that the overall trend of the concentration response for female animals is statistically significant (p = 0.006, two-tailed). The female pairwise comparisons for 0.5 and 1.0 ppm ozone also show statistically significant increases (p = 0.034 and p = 0.025,both two-tailed, respectively), whereas the male data exhibit no significant trend or pairwise differences. This gender pattern also was observed in some individual Project data as well, most notably in whole lung collagen content and collagen cross-links, in which a significant increase in collagen content was noted only in female animals. This combined analysis of centriacinar fibrosis also is fairly sensitive to the way collagen content is defined. For instance, an earlier analysis suggested a much stronger trend than the current analysis presents, mostly because collagen in the first analysis had been standardized by body weight rather than lung lobe weight; hence, it was confounded by ozone's effects on total body weight. That preliminary analysis also used different individual glycosaminoglycans to represent centriacinar fibrosis (Louise M. Ryan, personal communication).

Conclusions

Taken together, these analyses suggest that animals exposed to ozone showed structural, functional, and biochemical indicators of centriacinar fibrosis. These correlations are driven by the results from the exposures to 0.5 and 1.0 ppm ozone. Results for animals exposed to 0.12 ppm ozone are, in general, not significant. The process of bronchiolarization may be responsible for some or all of the biochemical and functional changes observed.

WHAT IS THE RELATION BETWEEN CHANGES IN THE NOSE AND CHANGES IN THE REST OF THE RESPIRATORY TRACT?

Mucosubstances within the epithelial cells of the nasal cavity and tracheobronchial tree are a primary source of the mucus that forms the lining layer for the conducting airways. As discussed earlier, repeated exposure to ambient levels of ozone have caused mucous cell metaplasia with increased amounts of intraepithelial mucosubstances in the nasal airways of laboratory animals (Harkema et al. 1987a,b, 1989; see the Nasal Morphometry section under Respiratory Tract Structure). Alterations in the amount of stored product or shifts in the distribution or numbers of cells making the mucosubstances could indicate significant biological changes occurring in the respiratory tract related to prolonged exposure to ozone. Because most of the rat airway epithelium normally contains only scant amounts of stored mucosubstances, significant increases in the volume densi-

Table 14. Selected Endpoints Used in the Composite Score for Centriacinar Fibrosis

Endpoint (Investigator)	Region	Rationale and Comments	Predicted Change After Exposure
Structure			
Interstitial volume (Pinkerton)	Centriacinar; entire ventilatory unit (pulmonary acinus); data for both cranial and caudal regions in each animal were averaged	Reflects centriacinar fibrosis because endothelial damage may precede the fibrosis	Increase
Collagen volume (Chang)	Centriacinar	Increase in fibrillar collagen is a classic aspect of fibrosis; provides a measure of collagen specific to location; with localized accumulation, this endpoint (measured at the point of localized damage) is a more sensitive measure of fibrotic response because it measures a focal change	Increase
Fibroblast volume (Chang)	Centriacinar	Predominant cell making the precursor of collagen; volumes of collagen and fibroblasts in the interstitium are important indicators of fibrosis	Increase
Interstitial macrophage volume (Chang)	Centriacinar	In progressive fibrotic lesions, these cells are expected to be found in the interstitium because they control fibroblast function; may suggest an active ongoing process that stimulates fibroblasts to continue synthesizing and depositing collagen precursors	Increase
Biochemistry			
Hydroxyproline content of the lung (per lobe) (Last)	Measured in whole lobe as global measure; not specific for a site within the lung	Measure of total lung collagen, possibly reflecting excessive fibrous accumulation in the lungs; an increase in lung collagen is a primary feature of fibrosis	Increase
Function			
Residual volume (Harkema-Pulmonary Function)	Whole lung, but affected by changes in the centriacinus	Set by the collapse of small airways during maximal exhalation; can decrease or increase by changes that make the small, noncartilaginous airways more or less stiff (resistant to collapse)	Decrease

(Table continues next page.)

Table 14. Selected Endpoints Used in the Composite Score for Centriacinar Fibrosis *(continued)*

Endpoint (Investigator)	Region	Rationale and Comments	Predicted Change After Exposure
Quasistatic chord compliance (Harkema-Pulmonary Function)	Whole lung	Indicates lung elasticity in the range of volume for tidal breathing; reflects general elasticity or stiffness of parenchyma	Decrease
Carbon monoxide diffusing capacity (Harkema-Pulmonary Function)	Whole lung	Indicates ease of gas diffusion across the alveolar-capillary membrane; reflects changes in the membrane thickness or surface area	Decrease

ties of stored mucosubstances have been used as a quantitative index of the magnitude of mucous cell metaplasia induced by ozone exposure.

Consistency Among Concentration-Response Relations

To determine if exposure to ozone had changed the amounts of stored intraepithelial secretory product, its volume was measured in the nasal cavity, trachea, and three different bronchi of different size, location, and generation number within the left lung lobe: the central, cranial, and caudal bronchi. Figure 20 shows levels of secretory product as a function of distance down the respiratory tract. After exposure to 1.0 ppm ozone, statistically significant increases in the levels of secretory product were noted in the nasal cavity, caudal bronchi, and cranial bronchi, and a statistically significant decrease in the level of secretory product was noted in the trachea.

The nose was particularly sensitive to structural and functional changes after exposure to ozone. In addition to increased levels of mucosubstances, rats exposed to 0.5 or 1.0 ppm ozone had significantly slower mucous flow rates compared with rats exposed to 0 or 0.12 ppm ozone. A marked mucous cell metaplasia in the transitional epithelium was noted in animals exposed to 0.5 or 1.0 ppm ozone. Bony atrophy also was observed in rats exposed to 0.5 or 1.0 ppm ozone. In contrast, structural changes in the lung were restricted to focal locations, and appeared to have only minor functional consequences.

Correlations Between Endpoints

Pairwise analyses were conducted to determine the correlation between mucous volume in each of the four nasal locations (proximal lateral meatus, distal lateral meatus, maxillary sinus, and nasopharyngeal meatus) and lung mucosubstances in each bronchus. Although significant correlations were not observed between the amounts of nasal mucosubstances and central bronchus mucus (mostly due to large variability in the values for central bronchus mucus), substantial correlation (and clear dependency on ozone concentration) was observed between the levels of nasal mucus and both caudal and cranial lung mucosubstances (Table 15).

Conclusions

Animals exposed to 0.5 or 1.0 ppm ozone showed alterations in the amount of mucosubstances in the nose and lung. Mucosubstances increased in nasal regions and in the caudal and cranial bronchi of the lung, and decreased in the trachea. The amount of mucosubstances in the caudal and cranial bronchi correlated with the amount of mucosubstances in the epithelium of the proximal and distal lateral meatus of the nose within each animal.

SUMMARY AND DISCUSSION

INTERPRETATION OF THE RESULTS FOR F344/N RATS

The results of these studies indicate that statistically significant changes were associated with ozone exposure in rats exposed to ozone for 20 months. Most of these changes occurred at ozone concentrations of 0.5 and 1.0 ppm. The changes related to ozone were restricted to the following regions: the nose, small airways, and the bronchiole–alveolar duct junction. One significant effect of 0.12 ppm ozone was found only at the specific respiratory region believed to be the most sensitive, the centriacinar region extending approximately 300 μm beyond the bronchiolar–alveolar duct junction (see Pinkerton et al. 1995). Areas further from this junction showed alterations only at higher concentrations of ozone (see Chang et al. 1995; Pinkerton et al. 1995). In general, very few changes of a functional or biochemical nature were seen in whole-lung or whole-lobe measurements.

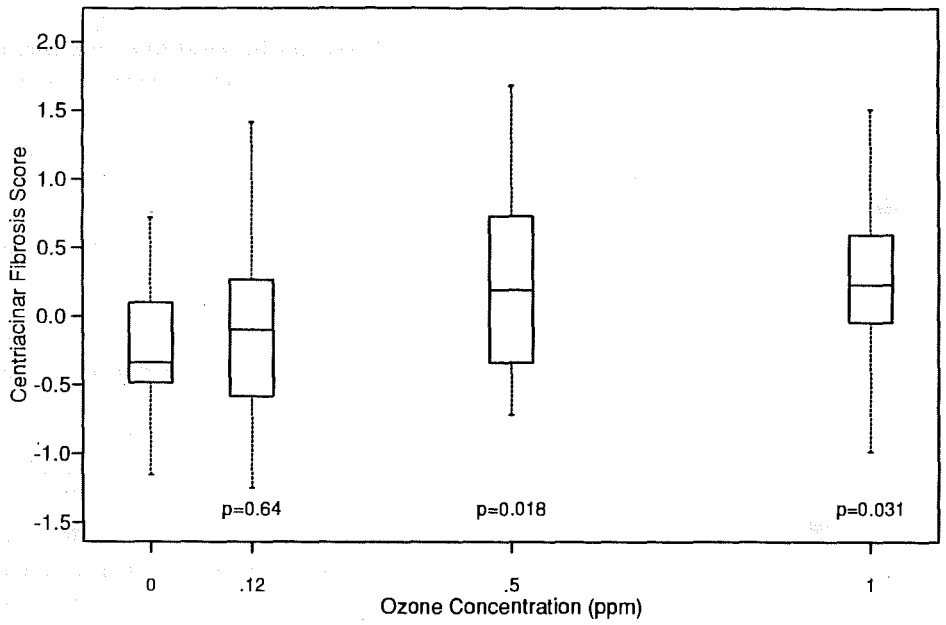

Figure 18. Combined analysis of centriacinar fibrosis in all animals. Significant concentration-response trend ($p = 0.0060$, two-tailed). p values given in the figure are for pairwise comparisons between the mean for each ozone concentration and the control value.

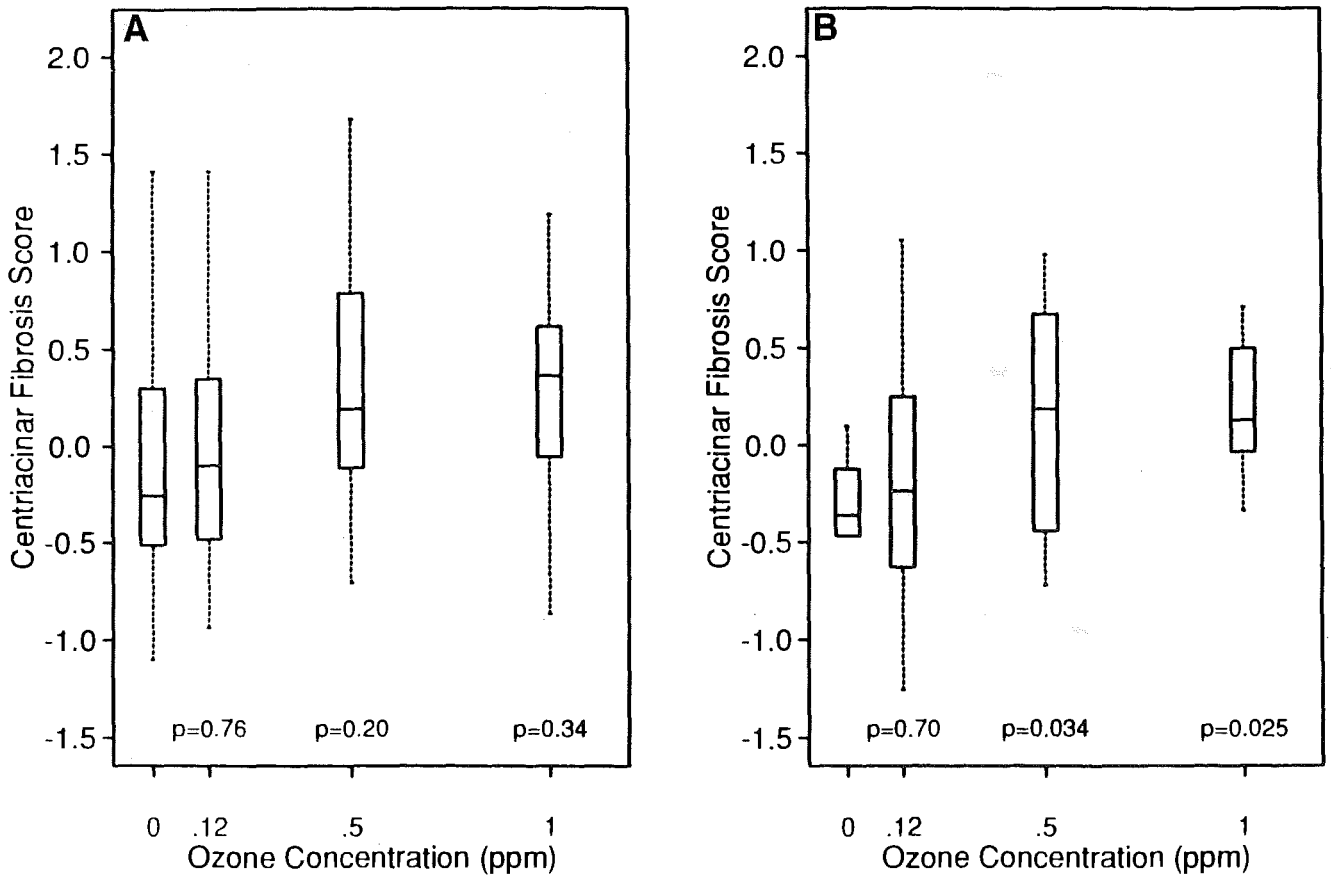

Figure 19. Combined analysis of centriacinar fibrosis by gender: A: Male data, concentration-response trend ($p = 0.30$, two-tailed). B: Female data, significant concentration response trend ($p = 0.0064$, two-tailed). p values given in the figure are for pairwise comparisons between the mean for each ozone concentration and the control value.

Pulmonary function and biochemical indices showed no significant changes at the lowest exposure level (0.12 ppm), and showed either no evidence or a slight trend at higher exposure levels (0.5 and 1.0 ppm).

On the basis of the results of the integrative analyses presented in this summary, the four framework questions can be answered as follows.

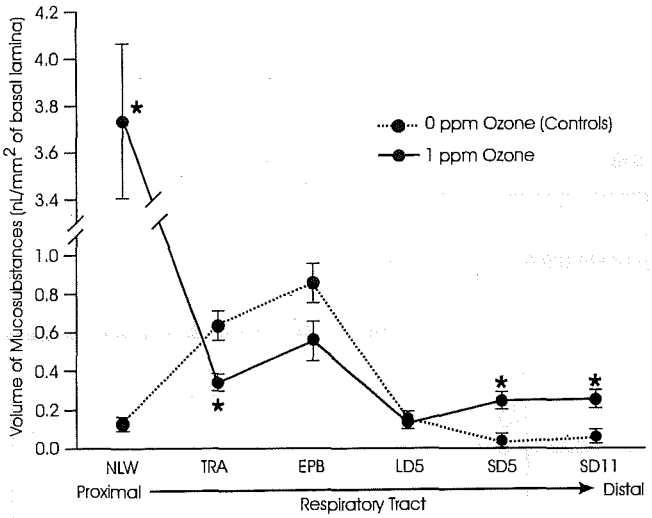

Figure 20. Volume of mucosubstances as a function of respiratory tract location. NLW = nasal lateral wall; TRA = trachea; EPB = extrapulmonary bronchus (generation 2); LD5 = large-diameter central bronchus (generation 5); SD5 = small-diameter cranial bronchus (generation 5); SD11 = small-diameter caudal bronchus (generation 11). An asterisk (*) indicates a value significantly different ($p < 0.05$) from the control value.

What changes in nasal function, if any, are correlated with the structural changes in the nose? Rats exposed to 0.5 or 1.0 (but not 0.12) ppm ozone had the structural and functional hallmarks of chronic rhinitis, including inflammation, mucous cell proliferation, increased intraepithelial mucosubstances, and dysfunction in mucous flow.

How are the structural, functional, and biochemical changes in the lung interrelated? Rats exposed to 0.5 or 1.0 (but not 0.12) ppm ozone had some structural hallmarks of chronic airway disease, including increases in stored mucus and secretory cells, in distal conducting airways. Focal structural changes also were noted in animals exposed to all exposure concentrations of ozone, including some at 0.12 ppm. Other symptoms of chronic airway disease, such as chronic inflammation and changes in in vivo functional measurements, were not present.

How are biochemical changes correlated with structural and functional indices of lung stiffness? Rats exposed to 0.5 or 1.0 (but not 0.12) ppm ozone showed structural, functional, and biochemical indicators of centriacinar fibrosis, although no in vivo functional changes were noted. Focal structural changes also were noted in animals exposed to 0.12 ppm ozone. The process of bronchiolarization may be responsible for some or all of the biochemical and functional changes observed.

What is the relation between changes in the nose and the rest of the respiratory tract? Nasal lesions induced by ozone in rats exposed to 0.5 or 1.0 (but not 0.12) ppm ozone correlated with alterations in the distal pulmonary airways. Therefore, examining nasal mucosa for alterations induced

Table 15. Correlation of Mucosubstances in Bronchi and Nose

Mucosubstances in Bronchus	Mucosubstances in Epithelium	*n*	Correlation Coefficient	*p* Value[a]
Central	Proximal lateral meatus	16	−0.06	0.83
	Distal lateral meatus	16	−0.12	0.66
	Maxillary sinus	16	−0.19	0.48
	Nasopharyngeal meatus	16	−0.03	0.92
Caudal	Proximal lateral meatus	17	0.60	0.011[b]
	Distal lateral meatus	17	0.56	0.020[b]
	Maxillary sinus	17	0.28	0.28
	Nasopharyngeal meatus	17	0.42	0.097
Cranial	Proximal lateral meatus	17	0.63	0.0068[b]
	Distal lateral meatus	17	0.68	0.0026[b]
	Maxillary sinus	17	0.47	0.057
	Nasopharyngeal meatus	17	0.36	0.15

[a]Based on a one-tailed test of significance assuming a positive correlation.

[b]Statistically significant correlation ($p \leq 0.025$).

by ozone may be a good predictor of damage in the lower airways.

Overall, animals showed structural alterations in the nose, tracheobronchial airways, and centriacinar region of the lung. With the tests used, functional impairment of the lung could not be demonstrated; however, functional changes were observed in the nose. Furthermore, the data strongly indicate that the animals developed tolerance in the lung, but not in the nose, to the injurious effects of ozone. This is supported by evidence of changes that could protect the airways, including increased mucosubstances and antioxidant enzymes in the airways, and replacement of sensitive cells with resistant cells in the centriacinar region. Significant remodeling of the nasal, tracheobronchial, and centriacinar regions took place in the animals in this Project. The long-term consequences of these changes and the mechanisms that produced them are still unknown.

FACTORS AFFECTING THE EXTRAPOLATION OF EFFECTS TO HUMANS

Patterns of Exposure

A long-term, regular pattern (6 hours per day, 5 days per week) of exposure was used in this Project. It does not reflect the seasonal or day-to-day variations that generally characterize human exposure in areas like Los Angeles, where ozone concentrations are elevated during part of the year (Lippmann 1989). Several findings from this Project suggest that caution should be used in extrapolating these results to human populations exposed sporadically or seasonally to the high ozone concentrations found in many cities. Some of the adaptive effects that have been reported in both animal and human studies could protect against damage from further exposures (tolerance); these protective effects would dissipate if exposure were stopped for a period of time. Thus, intermittent exposure might cause greater effects than a more constant exposure. The results of studies in this Project suggest that a pattern of consistent regular exposure to ozone leads to an increase in some indicators of tolerance: increased mucosubstances, increased levels of antioxidant enzyme activity, and reorganization of the most sensitive areas of the lung such that sensitive cell types are replaced with less sensitive cell types. These changes may prevent injury, and may be reflected in a lack of measurable changes in pulmonary function and in a lack of evidence for de novo expression of collagen. In fact, evidence from previous animal and human studies supports this suggestion that prolonged ozone exposure may lead to some degree of tolerance, and that this protection may not develop when exposures are periodic. In a study reported by Tyler and associates (1988), monkeys were exposed intermittently or daily to 0.25 ppm ozone for a period of 18 months. Monkeys exposed intermittently underwent approximately half the total exposure time as those exposed daily; however, when compared with the daily exposure group, these monkeys had significantly increased lung collagen content, chest wall compliance, and inspiratory capacity (Tyler et al. 1988). With continuous exposure, respiratory function changes may be attenuated. Tepper and coworkers (1989) reported initial changes, followed by attenuated responses, in respiratory function in F344/N rats exposed for a period of 2.25 hours per day over a five-day period to 0, 0.35, 0.5, or 1.0 ppm ozone. Progressive structural changes, including epithelial damage, inflammation in the bronchiolar region, and increases in lavageable protein, ascorbate, and glutathione, were observed during the five-day period.

Another way to compare the exposure conditions in this Project with those experienced by people is to ignore the patterns of exposure and compare the total ozone exposure (integrated over time) in this Project to the ambient situation. Table 16 shows annual and total exposure of rats in this Project and compares them to the typical exposure of people in Los Angeles in 1991.

Table 16. Integrated Ozone Exposures[a]

	Rats in This Project			People in Los Angeles[b]
Integrating Period	0.12 ppm	0.5 ppm	1.0 ppm	Ambient Levels (ppm)
Annual	187	468	936	50
Lifetime[c]	312	780	1560	--

[a] Values reflect total cumulative ozone exposure calculated as ppm × hours of exposure.

[b] Based on 1991 ozone levels (EPA 1992).

[c] Length of lifetime is considered to be 20 months (the duration of exposure) for rats in this Project.

When examined as an integrative exposure over a year, the rats in this Project were clearly exposed to greater amounts of ozone than people are. However, when examined as an integrative exposure over a "lifetime," the Project rats may have been exposed to less ozone than people residing in heavily polluted cities. In addition, these comparison values do not take ventilation into account. The rats in this Project were exposed during the day when, being nocturnal animals, they are relatively inactive. In contrast, a large number of human exposure studies have involved subjects who are exercising at levels ranging from very light to heavy. Results of human studies of short-term exposure indicate that, although the air concentration of ozone is the parameter that most strongly correlates with pulmonary function decrements, ventilation also plays a major role in the degree to which pulmonary function responds to ozone exposure (Hazucha 1987). Furthermore, recent studies that used ^{18}O-labeled ozone to compare the amount of ozone in exercising humans and laboratory rats suggest that higher concentrations of ozone reach the distal regions of the lungs of humans who are exercising than the lungs of sedentary rats. Therefore, ozone toxicity in inactive rats may underestimate the effects of ozone in exercising humans because rats have a lower-than-expected dose of ozone to the distal lung (Hatch et al. 1994).

Species and Gender Differences

Unlike humans, the rat is an obligate nose-breather. A large number of effects observed in this Project were observed in the various regions of the nose; however, at concentrations and exercise levels shown to induce changes in humans, breathing happens both orally and nasally. These factors need to be kept in mind when extrapolating effects from this Project to humans.

To the extent that responsiveness to ozone is genetically determined, any inbred animal strain will respond in a more uniform manner than a group of human subjects. This is another complicating factor in extrapolating from animals to humans. It is also important to consider the sensitivity of the F344/N rat in relation to other strains and species. A series of studies by Plopper and associates (1991) show that the monkey is more sensitive and more like humans than the rat in response to ozone exposure. Furthermore, the F344/N rat is not the strain of rat most sensitive to ozone; the Wistar and Sprague-Dawley rats show more sensitivity to ozone than F344/N rats (Pino et al. 1993).

Human studies indicate that responsiveness to short-term ozone exposure varies among individuals, but is reproducible (McDonnell et al. 1985; Linn et al. 1988). Although preliminary data for humans present a complex picture, it appears that genetic factors play a role in pulmonary function responsiveness (Slovik et al. 1994). This is supported by studies using genetically inbred mice by Kleeberger and coworkers (1993a,b), who report that one inbred strain is susceptible to and another one resistant to inflammation induced by ozone. Inflammation was determined by examination of bronchoalveolar lavage fluid. Separate genetic loci were found to control each strain's susceptibility to acute and subacute inflammation. Results of another study that examined the changes in ventilatory pattern that occur when animals are exposed to ozone suggest that the differences in inflammation found in susceptible and resistant strains may be due, in part, to changes in breathing patterns (Tankersley et al. 1993).

Although individual studies in this Project showed minor differences between male and female rats in the measurement of various parameters, an examination of all such differences showed no coherent trend. It therefore appears that the relation between gender and sensitivity to ozone exposure is not consistent throughout this Project.

STRENGTHS AND LIMITATIONS OF THE NTP/HEI COLLABORATIVE OZONE PROJECT

A number of strengths and limitations of this Project are evident. Strengths include the carefully executed procedures on a single set of animals, the multidisciplinary protocol (endpoints measured using morphology, biochemistry, physiology, and molecular biology in a single study), and the rigorous data analysis and review of individual studies and the combined results. The limitations reflected in the Project design include limited numbers of only one strain of rat that may be unusually resistant to ozone exposure, a single duration and pattern of exposure that does not represent ambient human conditions, and a recovery period that was too short to allow the reversibility of observed effects to be assessed. Nevertheless, because of this Project's strengths, when the results are evaluated in the context of the many ozone studies reported in the literature, important implications of ozone exposure may be evident.

A unique feature of the NTP/HEI Collaborative Ozone Project was the opportunity to study not only a broad spectrum of endpoints that would reveal the effects of prolonged ozone exposure, but also to study the relations among these endpoints. A critical component of the Project design was the integrative analysis that explored correlations between outcomes in order to derive an overall assessment of the adverse health effects of ozone. Furthermore, a combined analysis across all the studies examined the association between exposure to ozone and various disease surrogates. The goal behind performing correlative and

combined analyses of multiple and diverse endpoints was to increase the sensitivity of the analyses and reveal the possible mechanisms through which effects are induced. Although the results found through the combined analysis for chronic rhinitis could have been seen from the one study examining the nasal apparatus, the results of the analysis of chronic airway disease and centriacinar fibrosis would not have been evident from a single study.

Several important cautions must be expressed when examining the data from this Project. First, the multiplicity of endpoints could be problematic. Approximately 244 variables were examined in the total Project (see Table 1); therefore, results for some endpoints could appear to be statistically significant purely by chance. This is particularly likely for endpoints that are statistically significant at one ozone concentration, but that do not display a concentration-response relation. Similarly, interpreting the exploratory analysis of correlations must be approached cautiously. A total of 33 correlations were examined and ten were found to be statistically significant. However, it is possible that one or more of these correlations may have seemed to be statistically significant simply by chance.

SUMMARY AND CONCLUSIONS

Major structural changes were evident in the proximal region of the nose for animals exposed to 0.5 or 1.0 ppm ozone, including alterations to the surface epithelium, increases in intraepithelial mucus, and moderate mucosal inflammation. These structural alterations were associated with a dysfunction in mucous flow over the surface epithelium. Small, but statistically significant, biochemical changes were observed in whole lung tissue (when data were expressed on a per lung lobe basis) that might reflect structural changes in the centriacinar region. In addition, changes in antioxidant enzyme activity were noted within specific anatomical sites of the lung. They were most obvious in the centriacinar region, where the most marked cellular changes occurred. Subtle changes in lung structure were observed by morphometric evaluations of the centriacinar region at all concentrations of ozone tested, but mainly at 0.5 and 1.0 ppm ozone. These observations were consistent with routine histological findings of interstitial fibrosis in rats exposed to 0.5 or 1.0 ppm ozone. An effect of exposure to 0.12 ppm ozone was observed at only one focal region. This region, which extends approximately 300 μm beyond the bronchiole–alveolar duct junction, is believed to be particularly sensitive to ozone. Minimal or no functional changes in the Project animals could be detected by pulmonary function testing in anesthetized rats.

The integrative analyses reinforced these findings and, in fact, strengthened the correlation between the data for different endpoints. These results suggest that structural and biochemical findings, along with minimal functional alterations, demonstrate that the lungs of these animals became tolerant to the injurious effects of ozone as a result of continuous exposure. The appearance of inflammation in the nose of these animals is in striking contrast to the lack of inflammation in the rest of the respiratory system, and suggest that the nose did not develop tolerance to acute injury as did the lungs.

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REFERENCES

Aris RM, Christian D, Herne PQ, Kerr K, Finkbeiner WE, Balmes JR. 1993. Ozone-induced airway inflammation in human subjects as determined by airway lavage and biopsy. Am Rev Respir Dis 148:1363–72.

Barnes PJ. 1986. Neural control of human airways in health and disease. Am Rev Respir Dis 134:1289–1314.

Barr BC, Hyde DM, Plopper CG, Dungworth DL. 1988. Distal airway remodeling in rats chronically exposed to ozone. Am Rev Respir Dis 137:924–938.

Barr BC, Hyde DM, Plopper CG, Dungworth DL. 1990. A comparison of terminal airway remodeling in chronic daily versus episodic ozone exposure. Toxicol Appl Pharmacol 106:384–407.

Barry BE, Mercer RR, Miller FJ, Crapo JD. 1988. Effects of inhalation of 0.25 ppm ozone on the terminal bronchioles of juvenile and adult rats. Exp Lung Res 14:225–245.

Barry BE, Miller FJ, Crapo JD. 1985. Effects of inhalation of 0.12 and 0.25 parts per million ozone on the proximal alveolar region of juvenile and adult rats. Lab Invest 53:692–704.

Boorman GA, Schwartz LW, Dungworth DL. 1980. Pulmonary effect of prolonged ozone insult in rats: Morphometric evaluation of the central acinus. Lab Invest 43:108–115.

Boorman GA, Catalano PJ, Jacobson B, Kaden DA, Mellick PW, Nauss KM, Ryan LM. 1995. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part VI, Background and Study Design. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Brain JD. 1970. The uptake of inhaled gases by the nose. Ann Otol Rhinol Laryngol 79:529–539.

Calderon-Garcidueñas L, Osorno-Velasquez A, Bravo-Alvarez H, Delgado-Chavez, Barrios-Marquez R. 1992. Histopathologic changes of the nasal mucosa in southwest metropolitan Mexico City inhabitants. Am J Pathol 140:225–232.

Castleman WL, Dungworth DL, Schwartz LW, Tyler WS. 1980. Acute respiratory bronchiolitis: An ultrastructural and autoradiographic study of epithelial cell injury and renewal in rhesus monkeys exposed to ozone. Am J Pathol 98:811–840.

Catalano PJ, Rogus J, Ryan LM. 1995. Consequences of Prolonged Ozone Exposure on F344/N Rats: Collaborative Studies, Part X, Robust Composite Scores Based on Median Polish Analysis. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Chang L-Y, Huang Y, Stockstill BL, Graham JA, Grose EC, Ménache MG, Miller FJ, Costa DL, Crapo JD. 1992. Epithelial injury and interstitial fibrosis in the proximal alveolar regions of rats chronically exposed to a simulated pattern of urban ambient ozone. Toxicol Appl Pharmacol 115:241-252.

Chang L-Y, Stockstill BL, Ménache MG, Mercer RR, Crapo JD. 1995. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part VIII, Morphometric Analysis of Structural Alterations in Alveolar Regions. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Costa DL, Kutzman RS, Lehman JR, Popenoe EA, Drew ET. 1983. A subchronic multidose ozone study in rats. In: International Symposium on the Biomedical Effects of Ozone and Related Photochemical Oxidants (Lee SD, Mustafa MG, Mehlman MA, eds.). Princeton Scientific Publishers, Princeton, NJ.

Costa DL, Tepper JS, Stevens MA, Watkinson WP, Doerfler DL, Gelzleichter TR, Last JA. 1995. Functional evidence of restrictive lung disease in rats chronically exposed to a simulated urban profile of ozone. Am Rev Respir Crit Care Med (in press).

Cross CE, Halliwell B, Allen A. 1984. Antioxidant protection: A function of tracheobronchial and gastrointestinal mucus. Lancet 1:1328–1329.

Drazen JM, Austen KF. 1987. Leukotrienes and airway responses. Am Rev Respir Dis 136:985–998.

Drechsler-Parks DM, Bedi JF, Horvath SM. 1987. Pulmonary function responses of older men and women to ozone exposure. Exp Gerontol 22:91–101.

Dungworth DL. 1989. Noncarcinogenic responses of the respiratory tract to inhaled toxicants. In: Concepts of Inhalation Toxicology (RO McClellan, RF Henderson, eds.) pp. 273–298. Hemisphere Publishing Corp., New York, NY.

Eustis SL, Schwartz LW, Kosch PC, Dungworth DL. 1981. Chronic bronchiolitis in non-human primates after prolonged ozone exposure. Am J Pathol 105:121–137.

Evans MJ, Johnson LV, Stephens RJ, Freeman G. 1976. Cell renewal in the lungs of rats exposed to low levels of ozone. Exp Mol Pathol 24:70–83.

Fox SD, Adams WC, Brookes KA, Lasley BL. 1993. Enhanced response to ozone exposure during the follicular phase of the menstrual cycle. Environ Health Perspect 101:242–244.

Gillespie JR. 1983. Mechanisms that determine functional residual capacity in different mammalian species. Am Rev Respir Dis 128:S74–S77.

Grose EC, Stevens MA, Hatch GE, Jaskot RH, Selgrade MJK, Stead AG, Costa DL, Graham JA. 1989. The impact of a 12-month exposure to a diurnal pattern of ozone on pulmonary function, antioxidant biochemistry and immunology. In: Atmospheric Ozone Research and Its Policy Implications (Schneider T, Lee SD, Wolters GJR, Grant LD, eds.) pp. 535–544. Elsevier Science Publishing Co., New York, NY.

Gross KB, White HJ. 1987. Functional and pathologic consequences of a 52-week exposure to 0.5 ppm ozone followed by a clean air recovery period. Lung 165:283–295.

Harkema JR, Hotchkiss JA, Henderson RF. 1989. Effects of 0.12 and 0.80 ppm ozone on rat nasal and nasopharyngeal epithelial mucosubstances: Quantitative histochemistry. Toxicol Pathol 17:525–525.

Harkema JR, Mauderly JL. 1994. Consequences of Prolonged Ozone Inhalation on F344/N Rats: Collaborative Studies, Part V, Effects on Pulmonary Function. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Harkema JR, Morgan KT, Bermudez EG, Catalano PJ, Griffith WC. 1994. Consequences of Prolonged Ozone Inhalation on F344/N Rats: Collaborative Studies, Part VII, Effects on the Nasal Mucociliary Apparatus. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Harkema JR, Plopper CG, Hyde DN, St. George JA, Dungworth DL. 1987a. Effects of an ambient level of ozone on primate nasal epithelial mucosubstances: Quantitative histochemistry. Am J Pathol 127:90–96.

Harkema JR, Plopper CG, Hyde DN, St. George JA, Dungworth DL. 1987b. Response of the macaque nasal epithelium to ambient levels of ozone: A morphometric study of the transitional and respiratory epithelium. Am J Pathol 128:29–44.

Harkema JR, Plopper CG, Hyde DM, St. George JA, Wilson DW, Dungworth DL. 1993. Response of macaque bronchiolar epithelium to ambient concentrations of ozone. Am J Pathol 143(3):857–866.

Hatch GE, Slade R, Harris LP, McDonnell WF, Devlin RB, Koran HS, Costa DL, McKee J. 1994. Ozone dose and effect in humans and rats: A comparison using oxygen-18 labeling and bronchoalveolar lavage. Am J Respir Crit Care Med 150:676–683.

Hazucha MJ. 1987. Relationship between ozone exposure and pulmonary function changes. J Appl Physiol 62(4):1671–1680.

Hazucha MJ, Bates DV, Bromberg PA. 1989. Mechanism of action of ozone on the human lung. J Appl Physiol 67(4):1535–1541.

Hoaglin DC, Mosteller F, Tukey JW. 1983. Understanding Robust and Exploratory Analysis. John Wiley & Sons, New York, NY.

Holtzman MJ. 1991. Arachidonic acid metabolism: Implications of biological chemistry for lung function and disease. Am Rev Respir Dis 143:188–203.

Hotchkiss JA, Harkema JR, Henderson RF. 1991. Effect of cumulative ozone exposure on ozone-induced nasal epithelial hyperplasia and secretory metaplasia in rats. Exp Lung Res 15:589–600.

Hyde DM, Huckbard WC, Wong V, Wu R, Pinkerton K, Plopper CG. 1992. Ozone-induced acute tracheobronchial injury: Relationship to granulocyte immigration in the lung. Am J Respir Cell Mol Biol 6:481–497.

Kleeberger SR, Levitt RC, Zhang LY. 1993a. Susceptibility to ozone-induced inflammation: I. Genetic control of the response to subacute exposure. Am J Physiol: Lung Cell Mol Physiol 264(8): L15–L20.

Kleeberger SR, Levitt RC, Zhang LY. 1993b. Susceptibility to ozone-induced inflammation: II. Separate loci control responses to acute and subacute exposures. Am J Physiol: Lung Cell Mol Physiol 264(8):L21–L26.

Last JA. 1988. Biochemical and cellular interrelationships in the development of ozone-induced pulmonary fibrosis. In: Air Pollution, the Automobile, and Public Health (Watson AY, Bates RR, Kennedy D, eds.) pp. 415–440. National Academy Press, Washington, DC.

Last JA, Gelzleichter TR, Harkema JH, Hawk S. 1994. Consequences of Prolonged Inhalation of Ozone on Fischer-344/N Rats: Collaborative Studies, Part 1, Content and Cross-Linking of Lung Collagen. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Last JA, Greenberg DB, Castleman WL. 1979. Ozone-induced alterations in collagen metabolism of rat lungs. Toxicol Appl Pharmacol 51:247–258.

Last JA, Reiser KM, Tyler WS, Rucher RB. 1984. Long-term consequences of exposure to ozone: Lung collagen content. Toxicol Appl Pharmacol 72:111–118.

Leith DE. 1976. Comparative mammalian respiratory mechanics. Physiologist 19:485–510.

Linn WS, Avol EL, Shamoo DA, Peng RC, Valencia LM, Little DE, Hackney JD. 1988. Repeated laboratory ozone exposures of volunteer Los Angeles residents: An apparent seasonal variation in response. Toxicol Ind Health 4:505–520.

Lippmann M. 1989. Health effects of ozone: A critical review. J Air Pollut Control Assoc 39:672–695.

Mauderly JL, Gillett NA. 1992. Changes in respiratory function. In: Pathobiology of the Aged Rat, Vol. 1 (Mohr U, Dungworth DL, Capen CC, eds.). International Life Sciences Institute (ILSI) Press, Washington, DC.

McDonnell WF, Horstman DH, Abdul-Salaam S, House DE. 1985. Reproducibility of individual responses to ozone exposure. Am Rev Respir Dis 131:36–40.

Mercer RR, Anjilvel S, Miller FJ, Crapo. 1991. Inhomogeneity of ventilatory unit volume and its effects on reactive gas uptake. J Appl Physiol 70:2193–2205.

Moffatt RK, Hyde DM, Plopper CG, Tyler WS, Putney LF. 198. Ozone-induced adaptive and reactive cellular changes

in respiratory bronchioles of bonnet monkeys. Exp Lung Res 12(1): 57-74.

Moore PF, Schwartz LW. 1981. Morphological effects of prolonged exposure to ozone and sulfuric acid aerosol on the rat lung. Exp Mol Pathol 35:108–123.

Mosteller F, Tukey JW. 1977. Data Analysis and Regression. Addison-Wesley, Reading, MA.

National Toxicology Program. 1995. Technical Report on the Toxicology and Carcinogenesis Studies of Ozone and Ozone/NNK in Fischer-344/N Rats and B6C3F$_1$ mice. NTP Technical Report No. 440. NIH Publication No. 93–3371. U.S. Department of Health and Human Services, National Toxicology Program Information Office, Research Triangle Park, NC.

Nikula KJ, Wilson DW, Giri SN, Plopper CG, Dungworth DL. 1988. The response of the rat tracheal epithelium to ozone exposure: Injury, adaptation, and repair. Am J Pathol 131:373–384.

Overton JH, Graham RC, Miller FJ. 1987. A model of the regional uptake of gaseous pollutants in the lung: II. The sensitivity of ozone uptake in laboratory animal lungs to anatomical and ventilatory parameters. Toxicol Appl Pharmacol 88:418–432.

Parks WC. 1992. Localization of tropoelastin and type I procollagen expression in lungs after short-term ozone exposure. In: New Methods in Ozone Toxicology: Abstracts of Six Pilot Studies. HEI Communications Number 1. Health Effects Institute, Cambridge, MA.

Parks WC, Roby JD. 1994. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part IV, Effects on Expression of Extracellular Matrix Genes. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Penha PD, Werthamer S. 1974. Pulmonary lesions induced by long-term exposure to ozone: II. Ultrastructure observations of proliferation and regressive lesions. Arch Environ Health 29:282–289.

Pickrell JA, Hahn FF, Rebar AH, Horoda RA, Henderson RF. 1987. Changes in collagen metabolism and proteinolysis after repeated inhalation exposure to ozone. Exp Mol Pathol 46:159–167.

Pinkerton KE, Dodge DE, Cederdahl-Demmler J, Wong VJ, Peake J, Haselton CJ, Mellick PW, Singh G, Plopper CG. 1993. Differentiated bronchiolar epithelium in alveolar ducts of rats exposed to ozone for 20 months. Am J Pathol 142:947–956.

Pinkerton KE, Ménache MG, Plopper CG. 1995. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part IX, Changes in the Tracheobronchial Epithelium, Pulmonary Acinus, and Lung Antioxidant Enzyme Activity. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Pino MV, Hyde DM, Stovall MY. 1991. Strain differences in the response of the rat lung to an acute ozone exposure. Am Rev Respir Dis 143:A698.

Plopper CG, Chow CK, Dungworth DL, Brummer M, Nemeth TJ. 1978. Effect of low-level exposure on rat lungs: II. Morphological responses during recovery and re-exposure. Exp Mol Pathol 29:400–411.

Plopper CG, Chu FP, Haselton CJ, Peake J, Wu J, Pinkerton KE. 1994a. Dose-dependent tolerance to ozone: I. Tracheobronchial epithelial reorganization in rats after 20 months' exposure. Am J Pathol 144:404–421.

Plopper CG, Duan X, Buckpitt AR, Pinkerton KE. 1994b. Dose-dependent tolerance to ozone: IV. Site-specific elevation in antioxidant enzymes in the lungs of rats exposed for 90 days or 20 months. Toxicol Appl Pharmacol 127:124–131.

Plopper CG, Dungworth DL, Tyler WS, Chow CK. 1979. Pulmonary alterations in rats exposed to 0.2 and 0.1 ppm ozone: A correlated morphological and biochemical study. Arch Environ Health 34:390–395.

Plopper CG, Harkema JR, Last JA, Pinkerton KE, Tyler WS, St. George JA, Wong VJ, Nishio SJ, Weir AS, Dungworth DL, Barry BE, Hyde DM. 1991. The respiratory system of nonhuman primates responds more to ambient concentrations of ozone than does that of rats. In: Tropospheric Ozone and the Environment (Berglund RL, Lawson DR, McKee DJ, eds.) pp. 137–150. Air & Waste Management Association, Pittsburgh, PA.

Radhakrishnamurthy B. 1994. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part III, Effects on Complex Carbohydrates of Lung Connective Tissue. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Schlesinger RB, Gorczynski JE, Dennison J, Richards L, Kinney PL, Bosland MC. 1992. Long-term intermittent exposure to sulfuric acid aerosol, ozone, and their combination: Alterations in tracheobronchial mucociliary clearance and epithelial secretory cells. Exp Lung Res 18:505–534.

Schwartz LW, Dungworth DL, Mustafa MG, Tarkington BK, Tyler WS. 1976. Pulmonary responses of rats to ambient

levels of ozone: Effects of 7-day intermittent or continuous exposure. Lab Invest 34:565–578.

Seltzer J, Bigby BG, Stulbarg M, Holtzman MJ, Nadel JA, Ueki IF, Leikauf GD, Goetzl EJ, Boushey HA. 1986. O_3-induced change in bronchial reactivity to methacholine and airway inflammation in humans. J Appl Physiol 60(4):1321–1326.

Shore SA, Austen KF, Drazen JM. 1989. Eicosanoids and the lung. In: Lung Biology in Human Health and Disease, Vol. 41, Lung Cell Biology (Massar D, ed.). Marcel Dekker, New York, NY.

Slovik L, Bromberg PA, Rogers J, McDonnell WF, Hazucha MJ. 1994. Genetic influence in the lung function to ozone (abstract). Am J Respir Crit Care Med 149:A151.

Snedecor GW, Cochran WG (1989). Statistical Methods, 8th ed. Iowa State University Press, Ames, IA.

Stephens RF, Sloan MF, Evans MJ, Freeman G. 1974. Early response of lung to low levels of ozone. Am J Pathol 74:31–58.

Szarek JL. 1994. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part III, Mechanical Properties, Responses to Bronchoactive Stimuli, and Eicosanoid Release in Isolated Large and Small Airways. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Tankersley CG, Fitzgerald RS, Mitzner WA, Kleeberger SR. 1993. Hypercapnic ventilatory responses in mice differentially susceptible to acute ozone exposure. J Appl Physiol 75(6):2613–2619.

Tepper JS, Costa DL, Lehmann JR, Weber MF, Hatch GE. 1989. Attenuated structural and biochemical alteratons in the lung during functional adaptation to ozone. Am Rev Respir Dis 140:493–501.

Tepper JS, Weister MJ, Weber MF, Fitzgerald S, Costa DL. 1991. Chronic exposure to a simulated urban profile of ozone alters ventilation responses to carbon dioxide challenge in rats. Fundam Appl Toxicol 17:52–60.

Tyler WS, Tyler NK, Last JA, Gillespie MJ, Barstow TJ. 1988. Comparison of daily and seasonal exposures of young monkeys to ozone. Toxicology 50:131–144.

U.S. Environmental Protection Agency. 1991. National Air Quality and Emissions Trend Report, 1989. EPA/450/4-91-003. Environmental Protection Agency, Office of Air Quality Planning and Standards, Research Triangle Park, NC.

Wright ES, Kehrer JP, White DM, Smiler KL. 1988. Effects of chronic exposure to ozone on collagen in rat lung. Toxicol Appl Pharmacol 92:445–452.

APPENDIX A. List of All Endpoints Examined by HEI Investigators

Table A.1. Individual Endpoints by Structural, Biochemical, and Functional Parameters

Endpoint	Principal Investigator
Structure	
Wall area (in large and small airways; per segment length)	Szarek
Smooth muscle area (in large and small bronchi; per segment length; also as a percentage of wall area)	Szarek
Interstitial volume	Chang
Collagen volume[a]	Chang
Fibroblast volume[a]	Chang
Basement membrane volume[a]	Chang
Acellular space volume[a]	Chang
Macrophage volume[a]	Chang
Percentage of bronchiolarization[b]	Chang
Elastin volume[b]	Chang
Interstitial macrophage volume[b]	Chang
Ciliated cells[c]	Chang
Clara cells[c]	Chang
Brush cells[c]	Chang
Preciliated cells[c]	Chang
Unknown cells[c]	Chang
Terminal bronchiole diameter	Chang
Epithelial volume[d]	Pinkerton
Interstitial volume[d]	Pinkerton
Capillary volume[d]	Pinkerton
Air space volume[d]	Pinkerton
Macrophage volume[d]	Pinkerton
Airway epithelial cell thickness[e]	Pinkerton
Volume fraction and volume per unit of surface area of nonciliated bronchiolar epithelial cells[f]	Pinkerton
Stored intraepithelial cell secretory product[g]	Pinkerton, Harkema
Abundance of cell types in the nasal transitional epithelium	Harkema

(Table continued next page.)

Table A.1. Individual Endpoints by Structural, Biochemical, and Functional Parameters *(continued)*

Endpoint	Principal Investigator
Biochemistry	
4-Hydroxyproline (per lobe)	Last
Trifunctional cross-links hydroxypyridinium (per lobe)	Last
Difunctional cross-links dihydroxylysinonorleucine (per lobe)	Last
Hydroxylysinonorleucine (per lobe)	Last
Dihydroxylysinonorleucine:hydroxylysinonorleucine ratio (based on above)	Last
Hydroxypyridinium:4-hydroxyproline ratio (based on above)	Last
Total glycosaminoglycans (per milligram of dry-defatted tissue)	Radhakrishnamurthy
Hyaluronan[h]	Radhakrishnamurthy
Heparan sulfate[h]	Radhakrishnamurthy
Chondroitin 4-sulfate[h]	Radhakrishnamurthy
Chondroitin 6-sulfate[h]	Radhakrishnamurthy
Dermatan sulfate[h]	Radhakrishnamurthy
Heparin[e,h]	Radhakrishnamurthy
Collagen type I gene expression	Parks
Collagen type III gene expression	Parks
Elastin gene expression	Parks
Fibronectin gene expression	Parks
Interstitial collagenase gene expression	Parks
Catalase[i]	Pinkerton
Glutathione perioxidase[i]	Pinkerton
Glutathione S–transferase[i]	Pinkerton
Superoxide dismutase[i]	Pinkerton
Prostaglandin E_2 and leukotriene C_4 release from caudal airways	Szarek
Function	
Mucous flow rates (in 13 specific regions of the nasal cavity)	Harkema
Mucous volume (in 3 of the 13 regions)	Harkema
Breathing frequency	Harkema
Tidal volume	Harkema
Minute volume	Harkema
Dynamic lung compliance	Harkema
Total pulmonary resistance	Harkema
Quasistatic chord compliance	Harkema
Maximal compliance	Harkema

(Table continued next column.)

Table A.1. Individual Endpoints by Structural, Biochemical, and Functional Parameters *(continued)*

Endpoint	Principal Investigator
Total lung capacity	Harkema
Vital capacity	Harkema
Functional residual capacity	Harkema
Expiratory reserve volume	Harkema
Residual volume	Harkema
Carbon monoxide diffusing capacity	Harkema
Forced vital capacity	Harkema
Percentage of forced vital capacity expired in 0.1 second	Harkema
Peak expiratory flow rate	Harkema
Mean midexpiratory flow rate	Harkema
Flow rate at 50%, 25%, and 10% of forced vital capacity	Harkema
Slope of phase III of single-breath nitrogen washout	Harkema
Additional quantities and ratios derived from the preceding endpoints	Harkema
Mechanics and contractile responses of small and large bronchi	Szarek

[a] Measured in proximal and random alveolar regions.

[b] Measured in proximal alveolar region only.

[c] Measured in terminal bronchioles.

[d] Measured at 100 to 800 μm of alveolar duct path from septal tips along both short- and long-path airway segments.

[e] Measured in trachea, and cranial, central, and caudal bronchi.

[f] Measured in trachea; cranial, central, and caudal bronchi; and proximal and terminal bronchioles.

[g] Measured in nasal cavity; trachea; and cranial, central, and caudal bronchi.

[h] Measured per milligram of dry-defatted tissue and as the percentage of total glycosaminoglycans.

APPENDIX B. Summary of the Results of the NTP Portion of the NTP/HEI Collaborative Ozone Project

For the NTP studies, survival at the end of the 24-month and 30-month exposure periods was similar among the exposed groups and the control group. A slight decrease was evident in the mean body weight in the groups exposed to 1.0 ppm ozone, but no decrease in the groups exposed to 0.12 or 0.5 ppm ozone. All groups of animals exposed to ozone were hypoactive.

Increased incidence of metaplasia was observed in the nose and lungs of rats exposed to 0.5 or 1.0 ppm ozone for 24 months. In the nose, these lesions were

> . . . characterized by an increase in the number of goblet cells in the respiratory epithelium with mild squamous metaplasia of the cuboidal epithelium on the lateral wall. The increase in the number of goblet cells was found primarily in levels I and II epithelium occurring along the lateral wall and on the maxilloturbinates and nasoturbinates. The metaplasia in the lung was a patchy multifocal lesion consisting of extension of the bronchial epithelium into the alveoli of the centriacinar region. This may represent more an extension of the bronchial epithelium into the pulmonary parenchyma than an actual transition of one epithelial cell type into another. There were increased incidences of squamous metaplasia at the base of the epiglottis characterized by one or more layers of flattened epithelial cells where low cuboidal cells are normally found (National Toxicology Program 1995).

In addition, increased incidence of metaplasia was observed in the nose, larynx, and lungs of rats exposed to 0.5 or 1.0 ppm ozone for 30 months. The lung lesions were

> . . . multifocal, centriacinar and were characterized by the presence of cuboidal epithelium (ciliated and nonciliated) along the alveolar ducts where type I epithelium is normally present (National Toxicology Program 1995).

Inflammation and interstitial fibrosis also were observed in the lungs of ozone-exposed animals from this Project. No increase in alveolar/bronchial adenoma or carcinoma were seen in any of the exposure groups. These results are consistent with the observations in animals studied by HEI investigators.

In the cocarcinogenesis study, rats were exposed to 0.5 ppm ozone during or after an exposure to a known carcinogen, NNK. Exposure to ozone did not affect the occurrence of neoplastic or nonneoplastic lesions.

In the study with $B6C3F_1$ mice, survival at the end of the 24-month and 30-month exposure periods was generally similar among the exposed groups and the control group, although the 24-month survival rate was higher in female mice than male mice exposed to 1.0 ppm ozone. A slight decrease in the mean body weight and hypoactivity were noted in several groups of mice exposed to ozone. Increased incidence of metaplasia and hyperplasia were observed in the nose and lungs of mice exposed to 0.5 or 1.0 ppm ozone for 24 months. In the nose, metaplasia was characterized by " . . . increased thickening and extension of the squamous epithelium into the anterior portion of the nasal passage." Nasal hyperplasia was characterized by " . . . thickening of the noncuboidal (transitional) epithelium." In the lungs, metaplasia was characterized by an " . . . extension of the bronchial epithelium into the alveoli of the centriacinar region." An increased incidence of hyperplasia also was noted in the epiglottis of female mice, a change that was characterized by a " . . . minimal increase in the thickness of the epithelium." Increased levels of metaplasia and hyperplasia also were observed in the larynx.

In both the 24-month and 30-month studies with mice, evidence of possible malignancy was supported by marginal increases in the incidence of alveolar/bronchiolar adenoma or carcinoma (combined) in both genders exposed to 0.5 or 1.0 ppm ozone.

On the basis of these studies, the NTP concluded that no evidence of carcinogenic activity from exposure to ozone was found in male or female F344/N rats. However, the increased incidence of alveolar/bronchiolar adenoma or carcinoma provided some evidence of carcinogenic activity in female $B6C3F_1$ mice exposed to ozone. Evidence of carcinogenic activity also was found in male mice, but it was equivocal. The basis for these judgments regarding mice are presented in Table B.1.

Exposure to ozone was associated with nasal goblet cell hyperplasia, nasal squamous cell metaplasia, squamous metaplasia in the larynx, and both interstitial fibrosis and extension of the bronchial epithelium into the centriacinar alveolar ducts in the lungs of rats. Exposure to ozone was associated with nasal hyperplasia, inflammation, nasal squamous cell metaplasia, and extension of the bronchial epithelium into the centriacinar alveolar ducts in the lungs of mice.

The NTP also concluded that no evidence supported the hypothesis that exposure to 0.5 ppm ozone would enhance the incidence of pulmonary neoplasms induced by NNK in male rats.

Table B.1. Incidence of Combined Alveolar/Bronchiolar Adenomas and Carcinomas in Mice[a]

	24-Month Exposure		30-Month Exposure	
Ozone (ppm)	Males	Females	Males	Females
0	14/50	6/50	16/49	6/50
0.12	14/50	7/50	–	–
0.5	18/50	9/50	22/49	8/49
1.0	19/50	16/50	21/50	12/50

[a] Values are the number of mice with neoplasms/number of mice examined.

APPENDIX C. Correlative Analyses

In the following five figures, pairwise plots of rankits (the ranking of raw data that assumes an underlying normal distribution) are shown. The pairs of correlated parameters are given above each box plot. Open circles (O) indicate data for control animals and animals exposed to 0.12 ppm ozone; filled circles (●) indicate data for animals exposed to 0.5 or 1.0 ppm ozone. In each plot, smooth curves (constructed using a robust scatterplot smoother) are overlaid to give an indication of general trend.

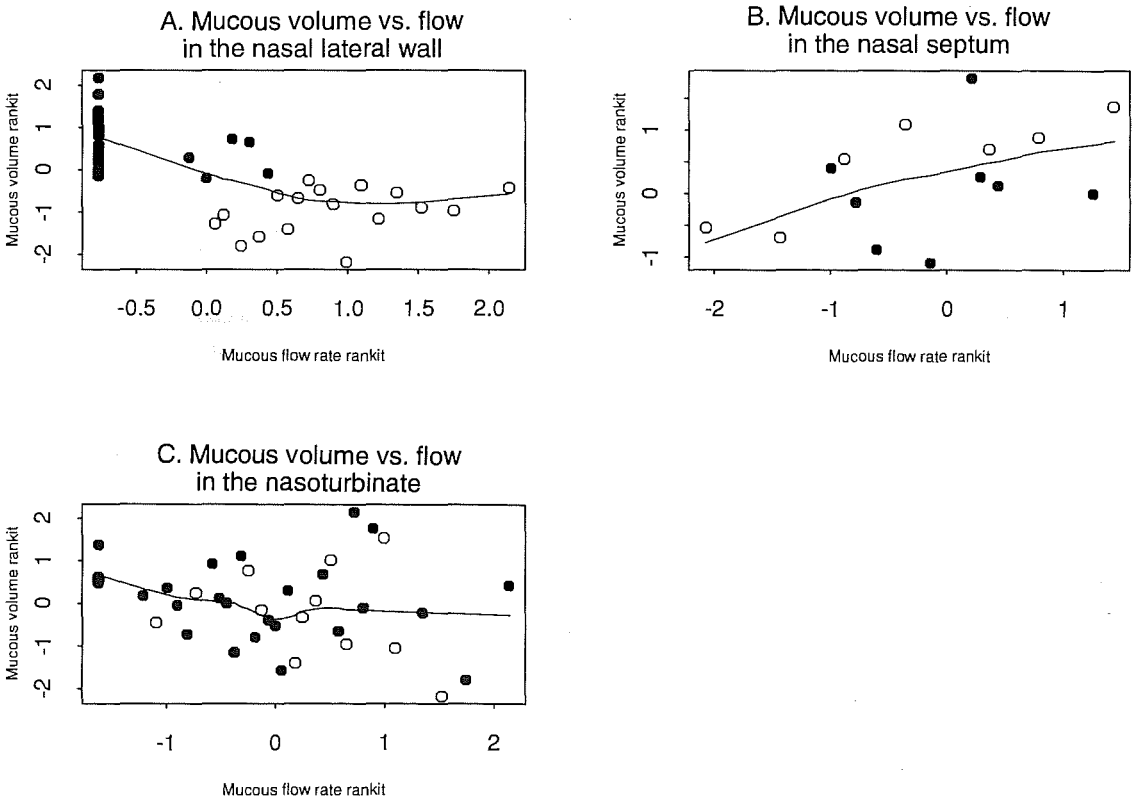

Figure C.1. Correlative analysis of structural function in the nasal apparatus.

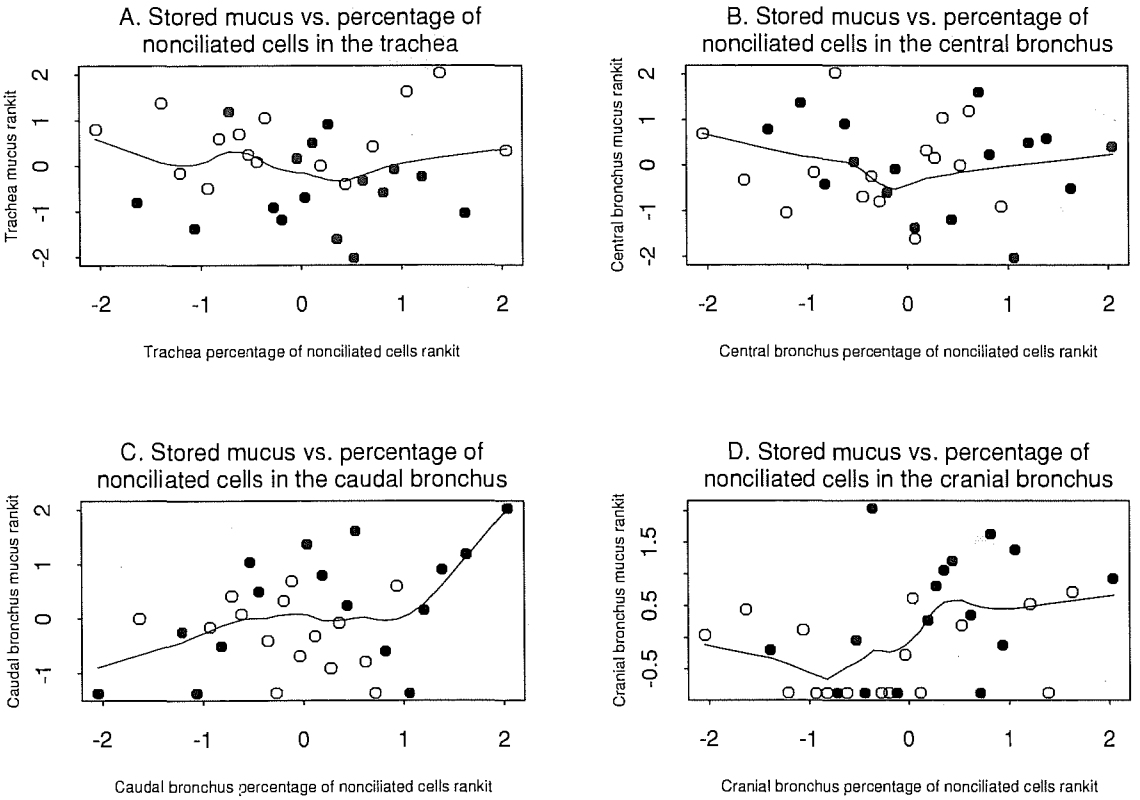

Figure C.2. Correlative analysis of structure versus function in the lung.

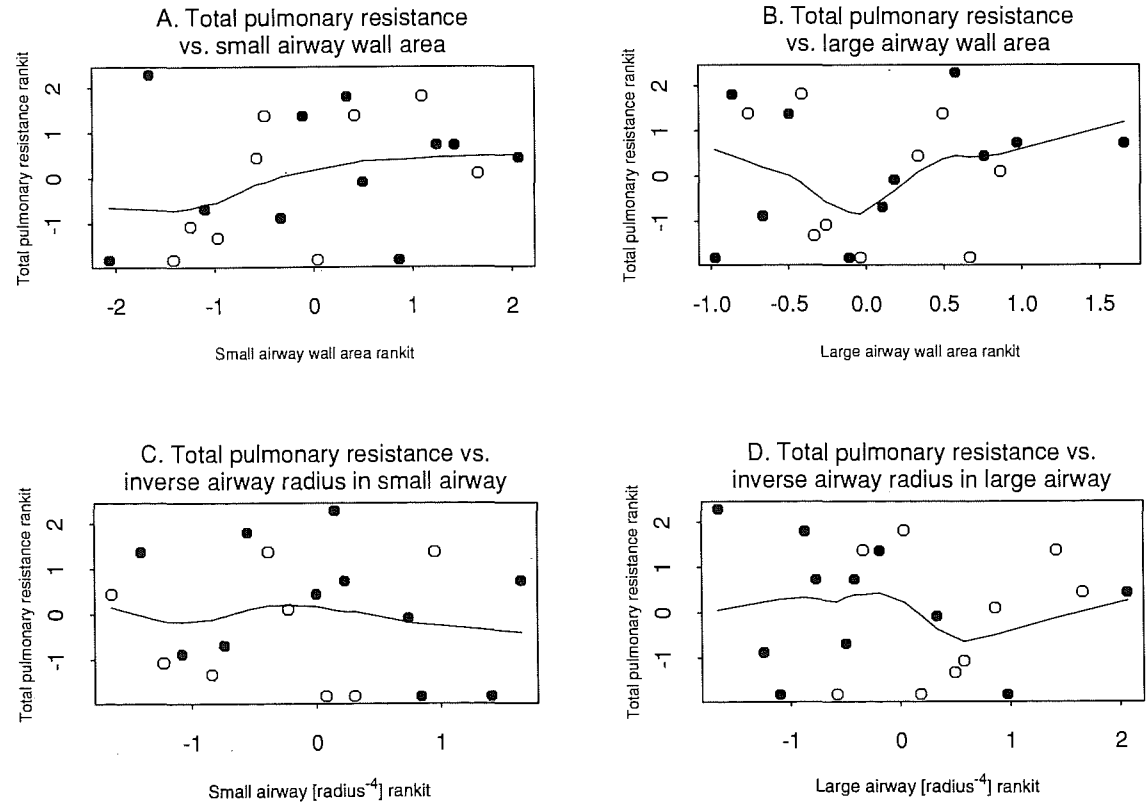

Figure C.3. Correlative analysis of function versus function in the lung.

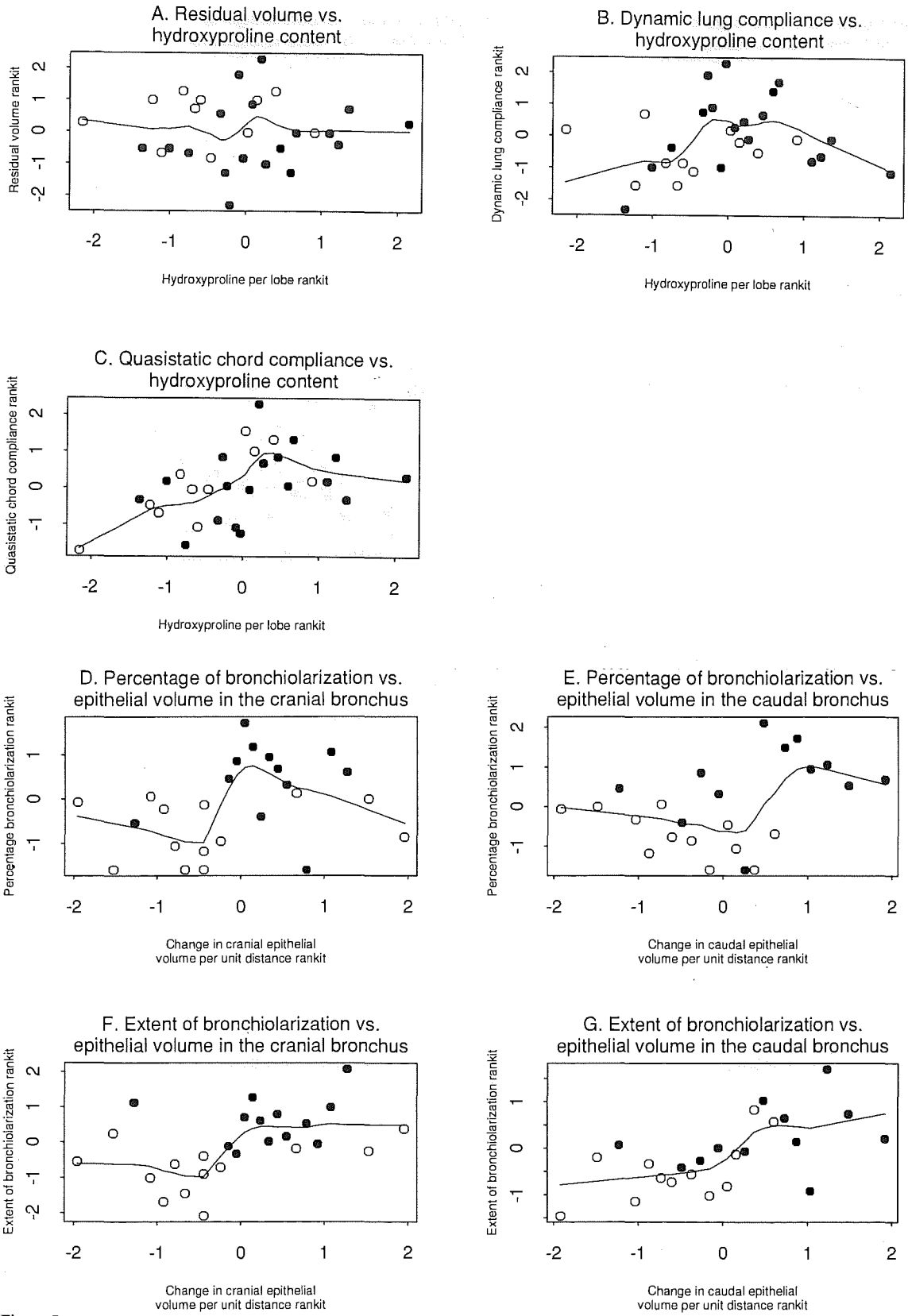

Figure C.4. Correlative analysis of biochemistry and functional measures *(Figure continues next page.)*

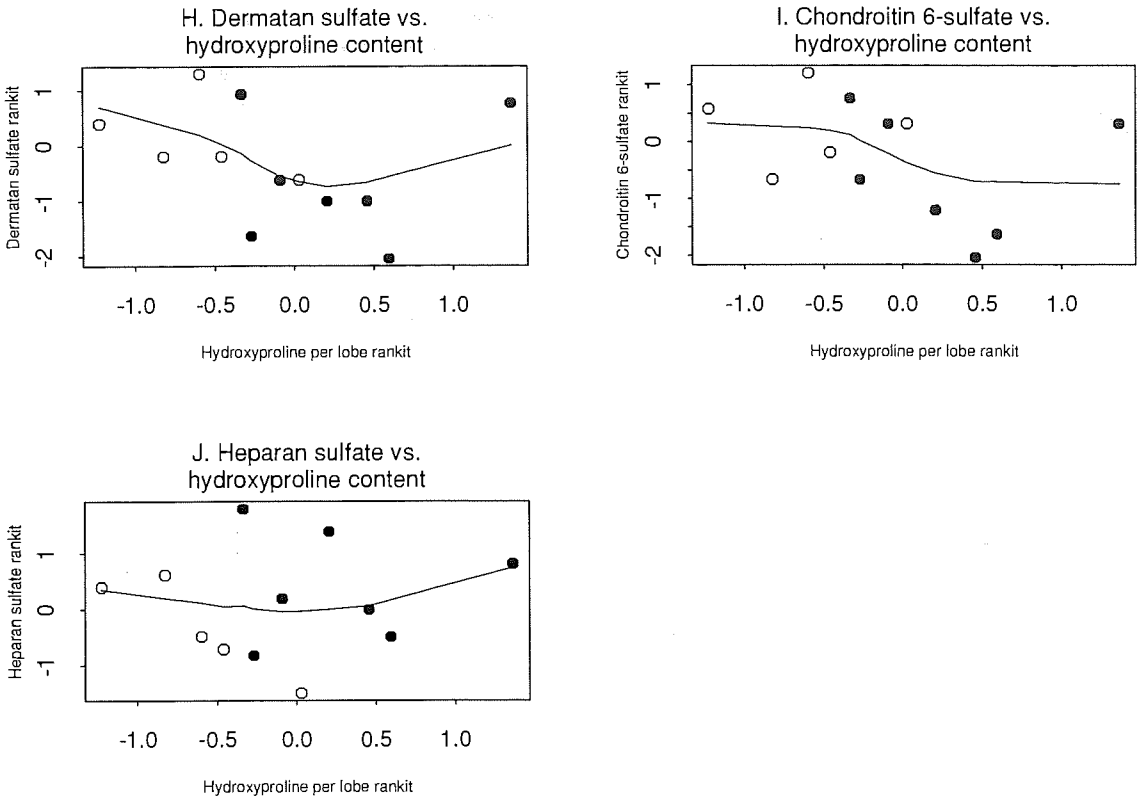

Figure C.4. Correlative analysis of biochemistry and functional methods *(continued).*

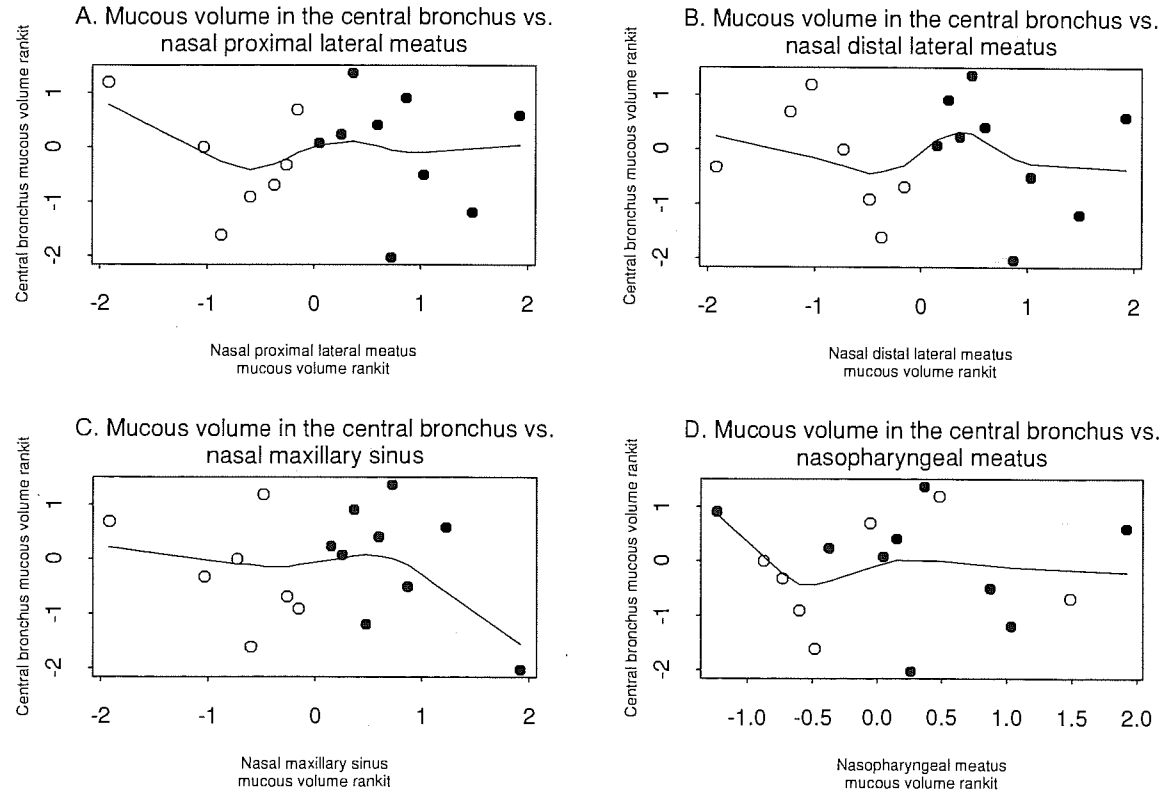

Figure C.5. Correlative analysis of volume of mucosubstances in the airways. *(Figure continues on next page.)*

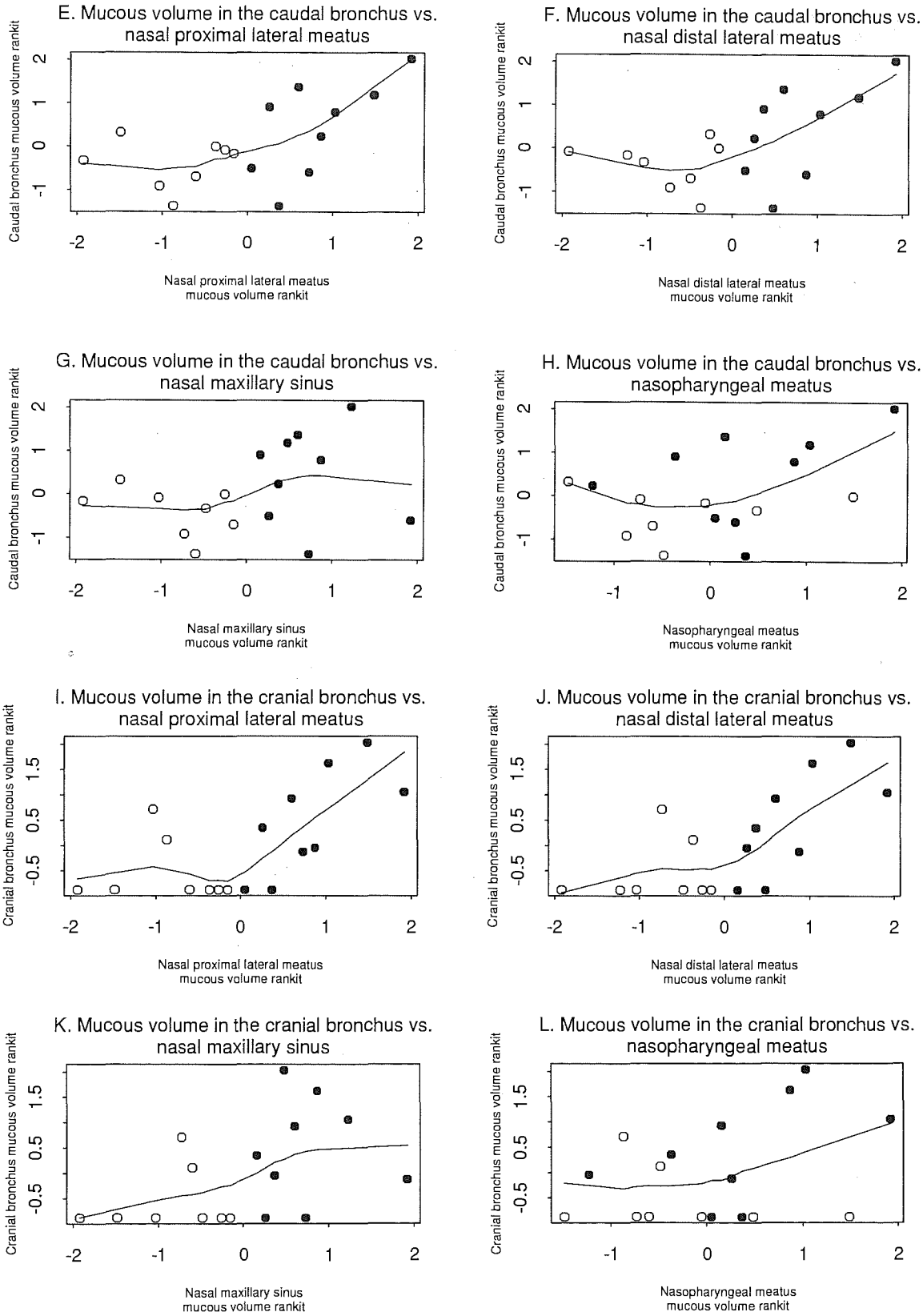

Figure C.5. Correlative analysis of volume of mucosubstances in the airways *(continued).*

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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 developing and applying 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.

Ling-Yi Chang received a Ph.D. in physiology in 1982 from North Carolina State University. She then joined the Pulmonary and Critical Care Medicine Division at Duke University as a Research Associate. She is currently Associate Research Professor of Medicine and Cell Biology. Her main research interests are pulmonary cell interactions with oxidants and their responses to oxidant stress, the effects on lung cell function and structure of air pollutants that generate free radicals, and the regulation of lung inflammation by molecules that adhere to the surface of alveolar epithelial cells.

Jack R. Harkema, D.V.M., Ph.D., is a Professor of Pathology at Michigan State University, College of Veterinary Medicine. 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, when he conducted the resarech reported here. He received his Ph.D. in comparative pathology at the University of California at Davis. He is a diplomate of the American College of Veterinary Pathologists. Dr. Harkema's primary research interest is the pathogenesis of airway epithelial cell injury, adaption, and repair after exposure to inhaled toxicants. He is currently investigating the cellular and molecular mechanisms involved in mucous cell hyperplasia and metaplasia in experimental models of chronic bronchitis.

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Dr. William C. Parks received his Ph.D. in anatomy and cell biology in 1982 from the Medical College of Wisconsin. He had postdoctoral training in carcinogenesis at Michigan State University with Drs. Veronica Maher and Justin McCormick, and in connective tissue biology at Washington University with Dr. Robert Mecham. Currently, Dr. Parks is an Assistant Professor in the Dermatology Division, Department of Medicine, and in the Department of Cell Biology and Physiology at Washington University. Research in his laboratory involves defining functional and regulatory domains of tropoelastin protein and mRNA, delineating the molecular and cellular mechanisms controlling tropoelastin and metalloproteinase expression, and characterizing the role of metalloproteinases and connective tissue proteins in pulmonary diseases and wound healing. Dr. Parks is a Career Investigator of the American Lung Association.

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Louise M. Ryan is an Associate Professor of Biostatistics at the Harvard School of Public Health and the Dana-Farber Cancer Institute. She received her Ph.D. in statistics from Harvard University in 1983. Her primary research interests include statistical methods for the design and analysis of carcinogenicity, developmental toxicity, and other toxicological experiments. She is particularly interested in statistical methods for analyzing multiple outcomes, especially in the context of dose-response modeling. Dr. Ryan also works on the statistical analysis for cancer clinical trials, and for epidemiologic studies in fertility and human reproduction.

John L. Szarek received his B.S. in biological sciences from the University of Illinois in Urbana, and a B.S. in pharmacy from the University of Illinois College of Pharmacy in Chicago. He received his Ph.D. in pharmaceutical sciences from the University of Kentucky College of Pharmacy in Lexington. He conducted postdoctoral research in the Department of Physiology and Biophysics in the College of Medicine at the University of Vermont in Burlington. Dr. Szarek is currently an Associate Professor at Marshall University School of Medicine in the Department of Pharmacology. His primary research interest is in the effects of oxidant stress on airway responsiveness.

ABBREVIATIONS

DHLNL	dihydroxylysinonorleucine
DL_{CO}	carbon monoxide diffusing capacity
EC_{50}	effective concentration or frequency of stimulus that elicits a half-maximal response
EPA	U.S. Environmental Protection Agency
FEV_1	forced expiratory volume in one second
HLNL	hydroxylysinonorleucine
mRNA	messenger RNA
NAAQS	National Ambient Air Quality Standards
NNK	4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone
NTP	National Toxicology Program
^{18}O	oxygen-18
ppm	parts per million
^{35}S	sulfur-35

INTRODUCTION TO THE NTP/HEI COLLABORATIVE OZONE PROJECT

The NTP/HEI Collaborative Ozone Project was a multi-institutional effort that drew on the expertise of many individuals to address the question of whether there is a linkage between prolonged ozone exposure and either the development of chronic respiratory diseases or damage to the respiratory tract that could contribute to such diseases. Collectively, chronic lung diseases are among the major contributors to morbidity and mortality in developed countries; they are the fourth leading cause of death in the United States. Chronic lung diseases include restrictive disorders such as interstitial fibrosis, and obstructive disorders such as emphysema, chronic bronchitis, and asthma. Cigarette smoking is the major contributor to emphysema and chronic bronchitis. Exposure to ozone or other air pollutants may contribute to or exacerbate these disorders.

The NTP/HEI Collaborative Ozone Project had its origins in HEI's efforts in 1986 to initiate a small research program to address the potential carcinogenicity of prolonged ozone exposure. Although HEI did not fund any animal carcinogenesis bioassays, the Institute's Health Research Committee thought that the issue was of sufficient importance to submit a nomination to the National Toxicology Program (NTP)* for chronic toxicity and carcinogenicity testing of ozone. The NTP coordinates the nation's testing of potentially toxic and hazardous chemicals. Over the years it has developed rigorous procedures for conducting and evaluating animal bioassays, especially the two-year carcinogenicity bioassays that form the basis of most risk assessments. Because ozone is potentially genotoxic and exposures to this pollutant are widespread, the NTP approved it for testing. The focus of the NTP studies was evaluating the carcinogenicity of ozone. However, both the NTP and HEI recognized that cancer was only one lung disorder of concern and that nonneoplastic endpoints should be studied as well. The NTP included 164 additional animals in its exposure chambers for HEI toxicity studies. The HEI implemented procedures to assure rigorous oversight and scientific review of the studies of nonneoplastic endpoints. Members of both organizations worked together and with academic and industrial scientists to develop research priorities and design studies.

* A list of abbreviations appears at the end of the Integrative Summary for your reference.

THE NATIONAL TOXICOLOGY PROGRAM BIOASSAY

The NTP conducted five long-term studies: a 24-month study exposing rats to ozone, a 24-month study exposing mice to ozone, a 30-month study exposing rats to ozone, a 30-month study exposing male mice to ozone, and a 24-month cocarcinogenesis study exposing rats to ozone and the lung carcinogen 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Details about the NTP ozone studies can be found in the *Technical Report on the Toxicology and Carcinogenesis Studies of Ozone and Ozone/NNK in F344/N Rats and B6C3F$_1$ Mice* (National Toxicology Program 1995; Boorman et al. 1995b) and in Part VI of this Research Report (Boorman et al. 1995a).

STUDIES FUNDED BY THE HEALTH EFFECTS INSTITUTE

Six groups of scientists participated in the HEI component of the NTP/HEI Collaborative Ozone Project:

- The Steering Committee consisted of members of the HEI Health Research Committee, HEI staff, the project manager for the NTP studies, and outside consultants. The main goal of this Committee was to develop research priorities.
- The HEI Health Research Committee issued Requests for Applications, made funding decisions, and provided guidance during the research stage of the Project.
- Scientists with expertise in pulmonary physiology, toxicology, cell biology, and biostatistics reviewed and ranked the research applications.
- The staff of Battelle Pacific Northwest Laboratories were the NTP contractors for the ozone exposures. They also facilitated the preparation and distribution of tissue samples and animals to the HEI investigators and conducted histopathologic investigations of all HEI animals.
- Eight investigator teams conducted the experimental studies; a ninth investigator team developed an animal allocation scheme, assisted some individual investigators in data analysis, and worked with all of the investigators to perform an integrative analysis of the data from all of the studies.
- Members of the HEI Health Review Committee, staff, and outside consultants reviewed the individual Investigators' Reports and the Integrative Summary. In this Commentary, the Health Review Committee provides

an evaluation of the investigators' integrative analysis and discusses the implications of these results for human health.

The Steering Committee was faced with the challenge of obtaining the maximum amount of information from a limited number of animals. To accomplish this, it set two major priorities to address the most important issues:

- Focus on studies that would provide information on whether ozone causes permanent changes in the respiratory tract of animals exposed to ozone; and
- Adopt an integrated approach that would subsequently allow correlation of the results of the biochemical, structural, and functional studies.

The HEI Health Research Committee incorporated these broad recommendations into a Request for Applications that was issued in 1990, entitled,"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." Twenty-six proposals were submitted in response to this Request for Applications and were evaluated by two ad hoc review panels. The HEI Health Research Committee selected eight investigator teams on the basis of the scientific merit of their applications, demonstrated expertise, and the likely contribution of the work to a coherent research program. Figure 1 of the Integrative Summary illustrates the objectives of the HEI studies and the relation of the NTP and HEI investigations. Throughout the course of the project, HEI conducted workshops and meetings to encourage investigators to interact and develop plans for individual and integrated analyses. Thus, the final project was a hybrid of HEI-managed and investigator-initiated research.

The following Commentary, prepared by the HEI Health Review Committee and staff, provides a background on the National Ambient Air Quality Standard (NAAQS) for ozone and summarizes the results of earlier studies of prolonged exposure of laboratory animals to ozone. The Commentary evaluates the strengths and limitations of the NTP/HEI Collaborative Ozone Project and the Integrative Summary. It concludes with a discussion of what the Health Review Committee thinks the results tell us about the potential for ozone to contribute to or exacerbate chronic lung diseases.

REGULATORY BACKGROUND

Ozone is an oxidant gas and a major component of photochemical smog. 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." In addition, Section 109 of the Clean Air Act provides for the establishment of air quality standards to protect the public health.

In 1971, the EPA promulgated an NAAQS of 0.08 parts per million (ppm) for photochemical oxidants (ozone and other oxidants such as nitrogen dioxide and hydrogen peroxide) that should not be exceeded for more than one hour, once per year. Because ozone is the principal oxidant in photochemical smog, and improved instrumentation for measuring ambient ozone had become available, the form of the NAAQS was changed in 1979 from "photochemical oxidants" to "ozone." The new standard was set at 0.12 ppm and retained the hourly average, which was also not to be exceeded more than once per year (reviewed by McKee 1993a). The EPA classifies nonattainment areas according to the degree to which they exceed the NAAQS and assigns a primary standard attainment date for each classification.

The Clean Air Act also requires the EPA to evaluate the air quality criteria to ensure that they reflect the latest scientific information relevant for each criteria pollutant every five years. For ozone, this was last done during the period from 1986 to 1992 (U.S. Environmental Protection Agency 1986, 1992). This review formed the basis for the EPA Administrator's decision in August of 1992 to not revise the existing one-hour primary and secondary standards. However, as part of a court mandate, the EPA is currently reevaluating the ozone standard. In April 1994, the Agency released a draft criteria document that reviewed the scientific data on ozone toxicity that had become available since 1986.

BASIS FOR THE CURRENT ONE-HOUR STANDARD

When the EPA selected the level for the current one-hour primary ozone standard in 1979, it considered the lowest levels of ozone that had been shown to cause adverse effects, potentially sensitive populations, the nature and severity of demonstrated health effects, and a reasonable margin of safety. Several controlled human exposure studies formed the basis of the Agency's decision. One key study was that of DeLucia and Adams (1977), who reported cough, dyspnea, and wheezing, together with small, statistically insignificant, decrements in lung function in adults with asthma exposed to 0.15 ppm ozone for one hour while exercising. Although quantitative information on human health effects of ozone inhalation was limited, qualitative

information suggested the possibility of adverse health effects occurring at ozone exposure concentrations below 0.15 ppm. Therefore, the EPA Administrator concluded that a standard of 0.12 ppm ozone was necessary and prudent to protect public health (reviewed by McKee 1993b).

PROPOSALS FOR A MULTIHOUR OR ANNUAL STANDARD

One rationale for the original one-hour ozone standard was to relate it to acute exposures of the type experienced by people in Los Angeles in the 1970s when ozone concentrations peaked rapidly during rush hour and then dropped. We now know that the typical ozone exposure pattern is not a one-hour peak. Ambient measurements have shown that during air pollution episodes, humans may be exposed to elevated ozone levels for several hours or even days, rather than to peaks lasting for one to two hours (Rombout et al. 1986, 1989; Lioy and Dyba 1989; van Bree et al. 1990).

The current ozone standard is largely based on data from laboratory and epidemiologic studies that demonstrate transient symptoms and pulmonary function changes in people exposed to ozone for relatively short periods of time (a few hours). In controlled exposure studies, short-term ozone exposures have been shown to produce a variety of transient pulmonary responses, including decreased inspiratory capacity, increased breathing frequency, nose and throat irritation, mild bronchoconstriction, airway hyperresponsiveness, cough, and the subjective symptoms of pain on deep inspiration and shortness of breath (reviewed by the U.S. Environmental Protection Agency 1986; Lippmann 1992, 1993). Individuals exposed to ozone show a reduction in some pulmonary function measurements including forced vital capacity and forced expiratory volume in one second (FEV_1). Changes in FEV_1 have been observed at ozone levels as low as 0.08 ppm in moderately exercising subjects who were exposed for 6.6 hours (Horstman et al. 1990). When subjects were exposed to ozone on several consecutive days, these symptoms abated (Hackney et al. 1977; U.S. Environmental Protection Agency 1986). The mechanisms by which the lungs appear to adapt to ozone exposure are unknown.

Epidemiologic studies also show that decrements in human pulmonary function occur during short-term exposure to ozone. For example, pulmonary function declined in children engaged in moderate activity in ambient air containing concentrations of ozone less than 0.12 ppm (Lioy et al. 1985; Spector et al. 1988, 1991). Despite the fact that the pulmonary function changes reversed as the levels of ambient ozone decreased, these observations raise concern that the current standard may not adequately protect human health.

Another concern is whether exposure to ozone damages cells that line the airways and precipitates an inflammatory response. These responses have, for the most part, been studied in animals. However, Seltzer and coworkers (1986) exposed healthy human subjects to 0.4 or 0.6 ppm ozone for two hours. They reported that the percentage of neutrophils increased nine-fold in samples of bronchoalveolar lavage fluid recovered from the subjects three hours after the end of the exposure period. Increased levels of neutrophils and other markers of inflammation were found in bronchoalveolar lavage samples of exercising human subjects after they had been exposed either for two hours to 0.4 ppm ozone (Koren et al. 1989) or for 6.6 hours to concentrations as low as 0.10 or 0.08 ppm ozone (Devlin et al. 1991). These observations have led to speculation that exposure to ozone may be a risk factor for pulmonary fibrosis, because repeated inflammatory episodes in the lung parenchyma are thought to be factors in the development of diffuse pulmonary fibrosis in humans (Crystal et al. 1991).

Humans are often exposed for several hours or days to ozone concentrations equal to or above those that have been shown to produce changes in lung function or the appearance of inflammatory markers. Because of this, it has been suggested that the NAAQS for ozone should be changed to incorporate a multihour (for example, eight-hour) or annual averaging time. Van Bree and associates (1990) proposed that lowering the NAAQS below 0.12 ppm for a multihour ozone standard might be more representative of ambient exposure conditions and therefore a better predictor of the harmful effects of daily exposures to ozone (Rombout et al. 1986; Van Bree et al. 1990; Lippmann 1993). Lippmann (1993) suggested that an annual or seasonal averaging time, in addition to a multihour standard for ozone, should be considered.

SCIENTIFIC BACKGROUND

EXPERIMENTAL APPROACHES TO UNDERSTANDING THE EFFECTS OF PROLONGED OZONE EXPOSURE

Despite the fact that ozone exposure has the potential to contribute to the development of chronic lung diseases, it is difficult to document that this occurs. Of most relevance would be human studies of ozone exposure. However, the field studies and controlled chamber studies, which have contributed important information on acute respiratory effects, do not provide information about the consequences of prolonged exposure, nor do they address whether ozone exposure causes or exacerbates pulmonary fibrosis, emphysema, asthma, or chronic bronchitis. The epidemiologic studies that have attempted to address these issues have

important limitations. For example, the Adventist Health Smog Study provided new and useful data (Hodgkin et al. 1984; Euler et al. 1987, 1988; Abbey et al. 1991a,b). The original conclusion of these studies was that residence in the South Coast Air Basin in California was estimated to cause a 15% increase in the prevalence of chronic obstructive pulmonary disease. However, no estimates of ozone exposure were available; therefore, the data were considered to be of limited value. When the data were analyzed taking into account time spent indoors, a small effect was seen for the cumulative prevalence of asthma and chronic bronchitis. A cumulative total suspended particle effect also was noticed, however, and it is very difficult to partition the effects between ozone and particles.

Currently, studies in laboratory animals provide the best source of information on the effects of prolonged exposure to ozone on the respiratory system. Because such studies are expensive and labor-intensive, only a limited number of bioassays have been conducted with laboratory animals that were exposed to ozone for longer than 6 months. Studies conducted in dogs (Freeman et al. 1973) used ozone concentrations (2 ppm) that were not relevant to ambient conditions for humans. Three more recent studies that have focused on long-term ozone exposure in animals are discussed in this Commentary:

- Investigators at the University of California at Davis conducted studies with rats and primates exposed to ozone for 90 days (Boorman et al. 1980; Barr et al. 1988) or one year (Last et al. 1984; Reiser et al. 1987) (referred to as the UC Davis studies).
- Researchers at the General Motors Research Laboratories exposed male F344/N rats to 0.125, 0.25, or 0.5 ppm ozone or clean air for 20 hours per day, seven days per week, for 52 weeks (Filipowicz and McCauley 1986; Gross and White 1987), after which time some animals were allowed to recover in clean, filtered air for 12 weeks (referred to as the General Motors studies).
- The EPA's Health Effects Research Laboratory conducted an 18-month ozone exposure study in male F344/N rats (Grose et al. 1989) (referred to as the EPA studies). The exposure regimen was designed to mimic urban ozone exposures. Rats were exposed to a continuous ozone background level of 0.06 ppm for 22 hours per day, seven days per week. For five days a week, a nine-hour ozone "ramped spike" (to a maximum concentration of 0.25 ppm, producing an integrated concentration 0.19 ppm) was superimposed on this background exposure. The study included morphometric (Chang et al. 1992) and functional (Tepper et al. 1991; Costa et al. 1995) studies designed to evaluate the progression of ozone-induced effects.

NASAL CAVITY AND CONDUCTING AIRWAYS

Data on the effects of prolonged ozone exposure on the nose and conducting airways indicate that the nose is very sensitive to ozone. The nose absorbs a large fraction of the ozone dose in obligate nose-breathers such as the rat. Harkema and colleagues (1987a,b) reported that the nasal transitional and respiratory epithelia and the cilia were damaged in monkeys exposed to 0.15 or 0.3 ppm ozone for 90 days. The number of mucous cells in both types of epithelia increased markedly, and the amount of intraepithelial mucous components increased in the transitional epithelium. Interestingly, the increased number of mucous cells depended on the duration of exposure rather than the ozone level. Thus, ozone exposure alters two components (mucus and cilia) that are essential for the proper function of the mucociliary clearance system, which removes particles and microorganisms deposited in the nose and bronchi.

Other studies examined the effects of chronic ozone exposure on the conducting airways of laboratory animals. Tracheal epithelial ciliated cells and cilia were damaged in rats exposed to 0.96 ppm ozone for 60 days (Nikula et al. 1988) and in bonnet monkeys exposed to 0.15 or 0.3 ppm ozone for 90 days (Dimitriadis 1993). Tracheobronchial mucociliary clearance became progressively slower in rabbits exposed to a mixture of 0.1 ppm ozone and 125 $\mu g/m^3$ sulfuric acid for up to 12 months (Schlesinger et al. 1992).

STRUCTURAL INDICES OF LUNG DAMAGE IN THE CENTRIACINAR REGION OF THE LUNGS

It has long been recognized that the centriacinar region of the lungs, which is located at the junction of the conducting airways and the gas exchange region, is particularly sensitive to ozone exposure (Boorman et al. 1980; Barry et al. 1985; Plopper et al. 1991). This region consists of a terminal bronchiole, respiratory bronchioles (present in primates but not in rodents), alveolar ducts, and adjacent alveoli (Weibel 1963). In the centriacinar region, the cross-sectional area of the lung expands, leading to a greater surface area for gas transport. The conducting airways absorb a large fraction of ozone fairly evenly over their length, and the relatively thick mucous layer provides protection to the underlying airway epithelium. However, the tissue dose of ozone increases appreciably in the terminal portions of the conducting airways where the mucous layer becomes thin and the area of the exposed lung surface increases. The highest tissue dose of ozone predicted by mathematical models (Miller et al. 1985; Overton and Graham 1989; Grotberg et al. 1990) is at the acinar entrance, the same region found to be the primary site of tissue damage in animals exposed to ozone (Stephens et al. 1974; Mellick et al. 1977).

In the UC Davis studies, Boorman and coworkers (1980) conducted a detailed morphometric evaluation of the effects of prolonged ozone exposure on the centriacinar region of rats exposed to clean air or ozone for periods of up to 90 days. Their major observations were that ozone exposure (0.5 or 0.8 ppm) caused accumulation of macrophages in the centriacinar region, and airway segments between the terminal bronchiole and the alveolar duct took on the appearance of respiratory bronchioles. The terminal bronchiole usually ends abruptly and the alveolar epithelium begins by lining an alveolar duct. In contrast to control animals, rats exposed to 0.8 ppm ozone showed a gradual transition between the terminal bronchiole and alveolar duct. The changes after 90 days of exposure were less severe than those seen after 7 days of exposure, suggesting that adaptation to ozone exposure may have occurred. Transmission electron microscopy showed that the air-blood barrier thickened in rats exposed to 0.8 ppm ozone for 20 or 90 days compared with control animals. An increase in the relative volume of the lung interstitium, which was characterized by increased numbers of fibroblast cells and prominent bundles of collagen, also was noted. The lesions induced by ozone in the centriacinar region diminished as exposure progressed; however, significant morphologic alterations persisted after exposure for 90 days to 0.5 or 0.8 ppm ozone. These findings agree with the results of other studies that reported epithelial thickening and inflammation in the centriacinar region of rats exposed to a subchronic ozone regimen (Crapo et al. 1984; Barry et al. 1985).

Chang and colleagues (1992) used rats from the EPA studies to evaluate the development of lung injury at several time points during and after 18 months of ozone exposure. They found progressive epithelial and interstitial tissue responses in the proximal alveolar region after prolonged ozone exposure. These included epithelial hyperplasia, fibroblast proliferation, and the accumulation of interstitial matrix. The content of basement membrane and collagen fibers increased. Interstitial matrix accumulation during ozone exposure was largely reversible but the thickened basement membranes did not change. Acute tissue reactions were evident after one week of exposure. These subsided after three weeks of exposure and then increased progressively with prolonged ozone exposure. Most parameters returned to normal values during a recovery period in clean air.

Animal studies also provide evidence that inflammation is associated with prolonged exposure to ozone. In the General Motors studies, an inflammatory response was found in rats exposed to 0.5 ppm ozone for 6 or 12 months (Gross and White 1987). These lesions were described as an accumulation of mononuclear cells and fibroblasts and a slight increase in the number of macrophages. The inflammatory response disappeared after a six-month recovery period in clean air.

Ozone-induced damage to the centriacinar region has been studied in primates also. Fujinaka and coworkers (1985) reported that the volume of respiratory bronchioles increased (a condition called respiratory bronchiolitis), and the diameter of the respiratory bronchiolar lumen decreased in bonnet monkeys exposed to 0.64 ppm ozone eight hours per day for one year. Tyler and colleagues (1988) also reported respiratory bronchiolitis in male monkeys exposed to either a daily or "seasonal" regimen of 0.25 ppm ozone for eight hours per day. (The seasonal regimen consisted of ozone exposure during alternate months for 18 months.)

BIOCHEMICAL INDICES OF LUNG DAMAGE

It has been speculated that diffuse interstitial pulmonary fibrosis may result from repeated exposure to air pollutants (Costa et al. 1991). In humans, pulmonary fibrosis is characterized by an increase in the deposition of extracellular matrix components (collagen, elastin, and glycosaminoglycans) (Bjermer et al. 1989; Phan 1989). Therefore, considerable interest has arisen in examining changes in connective tissue in animals exposed to ozone. Various biochemical studies of ozone's effects on lung collagen content, type, biosynthetic rate, and degree of cross-linking were discussed in the HEI Health Review Committee's Commentary on Part I of this Research Report (Last et al. 1994). The results of several investigations seem contradictory, because some investigators found an increase in collagen content or biosynthetic rate (see the UC Davis studies: Last et al. 1979, 1984; Reiser and Last 1981; Tyler et al. 1988) and others did not (see the General Motors studies: Filipowicz and McCauley 1986; Wright et al. 1988). The reason for the conflicting results may well be the fact that ozone injury is very focal, and increases in localized collagen levels would be difficult to detect in an assay of whole lung tissue.

Because ozone is a powerful oxidant, interest in measuring levels of antioxidants has increased. From the EPA studies, Grose and colleagues (1989) reported that the levels of α-tocopherol (a compound found in the lung's surfactant layer that provides a protective effect by inactivating free radicals) were lower in the bronchoalveolar lavage fluid from rats exposed to the simulated urban ozone profile for 12 months than in lavage fluid from control animals. One interpretation of this finding is that α-tocopherol reacted with ozone, which decreased its level in the surfactant layer. However, the investigators also observed increased levels of glutathione peroxidase and glutathione reductase,

which suggests an increase in antioxidant metabolic activity (another defense mechanism against ozone-induced cell damage).

LUNG FUNCTION

As discussed earlier, short-term exposure to ozone causes transient decrements in some pulmonary function measurements in exercising or sensitive people. It also can cause inflammatory cells and other markers of inflammation to appear in bronchoalveolar lavage fluid. What is not known is whether these acute effects have a long-term impact on lung function. The results of studies that have addressed this issue are conflicting (see the HEI Health Review Committee's Commentary in Part V of this Research Report [Harkema and Mauderly 1994]).

As part of the EPA studies, Costa and coworkers (1995) evaluated static and dynamic lung function in rats. Male F344/N rats were evaluated after being exposed to the simulated urban ozone profile for 1, 3, 13, 52, or 78 weeks. In addition, cohorts from the 13-, 52-, and 78-week exposure groups were evaluated after an additional 6, 27, and 17 weeks, respectively, of breathing clean air. Small (less than 10%), but statistically significant, reductions in total lung capacity and residual volume were observed after 13, 52, and 78 weeks of ozone exposure. Exposure to clean air did not completely reverse the reduction in total lung capacity. Also, respiratory system compliance was not affected by ozone exposure, but it decreased as a function of the recovery time in clean air. No effects on collagen related to ozone exposure were noted. The authors postulated that near life-long exposure of rats to ozone resulted in functionally restrictive or stiffened lungs without pulmonary fibrosis.

Tepper and coworkers (1991) studied changes in lung function (under conditions of ventilatory stress) in rats from the same EPA studies. During a challenge with carbon dioxide after ozone exposure, the investigators observed small, but statistically significant, changes in breathing patterns and breathing mechanics in unanesthetized rats after exposure to ozone for 1, 3, 13, 52, or 78 weeks, when compared with control rats. They also noted that ozone exposure caused an overall increase in expiratory resistance, possibly accounting for the rats decreased ventilation during challenge with carbon dioxide when compared with control animals.

In the General Motors studies, Gross and White (1987) measured pulmonary function (including measurements of lung volume, expiratory air flow, and the diffusing capacity of the lung for carbon monoxide [DL_{CO}]) before exposure, after 26 and 52 weeks of exposure, and after a 12-week recovery period at the end of exposure. The 52-week ozone exposure produced small, but statistically significant, increases in functional residual capacity and residual volume, and a decrease in DL_{CO}. All measurements returned to control levels after the 12-week recovery period, suggesting that the functional changes seen in the lungs in response to ozone were reversible.

In summary, studies of pulmonary function that have been conducted on animals exposed to ozone for many months have produced conflicting results. Small increases in residual volume have been reported in rats exposed to ozone for six months or more in some studies (Gross and White 1987), but in others, the change was in the opposite direction (Costa et al. 1995).

TECHNICAL EVALUATION

PROJECT DESIGN AND METHODS

The overall design of the NTP/HEI Collaborative Ozone Project was to expose 164 male and female F344/N rats to filtered air (0 ppm ozone) or to one of three concentrations of ozone (0.12, 0.50, or 1.0 ppm) (see Part VI of this Research Report [Boorman et al. 1995a] for details of the study design and protocol). The exposure regimen was six hours per day, five days per week, for 20 months. The ozone concentrations were selected to include the maximum concentration the animals would tolerate (1.0 ppm), the current NAAQS for ozone (0.12 ppm), and an intermediate concentration. It should be pointed out, however, that the cumulative ozone dose at the lowest exposure concentration is substantially higher than people living in heavily polluted areas would receive. One strength of the study, and one way it differed from many previous bioassays, is that the exposure protocol permitted an evaluation of concentration-response relations for both male and female animals after exposure to ozone throughout a substantial portion, if not all, of their lifetimes.

Limitations of the Study Design

The design of the HEI studies was directed, to some extent, by the NTP's protocol and procedures. This affected the number of studies that could be conducted and produced small sample sizes in some experimental groups. For example, the small number of animals exposed to 0.12 ppm ozone and the diverse set of parameters measured in this multi-investigator study did not allow optimal sample sizes for statistical power to be achieved in all instances. Also, experimental outcomes were measured at only one time point (20 months). The protocol required daytime exposure; however, because rats are nocturnal, their lack of relative activity during the daytime may have decreased the

effective dose of ozone to their respiratory tract. The HEI requested that exposure of the animals for its studies be terminated after 20 months to avoid confounding the results with the effects from leukemia and other naturally occurring degenerative diseases found in aged rats. All of the above restrictions limit, to some extent, the interpretation of the results. Most notably, conclusions on the progression of effects cannot be derived from this study.

The choice of species is always a major consideration for studies from which the results will be extrapolated to humans. Primates would be expected to resemble humans in their responses to ozone more closely than rodents. However, large-scale experimentation using primates is costly and difficult. Therefore, species differences must be kept in mind when interpreting the results of these studies. For example, rats are obligate nose breathers and humans breathe oronasally. Because of interspecies differences in the anatomy of the respiratory tract, differences also exist between rats and humans in the effective dose of ozone delivered to different regions of the respiratory tract.

One of the decisions that the HEI Research Committee made was to conduct most of the tests on the HEI animals one week after the ozone exposures terminated. During that one-week period, all animals were exposed to clean air. The Committee made this decision because it was not interested in measuring transient changes that are characteristic of acute responses to ozone. However, some of the observed effects might have reversed if the recovery period had been longer. Therefore, we cannot be certain that the ozone-induced changes observed in this study were permanent.

Ozone Exposures

The ozone exposures were carried out under rigorously controlled conditions and every effort was made to assure uniformity and accuracy of the ozone concentrations in the exposure chambers. Details on the generation and monitoring of ozone can be found in Part VI of this Research Report (Boorman et al. 1995a). A high degree of confidence can be placed in the reported ozone concentrations, which were 0.120 ± 0.006 ppm, 0.501 ± 0.023 ppm, and 0.998 ± 0.040 ppm (means ± SD) (Boorman et al. 1995a).

Quality Assurance

The exposure and necropsy procedures of the NTP/HEI Collaborative Ozone Project were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 24-month and 30-month studies were submitted to the NTP archives, these studies were audited by an independent quality assurance contractor. Rodents used in the Carcinogenesis Program of the NTP are produced and housed in optimally clean facilities to eliminate potential pathogens that may affect study results. The disease status of the rodents was monitored via serology from extra (sentinel) animals in the study rooms (Boorman et al. 1995b). There was no evidence of infection by common rodent pathogens.

Animal Allocation and Necropsy

The animal allocation scheme that was developed by Drs. Catalano and Ryan was a strength of the NTP/HEI Collaborative Ozone Project. Tissue samples from a single animal were shared by multiple investigators and many functional and biochemical studies were conducted on the same animal. This scheme allowed sufficient overlap among individual studies to facilitate an integrative analysis of data for the multiple outcomes without compromising the sample sizes for individual investigations. The animal allocation scheme is described in detail in Part VI of Research Report Number 65 (Boorman et al. 1995a).

In summary, the NTP/HEI Collaborative Ozone Project was carefully designed and well-executed. The selection of ozone exposure concentrations was based on a solid rationale and the inclusion of four exposure groups provided an opportunity to make concentration-response comparisons across a range of ozone levels. The exposure and necropsy procedures were carried out under rigorously controlled conditions that included appropriate quality assurance procedures. Moreover, the animal allocation scheme enhanced the study by providing the ability to integrate the results and to correlate the findings among the individual studies.

All of the HEI investigators were experienced with the methods selected for their individual studies and used the techniques appropriately. Thus, one can have a high degree of confidence in the findings. The collaboration among the investigators, coordinated by Dr. Kaden, resulted in an Integrative Summary that is scientifically stronger than the individual studies.

RESULTS AND INTERPRETATION

Parts I through X of the Research Report Number 65 on the NTP/HEI Collaborative Ozone Project contain details about the design and results of the individual studies. This section discusses the key results of the Project, especially those used by the investigators for their integrative analyses. (In addition to the individual Investigators' Reports, the key results are highlighted in the Integrative Summary and in Table 1 of this Commentary.) The section continues with an analysis of how the major results correlate with the three disease surrogates selected by the investigators (chronic

Table 1. Summary of Key Findings from the NTP/HEI Collaborative Ozone Project

Endpoint[a]	Ozone Concentration[b] (ppm) 0.12	0.5	1.0	Comments
Nose[1]				
Total epithelial cells	–	–	↑↑	Lateral walls and turbinates of the proximal nasal mucosa
Mucous cells	–	↑	↑↑	Lateral walls and turbinates of the proximal nasal mucosa
Stored mucosubstances	–	↑↑	↑↑↑	Lateral walls and turbinates of the proximal nasal mucosa
Mucous flow	–	↓	↓↓	Lateral walls and turbinates of the proximal nasal mucosa
Trachea[2]				
Stored secretory product	–	–	↓	Distal trachea
Nonciliated cell volume	–	–	–	
Epithelial thickness	–	–	–	
Antioxidant enzymes	ND	–↑	–↑	Increased for superoxide dismutase, not for glutathione peroxidase and glutathione S–transferase
Bronchus				
Morphometry and Histochemistry				
Stored secretory product[2]	–	–	↑	Increased for cranial and caudal bronchi, not central bronchus
Nonciliated cell volume[2]	–	–	–	
Epithelial thickness[2]	–	–	–	
Antioxidant enzymes[2]	ND	–↕	–↕	No change for superoxide dismutase and glutathione S–transferase; glutathione peroxidase decreased in the major daughter bronchus, and increased in the minor daughter bronchus
Area of large airway walls[3]	–	–	–	
Area of smooth muscle in large airways[3]	–	–	–	
Area of small airway walls[3]	–	↑	–	Increased in male rats only
Area of smooth muscle in small airways[3]	–	↑	–	
In Vitro Responses of Isolated Large Airways[3]				
Tension	–	–	↑	Increased for passive but not active tension
Stress	–	–	↑	Increased for passive but not active stress
Contractile response (tension)	–	–	–	
Contractile response (stress)	–↕	–	–↕	Variable responses to different stimuli
Prostaglandin E_2 release	–	–	–↑	Increased in percentage but not in total amount
Leukotriene C_4 release	–	–	–	

(Table continues next page.)

[a] Footnote numbers indicate the Investigator's Report that discusses this endpoint: 1 = Harkema et al. 1994; 2 = Pinkerton et al. 1995; 3 = Szarek 1994; 4 = Chang et al. 1995; 5 = Last et al. 1994; 6 = Radhakrishnamurthy 1994; 7 = Parks and Roby 1994; 8 = Harkema and Mauderly 1994.

[b] ↑ = statistically significant increase; ↓ = statistically significant decrease; – = no change; ( ) = changes that were either not statistically analyzed or not statistically significant; ↕= variable results; ? = inconsistent results; ND = not determined. The number of arrows indicates the relative magnitude of the findings.

Table 1. Summary of Key Findings from the NTP/HEI Collaborative Ozone Project *(continued)*

Endpoint[a]	Ozone Concentration[b] (ppm) 0.12	0.5	1.0	Comments
In Vitro Responses of Isolated Small Airways[3]				
Tension	–	–	–	Active and passive tension
Stress (passive)	↓	–	–	
Stress (active)	–	↓	↓	
Contractile response (tension)	–	–	–	
Contractile response (stress)	–↕	↓	–	Decreased stress at 0.5 ppm ozone for 4 of 5 stimuli in male rats only
Proximal Bronchioles				
Epithelial thickness[2]	–	–	–	
Nonciliated epithelial cells[2]	–	–	–	
Terminal Bronchioles				
Epithelial thickness[2]	–	–	–	
Nonciliated epithelial cell volume[2]	–	–	↑	
Diameter[4]	–	–	–	
Epithelial thickness[4]	–	–	–	
Number of cells[4]	–	–	↓	
Surface density of cilia[4]	–	–	↓	
Antioxidant enzymes[2]	ND	↑	↑	Superoxide dismutase and glutathione peroxidase
Centriacinar Region[2]				
Septal tip thickness	–	(↑)	(↑)	Combined data for cranial and caudal regions presented; increased in male rats exposed to 0.5 ppm ozone; no statistical analysis performed
Interstitial volume	–	–	↑	Combined data for cranial and caudal regions presented
Epithelial thickness (measured)	?	(↑)	(↑↑)	Combined data for cranial and caudal regions presented; ↑ to 300–600 μm in male rats exposed to 0.5 or 1.0 ppm ozone; questionable increase to 200 μm (observed in male rats exposed to 0.12 ppm ozone by one method of analysis, not confirmed by another); no statistical analysis performed
Epithelial thickness (model)	?	↑	↑↑	Cranial alveolar duct only: statistically significant increase in both genders exposed to 1.0 ppm ozone, and in male rats exposed to 0.12 or 0.5 ppm ozone
Extension of nonciliated columnar cells down the alveolar duct (visual assessment)	(↑)	(↑)	(↑↑)	Cranial alveolar duct: apparent increase in both genders exposed to 1.0 ppm ozone, and in female rats exposed to 0.5 ppm ozone Caudal alveolar duct: apparent increase in both genders exposed to 0.12 or 1.0 ppm ozone, and in male rats exposed to 0.5 ppm ozone No statistical analysis performed

(Table continues next page.)

[a] Footnote numbers indicate the Investigator's Report that discusses this endpoint: 1 = Harkema et al. 1994; 2 = Pinkerton et al. 1995; 3 = Szarek 1994; 4 = Chang et al. 1995; 5 = Last et al. 1994; 6 = Radhakrishnamurthy 1994; 7 = Parks and Roby 1994; 8 = Harkema and Mauderly 1994.

[b] ↑ = statistically significant increase; ↓ = statistically significant decrease; – = no change; ( ) = changes that were either not statistically analyzed or not statistically significant; ↕= variable results; ? = inconsistent results; ND = not determined. The number of arrows indicates the relative magnitude of the findings.

Table 1. Summary of Key Findings from the NTP/HEI Collaborative Ozone Project *(continued)*

Endpoint[a]	Ozone Concentration[b] (ppm) 0.12	0.5	1.0	Comments
Proximal Alveolar Region[4]				
Bronchiolarization	–	↑	↑↑	
Epithelial metaplasia	–	↑	↑↑	
Interstitial volume	–	↑	↑↑	
Interstitial macrophages	–	–	–	
Alveolar macrophages	–	–	↑	
Random Alveolar Regions[4]				
Bronchiolarization	–	–	–	
Epithelial thickness	–	–	–	
Interstitial volume	–	–	–	
Inflammatory cells	–	–	–	
Biochemical Changes				
Total collagen (males)[5]	–	–	–	
Total collagen (females)[5]	–	(↑)	(↑)	Statistically significant trends in female rats for 4-hydroxyproline, hydoxypyridinium, and cross-link ratios when data expressed on a per lobe basis
Total glycosaminoglycans[6]	–	↓	↓	
Individual glycosaminoglycans[6]	–	↑↓	↑↓	One increased and three decreased
Tropoelastin messenger RNA[7]	ND	ND	–	Results of small pilot study indicated increases after 2 months of exposure to 1.0 ppm ozone
α1(I) Collagen messenger RNA[7]	ND	ND	–	Results of small pilot study indicated increases after 2 months of exposure to 1.0 ppm ozone
Pulmonary Function[8]				
Airway function (total pulmonary resistance, 50% forced midexpiratory flow, forced vital capacity, mean midexpiratory flow)	–	–	–	
Lung parenchyma (vital capacity, quasistatic chord compliance, total lung capacity, functional residual capacity)	–	–	–	
Residual volume	(↓)	↓	(↓)	Subanalysis by gender statistically significant for female rats exposed to 0.5 ppm ozone
Gas transfer (DL_{CO}, DL_{CO}/alveolar lung volume)	–	–	–	

[a] Footnote numbers indicate the Investigators' Report that discusses this endpoint: 1 = Harkema et al. 1994; 2 = Pinkerton et al. 1995; 3 = Szarek 1994; 4 = Chang et al. 1995; 5 = Last et al. 1994; 6 = Radhakrishnamurthy 1994; 7 = Parks and Roby 1994; 8 = Harkema and Mauderly 1994.

[b] ↑ = statistically significant increase; ↓ = statistically significant decrease; – = no change; ( ) = changes that were either not statistically analyzed or not statistically significant; ↕ = variable results; ? = inconsistent results; ND = not determined. The number of arrows indicates the relative magnitude of the findings.

rhinitis, chronic airway disease, and centriacinar fibrosis), and concludes with a discussion of the implications of the findings for human health.

General Findings

The survival rates of rats exposed to the three concentrations of ozone for the NTP bioassay were similar to those of the control animals (Boorman et al. 1995b). Of the rats assigned to HEI studies, 5 of 44 control rats, 8 of 32 rats exposed to 0.12 ppm ozone, 5 of 44 rats exposed to 0.5 ppm ozone, and 2 of 44 rats exposed to 1.0 ppm ozone died either before the end of the 20-month exposure period or during the one-week recovery period in clean air. All rats were necropsied. The mean body weights and rate of body weight gain were slightly lower in the rats exposed to 1.0 ppm ozone throughout most of the study. This indicates that the 1.0-ppm dose was close to the maximal dose that could be tolerated by this strain of rat. The NTP scientists also noted that animals exposed to ozone, particularly those exposed to 1.0 ppm, were less active during and immediately after exposure, than those exposed to clean air (Boorman et al. 1995b).

Two other general findings are relevant to the interpretation of the results. First, mononuclear cell leukemia, which is common in aged rats, was observed in 25% of the animals. The incidence of leukemia was similar in control rats and rats exposed to ozone, which suggests that ozone did not induce a leukemic effect. In approximately half of the rats with leukemia, the disease was considered to be advanced and to have marked pulmonary involvement (Boorman et al. 1995b). It is not known what effect, if any, the mononuclear cell leukemia may have had on the data generated from these rats by different investigators. Second, no positive serologic reactions for a variety of rat pathogens were observed during the 20-month exposure period. Thus, we can be reasonably sure that pulmonary infection was not a confounding factor in the interpretation of the results.

Nasal Cavity

Some of the most striking effects of prolonged ozone exposure were found in the nasal cavity (Harkema et al. 1994; Tables 2 and 3, and Figures 5 and 13 of the Integrative Summary). A clear relation was established between ozone exposure levels and changes in nasal function and structure. Measurements performed in three regions of the proximal nasal passage (the lateral wall, the septum, and the nasoturbinates), which are known from short-term exposure studies to be particularly sensitive to ozone, showed that rats exposed to 1.0 ppm ozone had decreased mucous flow rates and increased volume densities of intraepithelial mucosubstances (mucous hypersecretion), compared with rats exposed to 0 or 0.12 ppm ozone. Rats exposed to 0.5 ppm ozone showed the same changes, but to a lesser degree. Mucous cell metaplasia (the transformation of an epithelium with no mucous cells into an epithelium with numerous mucous cells) was seen in the nasal transitional epithelium of rats exposed to 0.5 or 1.0 ppm ozone. No changes were seen in animals exposed to 0.12 ppm ozone. In general, the changes induced by ozone that were observed in the nasal passages of the NTP/HEI animals resembled those seen in other studies of ozone's acute effects on the nasal passages of monkeys and rats (Harkema et al. 1987a,b, 1989).

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 a bony atrophy in both male and female rats. Instead, mucous cell metaplasia may have contributed to these conditions by producing an excessive amount of mucosubstances that may have altered the normal properties of the nasal mucus. The investigators' results suggest that exposure to concentrations of at least 0.5 ppm ozone can decrease the rate of mucociliary clearance, an important defense mechanism that protects the upper respiratory tract against infection and aids in protecting the lung from inhaled pollutants.

Extrapolating the findings in the nasal cavity of the rat to humans is difficult. First, rats are obligate nose-breathers and humans breath through both mouth and nose; moreover, several differences exist between the gross architecture of rodent and primate nasal passages. These are chiefly related to the structure and number of nasal turbinates (structures projecting from nasal walls that compose the primary air filtration network in the nasal cavity). Rats have a complex turbinate structure, in contrast to the turbinate structure in primates, which is minimal (Plopper et al. 1991). Thus, the amount of ozone removed from incoming air in the nasal passages differs between rodents and primates. Several other potential differences between the nasociliary apparatus of rodents and humans may make direct comparisons between the two species difficult. For example, the rate of repair of nasal mucosal cells during recovery from exposure may be different. The sensitivity of the glandular elements to ozone may influence the relative amounts of mucosubstances produced. Finally, differences in host responses to secondary infections, which affect the extent and degree of inflammatory changes seen in the nose, may influence comparisons.

Conducting Airways

Few effects of prolonged ozone exposure were observed in the large airways. The most notable finding was a de-

crease in the amount of stored secretory products containing carbohydrates in the distal trachea of rats exposed to 1.0 ppm ozone compared with control rats. Also, the activity of one antioxidant enzyme, superoxide dismutase, increased in rats exposed to 0.5 or 1.0 ppm ozone. (Antioxidant enzyme activities were not measured in rats exposed to 0.12 ppm ozone.) No changes were found in the activity of glutathione peroxidase and glutathione S–transferase (Pinkerton et al. 1995; Figures 12 and 20 in the Integrative Summary).

Rats exposed to 0.12, 0.5, or 1.0 ppm ozone showed no statistically significant structural or functional changes in isolated large bronchi compared with rats breathing clean air (Szarek 1994; Pinkerton et al. 1995). However, changes related to ozone exposure were seen in the small bronchi. At the highest ozone concentration, statistically significant increases in the amount of stored secretory products were seen in the bronchi in the cranial and caudal regions of the lungs compared with levels in the control animals.

Szarek (1994) isolated bronchial segments and measured changes in the area of the airway wall and in the amount of smooth muscle. He reported statistically significant increases in these parameters at some, but not all, ozone concentrations. He also used a variety of stimuli in vitro to measure the mechanical properties and the contractile responses of isolated small bronchial airways. The contractile responses were expressed as either tension (force generated per unit length of airway) or stress (force generated per unit area). For each parameter (tension or stress), the responses were expressed in two ways: maximal response, or the effective concentration or frequency of stimulant that elicited a half maximal response. Therefore, in interpreting the authors' conclusions about the consistency among concentration-response relations for functional effects in the small bronchi (see the section in the Integrative Summary entitled "How are the structural, functional, and biochemical changes in the lung interrelated?"), the reader should be cautioned that multiple endpoints were generated by this study and consistent effects were found only for the maximal stress values in male rats exposed to 0.5 ppm ozone (Table 1 in the Health Review Committee's Commentary in Szarek 1994). From a physiologic standpoint, it is not clear how the measurements of altered structure and contractility in isolated small airways relate to airway function in vivo. Despite an apparent increase in the amount of smooth muscle in the walls of the small airways of rats exposed to one concentration of ozone, the stress parameters decreased rather than increased, as would have been expected from an increase in muscle mass. In addition, no changes in active or passive tension were noted. In summary, only one parameter of airway contractile function was altered by ozone exposure and the observed changes were not related to concentration. This suggests that ozone exposure in this study had minimal, if any, important effects on smooth muscle function.

In the section of the Integrative Summary entitled "Mechanical Properties of Airways and Contractile Responses," the significance of the eicosanoid results from isolated large bronchi could be misinterpreted. As indicated in Table 6 of the Integrative Summary, what is noted as a statistically significant two-fold increase in the release of prostaglandin E_2 induced by a calcium ionophore in large airways isolated from rats exposed to 1.0 ppm ozone actually represents an increase in the percentage of change in the amount released, not a change in the total amount of prostaglandin E_2 that was released. The increase in the percentage of change was driven as much by the low basal levels of prostaglandin E_2 in rats exposed to 1.0 ppm ozone as by increased synthesis. These factors limit the biological significance of this observation.

Centriacinar Region

As expected from earlier studies (Boorman et al. 1980; Crapo et al. 1984; Barry et al. 1985; Barr et al. 1988; Chang et al. 1992), a major effect of ozone exposure was on the epithelium that lines the centriacinar region of the lungs. At the light microscopic level, histopathologic changes were seen in the centriacinar region of the lungs of animals exposed to 0.5 or 1.0 ppm ozone (Boorman et al. 1995a,b). These changes consisted of interstitial thickening and the appearance of ciliated and secretory bronchiolar epithelial cells in the alveolar ducts and adjacent alveoli. The Battelle Pacific Northwest Laboratory pathologists characterized these lesions as minimal to mild in the animals exposed to 0.5 ppm ozone, and as mild to moderate in those exposed to 1.0 ppm ozone. Minimal to mild macrophage infiltration also was present in most of the rats exposed to 1.0 ppm ozone.

Drs. Chang and Pinkerton (Parts VIII and IX of Research Report Number 65, respectively) used both light microscopy and electron microscopy to examine different sites in the centriacinar region. Very few changes were seen in the terminal bronchioles by either investigator. Of the many different morphometric parameters examined, the only statistically significant findings in the terminal bronchioles occurred in animals exposed to 1.0 ppm ozone (Pinkerton et al. 1995).

In the proximal alveolar region, however, changes induced by ozone in the epithelium and interstitium were readily apparent in rats exposed to 0.5 or 1.0 ppm ozone (Chang et al. 1995). The thin, squamous type of epithelium in the alveolar ducts was replaced with a well-differentiated bronchiolar epithelium in a process known as bron-

chiolarization. The bronchiolarized epithelium consisted of ciliated bronchiolar cells and nonciliated bronchiolar Clara cells in rats exposed to 0.5 or 1.0 ppm ozone. Remodeling extended for as many as five generations of branching into the pulmonary acinus, and was observable at a distance of up to 1000 μm into the alveolar duct in rats exposed to 1.0 ppm ozone (Pinkerton et al. 1993, 1995; Figures 6, 7, and 8 in the Integrative Summary).

A mild fibrotic response, which was characterized by increased connective tissue matrix constituents, including collagen, elastin, and basement membrane, occurred in the proximal alveolar region of rats exposed to 0.5 or 1.0 ppm ozone (Chang et al. 1995; Figure 9 in the Integrative Summary). The increased volume of extracellular matrix was accompanied by an increased volume of fibroblasts. The only sign of an inflammatory response was an increase in the volume of alveolar macrophages in animals exposed to 1.0 ppm ozone. No macrophages were found in the interstitium. The extracellular matrix of the rats exposed to 0.12 ppm ozone did not differ from that of animals exposed to clean air (Chang et al. 1995; Pinkerton et al. 1995).

Pinkerton and colleagues (1995) measured bronchiolarization using three different parameters: epithelial thickness, epithelial volume, and the degree to which ciliated cells extended down the alveolar duct. In agreement with the findings of Chang and associates (1995), these investigators also observed bronchiolarization of the alveolar ducts in rats exposed to 0.5 or 1.0 ppm ozone. However, in addition to increases in the centriacinar region of rats exposed to 0.5 or 1.0 ppm ozone, they also reported statistically significant epithelial changes in regions closer to the junction of the bronchioles and alveolar ducts after exposure to 0.12 ppm ozone. In contrast, Chang and colleagues did not detect any changes in rats exposed to 0.12 ppm ozone. Pinkerton and coworkers used a sophisticated sampling strategy, and their choice of multiple sampling locations may have enabled them to detect changes after exposure to 0.12 ppm ozone that would not have been detected by other sampling methods. They examined the whole centriacinar region from the bronchiole–alveolar duct junction to the most distal alveolar ducts as a function of distance. Chang and colleagues studied cross-sections of the terminal bronchiole and of small airways from the level at which the first generation alveolar duct branches (which they referred to as the proximal alveolar region). Because Chang and colleagues did not examine regions closer to the bronchiole–alveolar duct junction (which were included in the study of Pinkerton and coworkers), their method could not have detected changes induced by 0.12 ppm ozone that did not extend to the duct bifurcation.

However, the interpretation of the observations of Pinkerton and colleagues (1995) is complicated by three factors. First, the conclusion that exposure to 0.12 ppm ozone had an effect on bronchiolarization was based primarily on the investigators' use of a small subset of the large amount of experimental data (specifically, epithelial volume density) to construct a mathematical model. Using their model, Dr. Pinkerton and colleagues (1995) reported statistically significant increases in the volume density of epithelial cells in alveolar ducts from the cranial region of lungs of male rats at all ozone exposure concentrations. In contrast, statistically significant increases were seen only in the female rats exposed to 1.0 ppm ozone. The statistically significant effect seen in the cranial region in male rats exposed to 0.12 ppm ozone appears to be influenced by aberrantly high epithelial cell volume densities within the first 200 μm down the alveolar ducts in control rats (Table 25 in Pinkerton et al. 1995). In the first 200 μm of the cranial region of male rats, epithelial cell volume density and, therefore, bronchiolarization was greater in the control group than in rats exposed to ozone. In addition, these control values for the first 200 μm of the cranial region of male rats were almost twice as high as the control values for the same 200 μm of the cranial region of female rats and of the caudal regions of both male and female rats. Thus, the values on which a statistically significant effect was established may be outliers; however, no test for outliers was performed to assess this possibility.

Second, according to the analysis of the model, effects were seen in the alveolar ducts in the cranial, but not the caudal, region of male rats exposed to 0.12 ppm ozone. However, effects induced by ozone on nonciliated epithelial cell volume in the terminal bronchioles were seen in the caudal, but not the cranial, region in both genders. Other data on bronchiolarization also show variability according to site. Thus, the consistency of effects seen in animals exposed to 0.12 ppm ozone is uncertain. Moreover, allowance for multiple testing, which could produce statistically significant differences purely by chance, would considerably reduce the statistical significance of these results.

Third, Dr. Pinkerton and colleagues collected a large amount of experimental data on bronchiolarization that was not used to construct their mathematical model. Although they did not perform statistical analyses on these data, qualitative examination suggests that the data provide weak support for the conclusion that significant bronchiolarization occurred in male rats exposed to 0.12 ppm ozone.

Because of these concerns, the biological significance of the effects seen in male rats exposed to 0.12 ppm ozone is

uncertain. The variable and minimal effects of exposure to 0.12 ppm ozone on the structure of the centriacinar region require further evaluation.

In general, the overall results of the structural studies agree with earlier observations of the effects of prolonged exposure to ozone on the structure of the centriacinar region of laboratory animals (Fujinaka et al. 1985; Barr et al. 1988; Chang et al. 1992). Specifically, the progressive epithelial and interstitial tissue responses that included epithelial hyperplasia, bronchiolarization of alveolar duct epithelium, and the accumulation of interstitial matrix constituents seen after prolonged exposure of rats to ozone in other studies were confirmed by the studies of Drs. Chang and Pinkerton and their colleagues for rats exposed for 20 months to 0.50 or 1.0 ppm ozone.

One difference between these results and those that have been published earlier is that the structural changes were less pronounced in the animals from this Project than those that have been reported. For example, in the EPA studies, Chang and colleagues (1992) observed morphologic changes in type I and type II epithelial cells in the alveolar epithelium of rats exposed to an ambient ozone pattern (continuous background concentration of 0.06 ppm ozone, on which an ozone spike [0.25 ppm with an integrated concentration under the peak of 0.19 ppm] was superimposed). The same group of investigators found no differences between the animals exposed to 0 ppm ozone and those exposed to 0.12 ppm in the NTP/HEI Collaborative Ozone Project. Also, inflammatory responses were reported in the centriacinar region of rats exposed to 0.5 ppm ozone for 90 days (Boorman et al. 1980) or 52 weeks (Gross and White 1987). Even at the highest ozone exposure concentrations, minimal or no signs of inflammation were seen in the lungs of the animals in this Project. It is possible that the extended exposure period in the NTP/HEI Collaborative Ozone Project allowed sufficient time for adaptation to occur. For example, the ozone-induced structural changes may have been present at 3 and 12 months, but not evident at 20 months. Or, changes induced by ozone may have regressed during the one-week recovery period. Alternatively, the animal husbandry conditions may have been a factor. The rats used in the NTP programs were produced in optimally clean facilities and housed under barrier conditions to eliminate potential pathogens that might affect the study results. In the past, pneumonia has been a problem in rat colonies (Brownstein 1985; Schoeb and Lindsey 1985), and this factor may have contributed to some of the histopathological lesions observed in earlier studies in which the ozone exposure may have been superimposed on a low-grade chronic infection.

Lung Biochemistry

It is known that the lung damage caused by exposure to ozone is very focal; therefore, small localized changes in some biochemical parameters may not be measurable in assays of whole tissues.

Last and colleagues (1994) reported that total lung collagen and the content of the hydroxypyridinium cross-link (a characteristic of mature collagen) did not change in male rats exposed to any concentration of ozone. However, they did observe statistically significant elevations in these two parameters in female rats exposed to 1.0 ppm ozone when the data were expressed per lung lobe (not per lung weight). (The data on total lung collagen are shown in Figure 10 of the Integrative Summary.) The expression of genes that code for two connective tissue proteins, $\alpha 1$(I) procollagen (one of the chains of type I collagen) and tropoelastin (the monomer of elastin), was not increased in lung tissue from rats exposed to 1.0 ppm ozone for 20 months (Parks and Roby 1994). These findings suggest that continuous basal synthesis of collagen and elastin were not increased during prolonged exposure to ozone.

Another important class of connective tissue matrix components are glycosaminoglycans, most of which are components of larger molecules called proteoglycans. Radhakrishnamurthy (1994) found that the total content of glycosaminoglycans in lung lobes from rats exposed to 0.5 or 1.0 ppm ozone decreased to about 80% of that seen in control rats. The level of hyaluronan, the major glycosaminoglycan in normal rat lung, fell to approximately 68% and 59% of control values in the groups exposed to 0.5 or 1.0 ppm ozone, respectively. Statistically significant decreases in the content of two minor glycosaminoglycans, chondroitin 4-sulfate and chondroitin 6-sulfate, also were observed in rats exposed to 0.5 or 1.0 ppm ozone (Figure 11 in the Integrative Summary). The decreased content of glycosaminoglycans was in the opposite direction to that expected in diffuse pulmonary fibrosis. Therefore, the biological significance of these findings is uncertain.

Respiratory Function

Evaluating respiratory function is one of the best ways of interpreting the biological relevance of small structural and biochemical changes. Therefore, the Health Research Committee identified respiratory function studies as a high priority and issued a separate Request for Applications (90-1, Part A) soliciting applications for such studies in order to have the appropriate expertise for this component of the Project. It should be noted that this group had the

largest sample size, which provided the investigators with adequate statistical power to detect small effects if they were present.

One to six days after ozone exposure ceased, Drs. Harkema and Mauderly (1994) measured or calculated over 30 respiratory function parameters on anesthetized rats. These included breathing patterns, dynamic lung mechanics, lung pressure-volume characteristics, intrapulmonary gas distribution, expiratory flow limitation, and alveolar-capillary gas transfer. The most notable finding was that very few pulmonary function parameters were affected by ozone exposure. No effects of ozone exposure were detectable in tests of airway function (total pulmonary resistance, forced expiratory flow, forced vital capacity, mean midexpiratory flow), lung parenchymal function (vital capacity, quasistatic chord compliance, total lung capacity, functional residual capacity, residual volume), or gas transfer (DL_{CO}, DL_{CO}/alveolar lung volume). The only consistent ozone-related effect was a small reduction of residual volume. This trend was observed in most exposure groups, but was statistically significant only in rats exposed to 0.5 ppm ozone.

The finding of a small decrease in residual volume agrees with the result of Costa and coworkers (1995), but is in the opposite direction from that of Gross and White (1987), who observed a statistically significant increase in residual volume after exposing male F344/N rats to 0.5 ppm ozone for one year; residual volume returned to normal after the rats breathed clean air for 12 weeks.

Drs. Harkema and Mauderly (1994) hypothesized that the decrease in residual volume might be attributable to fibrotic changes in the terminal airways resulting in increased stiffness, which allowed more air to be expelled from the gas exchange region during exhalation before these small airways closed. However, this finding was significant only in rats exposed to 0.5 ppm ozone, and measurements of lung compliance gave no indication of increased lung stiffness. The investigators also suggested that the focal tissue stiffening induced by ozone exposure may not have been sufficiently severe or widespread to influence total lung elastic recoil by a measurable amount. Furthermore, they suggested that no statistically significant changes in residual volume were noted in the animals exposed to 1.0 ppm ozone because the increased stiffness that maintained the patency of the walls of the terminal airways was offset by a narrowing of the lumina caused by thickening of the walls in these same airways. Rats exposed to 1.0 ppm ozone had moderate to marked levels of stainable centriacinar collagen when compared with control rats, but rats exposed to 0.5 ppm ozone had variable amounts of this material (Last et al. 1994). Although the finding of increased collagen supports the hypothesis of an exposure-related stiffening of the terminal bronchiole–alveolar duct region, it does not explain why the residual volume values did not decrease in a concentration-dependent manner.

Drs. Harkema and Mauderly (1994) appropriately cautioned that the effects of ozone exposure in humans may not be as benign as those observed in the rats because the duration of tissue exposure is much longer in the course of a human lifetime. In addition, human exposures to ozone occur in association with other insults to lungs, including other air pollutants and tobacco smoke, and in the presence of existing lung disease. These factors could be important contributors to the extent and severity of the responses by the lungs to ozone exposure.

Nevertheless, the important conclusion of this Project is that the biochemical and structural changes observed in rats exposed to ozone were not severe enough to have a measurable impact on overall lung function, using the techniques available to detect abnormalities of pulmonary function in humans.

Integrative Analyses

The NTP/HEI Collaborative Ozone Project generated a powerful data base containing over 240 carefully measured endpoints. Although each study was designed to be interpreted as an individual entity, because of the careful tissue allocation scheme the Project provided a unique opportunity to integrate the results of individual studies and to evaluate the effects of ozone exposure on a broad spectrum of endpoints. During the course of the Project and after the laboratory studies had been completed, the investigators selected endpoints from their studies that were used for integrative analyses. The investigators used these endpoints to study consistency among concentration-response relations, to perform correlative analyses, and to group together selected endpoints to represent three disease surrogates. The meaning of the term "disease surrogates," as it is used in this Commentary, should be noted. The investigators grouped certain endpoints around some signs or characteristics of three diseases in order to provide a structure for their integrative analyses. The term disease surrogate does not imply that the animals actually developed the diseases. Instead, it refers to a grouping of endpoints that were considered by the investigators to be potentially related to different pathogenic processes. These disease surrogates, as defined by the investigators, are centriacinar fibrosis ("an increase in some of the interstitial components of the alveoli of the most proximal portions of the pulmonary acinus"), chronic airway disease ("a set of conditions that could impede airflow to and from the lungs by one or several mechanisms, alone or in combination"), and chronic rhinitis ("a set of pathological alterations in the nose, including

mucosal inflammation and proliferation of mucous cells, which results in an increase in intraepithelial and luminal mucus").

The section of the Integrative Summary entitled "Analytical Approaches" describes the three methods of analysis that the investigators used to integrate the results of the individual studies. In the first analytical approach, the consistency among concentration-response relations was evaluated. This type of comparison could be used when specific endpoints were not measured on the same animals, as well as when they were. The authors point out that, although different animals were used for different analyses, they had all been treated with identical exposure protocols. Thus, changes in endpoints observed on different sets of animals could be compared, and interpretations could be drawn from these comparisons. In the second analytical approach, they determined the extent to which different endpoints were correlated. When two endpoints were measured on the same animal, the authors were able to use direct pairwise correlations to evaluate the degree to which concurrent changes were present. This provided a quantitative assessment of the interrelations among some of the biochemical, structural, and functional measurements. The third analytical approach, referred to as "combined analyses," used a technique called median polish analysis that draws on the ideas of Tukey (1977). This technique allowed the investigators to combine data across studies when the relevant endpoints had not been measured on the same animals. The composite variables that were produced by the median polish analysis then were analyzed with standard statistical techniques. This approach was used to integrate the multiple endpoints related to each disease surrogate. A detailed description of the median polish analysis method and a Commentary on its use can be found in Part X of Research Report Number 65 (Catalano et al. 1995).

The authors posed four questions as a framework for presenting the results of their integrative analyses. Although these questions provide a reasonable format for this purpose, they are only a few of many that can be asked of these data. These four questions are discussed in the order in which they are presented in the Integrative Summary, and the results of each of the three analytic approaches are outlined for each question.

What Changes in Nasal Function, if Any, Are Correlated with the Structural Changes in the Nose? Rats exposed to 0.5 or 1.0 ppm ozone had mucous cell metaplasia, significantly greater volume densities of intraepithelial mucosubstances, and slower mucous flow rates than rats exposed to 0 or 0.12 ppm ozone. Each endpoint had a clear concentration-response relation.

Table 8 in the Integrative Summary illustrates the three correlations of structural and functional parameters that the investigators conducted for their nasal studies. The investigators found a statistically significant relation between mucous flow rate in the lateral wall and the amount of mucosubstances in the lateral wall. This region was most sensitive to ozone exposure. The correlations were not statistically significant for regions unaffected by ozone exposure (the septum and the medial aspect of the nasoturbinate).

The authors used two structural endpoints and one functional endpoint (see Table 9 in the Integrative Summary) for the combined analyses of chronic rhinitis. This analysis showed a highly statistically significant trend ($p < 0.0001$) toward an increased response associated with increasing ozone levels (see Figure 15 in the Integrative Summary). However, examination of the data indicates that the trend does not increase monotonically. The composite score for the rats exposed to 0.12 ppm ozone did not differ from the score for control rats; nevertheless, the responses of the groups exposed to 0.5 or 1.0 ppm ozone were clearly greater than responses in the control group, and these paired comparisons were statistically significant.

Compared with the chronic airway disease and centriacinar fibrosis disease surrogates discussed below, the structural and functional endpoints associated with chronic rhinitis showed the most pronounced changes resulting from prolonged exposure to ozone.

How Are the Structural, Functional, and Biochemical Changes in the Lung Interrelated? As examples of consistency among concentration-response relations, the authors cited several morphometric, biochemical, and functional endpoints that have already been addressed in the discussion of the results of the individual studies. In the following comments, the endpoints that the authors cite as supporting consistency among concentration-response relations are presented in normal type. Italic type is used for comments from the Health Review Committee on the Investigators' conclusions. Most of the selected endpoints were structural changes as determined by morphometric studies; only one pulmonary function test was considered.

STRUCTURAL CHANGES

- Increased amounts of stored secretory product in the small bronchi of rats exposed to 1.0 ppm ozone.

- Statistically significant concentration-related trends in the amounts of stored secretory product in the trachea and caudal bronchus. *Paired comparisons showed increases only in the 1.0 ppm exposure group.*
- Increased smooth muscle area in small bronchi isolated from rats exposed to 0.5 ppm ozone. *No increases were seen in groups exposed to 1.0 ppm ozone. Therefore, no concentration-response trend for this endpoint was evident.*
- Increased volumes of interstitium and epithelium in the proximal alveolar region of rats exposed to 0.5 or 1.0 ppm ozone. *This was one of the strongest concentration-response relations found in this Project.*
- Bronchial epithelial metaplasia in proximal alveolar ducts. *This response was dependent on site and gender in the animals exposed to 0.12 ppm ozone.*

FUNCTIONAL CHANGES IN VITRO

- A reduction in maximal stress in small bronchi isolated from rats exposed to 0.5 ppm ozone, but not in bronchi from rats exposed to 0.12 or 1.0 ppm ozone.

FUNCTIONAL CHANGES IN VIVO

- A reduced residual volume in female rats exposed to 0.5 ppm ozone. *As noted by the investigators, most pulmonary function parameters showed little or no change in response to ozone exposure.*

BIOCHEMICAL CHANGES

- Rats exposed to 0.5 or 1.0 ppm ozone had increased levels of the antioxidant enzymes superoxide dismutase and glutathione peroxidase in the bronchi and trachea.

On the basis of these findings, one can conclude that a concentration-response relation for some structural and biochemical endpoints was evident; however, this relation was not seen with the functional changes.

For their correlative analyses, the authors selected several structural and functional endpoints related to cell type and stored mucus (see Table 10 in the Integrative Summary). As was true for the nasal region, the correlations between the epithelial cells and stored mucus were statistically significant for anatomically discrete regions (caudal and cranial regions of the lungs). The authors also correlated pulmonary function parameters with airway caliber in the lungs. However, none of the correlations between total pulmonary resistance and small airway dimensions were statistically significant. Thus, these analyses showed statistically significant correlations for mucus-producing cells and stored mucus, but not for airway structure and function.

The authors chose ten endpoints for their combined analysis of the chronic airway disease surrogate (see Table 12 in the Integrative Summary). Of these, seven were structural measurements, one was a biochemical measurement, one was an in vitro measurement of lung function, and one was an in vivo measurement of lung function. It is not clear why pulmonary resistance, which is a generally accepted endpoint for evaluating chronic airway disease in humans and was included in the correlative analysis, was not included in the combined analysis.

Figures 16 and 17 in the Integrative Summary present the results for the combined analysis of endpoints related to the chronic airway disease surrogate. Statistically significant trends toward an increased response with increasing ozone levels were obtained for all rats ($p = 0.014$) and for male rats alone ($p = 0.033$). If an adjustment for multiple testing had been done, the trend in the males would have been reduced to marginal significance ($p = 0.066$). The data for males do not display a trend; rather, the response of male rats exposed to 0.12 ppm ozone was lower than males exposed to 0 ppm ozone. In contrast, the responses of male rats exposed to 0.5 or 1.0 ppm ozone were slightly elevated, compared with male control animals. These paired comparisons did not achieve statistical significance; however, they were most likely responsible for the statistical significance of the trend analysis. Thus, the results of the trend analysis must be interpreted with caution.

The authors have not given a clear answer in response to the question "How are the structural, functional, and biochemical changes in the lung interrelated?" They point out that rats exposed to 0.5 or 1.0 ppm ozone had some indicators of chronic airway disease (increased stored mucus and secretory cells in distal conducting airways) and that focal structural changes were observed in rats exposed to 0.12 ppm ozone. Several concerns limit confidence in the relation among the three sets of endpoints, however. First, as discussed above, the bronchiolarization results obtained in rats exposed to 0.12 ppm ozone have weak statistical support. Second, the positive correlations reported in Table 10 of the Integrative Summary refer to structural and histochemical parameters. It is not surprising that a correlation was found between increased mucus and an increase in the number of mucus-producing cells. Third, none of the four correlations between structural and functional parameters related to airway caliber (Table 11) were statistically significant. Fourth, although the 10 components selected for the combined analysis of the chronic airway disease surrogate are, strictly speaking, related to "conditions that could

impede airflow to and from the lungs," they may not be the key components that lead to clinical chronic airway disease in humans. A large percentage of the endpoints for this disease surrogate were derived from morphometric studies. The functional endpoints selected for the combined analysis include only one in vivo parameter (mean midexpiratory flow, but not airway resistance) and measurements of in vitro airway responsiveness.

In contrast, clinical assessment of chronic airway disease in humans relies heavily on functional parameters. Because each endpoint was given equal importance in the median polish analysis, in vivo functional measurements, which are generally considered to be critical in clinical diagnosis of chronic airway disease, had less importance than the morphometric measurements. Thus, the authors' conclusions describe relations among the morphologic endpoints rather than relations among structure, biochemistry, and function. Nevertheless, the observation that morphologic and biochemical changes did not affect overall pulmonary function is critical to the authors' conclusion that the lungs of rats exposed to ozone for 20 months developed tolerance to this pollutant.

How Are Biochemical Changes Correlated with Structural and Functional Indices of Lung Stiffness? As examples of consistency among concentration-response relations, the authors cite the following:

- The percentage of bronchiolarization in the centriacinar region of rats exposed to 0.12, 0.5, or 1.0 ppm ozone was larger than in control animals. *This was not a general effect. Rather, it was found in certain regions of the lung, was specific to gender, and was most uncertain in the group exposed to 0.12 ppm ozone.*
- Mild centriacinar fibrosis in the terminal bronchiole–alveolar duct region in rats exposed to 0.5 or 1.0 ppm ozone.
- A reduced residual volume in rats exposed to 0.5 ppm ozone. *No concentration-response relation was evident for this endpoint. Also, no changes were seen for the other pulmonary function tests.*

Again, this analysis is heavily dependent on morphometric measurements. Concentration-response effects were consistently observed for the structural endpoints, but not for indices of pulmonary function.

The authors explored the relation between measures of lung function and centriacinar fibrosis by examining correlations between each of three functional endpoints (residual volume, dynamic lung compliance, and quasistatic chord compliance) and lung collagen content (as measured by the amount of 4-hydroxyproline) (see Table 13 in the Integrative Summary). Dynamic lung compliance and quasistatic chord compliance are indices of lung elasticity. Because collagen is often associated with the glycosaminoglycan dermatan sulfate in the lung extracellular matrix, and the glycosaminoglycans chondroitin 6-sulfate and heparan sulfate are also constituents of the matrix, correlations between the content of 4-hydroxyproline and these glycosaminoglycans also were included. Of this group of correlative analyses, only the comparison between hydroxyproline and quasistatic chord compliance was statistically significant. The authors note that because sample sizes for these comparisons were small, the statistical power for these correlations was limited.

Table 13 also presents the results of correlations between the percentage of bronchiolarization and the volume of epithelial cells in the cranial and caudal bronchi, and between the extent of bronchiolarization and the volume of epithelial cells in the cranial and caudal bronchi. Only the latter two correlations achieved statistical significance.

For the combined analysis of centriacinar fibrosis, the authors chose four structural endpoints, one biochemical endpoint, and three functional endpoints (see Table 14 of the Integrative Summary). Two other biochemical endpoints, changes in specific glycosaminoglycans, which were included in the original combined analysis for this disease surrogate (see Table 13 in Catalano et al. 1995), were excluded from the Integrative Summary. The authors' rationale for excluding the data on glycosaminoglycans was that, after further consideration of the appropriate endpoints by a larger group of investigators, they could not find sufficient justification for including these data. With the two glycosaminoglycans excluded from the analysis, a statistically significant trend toward centriacinar fibrosis was seen across ozone concentrations, and statistically significant increased responses for the pairwise comparisons of rats exposed to 0.5 or 1.0 ppm ozone with control animals were observed (see Figure 18 in the Integrative Summary). An analysis of gender subgroups showed a similar result for female rats, but not for male rats. As the authors point out, the major reason for the sensitivity to the inclusion or exclusion of the two glycosaminoglycans is that they responded differently (a decrease) than had originally been postulated. An important feature of the median polish technique, which was used for the combined analysis, is that the variables and the expected direction of their change must be selected a priori (Catalano et al. 1995).

The authors interpret the results of their three analytic approaches as indicating that rats exposed to 0.5 or 1.0 ppm ozone showed structural, functional, and biochemical changes indicative of centriacinar fibrosis. Therefore, they concluded that structural and functional measurements of lung stiffness are related to biochemical changes. Two concerns arise

regarding this conclusion. First, one must weigh the importance of the functional changes considered by the authors. Because only one of thirty measurements of in vivo lung function changed after ozone exposure, one can conclude that the most important finding was that exposure to ozone did not cause changes in overall lung function. In addition, the reduction in residual volume was statistically significant only in rats exposed to 0.5 ppm ozone. Thus, there was not a true concentration-response relation. Tests of lung function have different degrees of sensitivity; however, these analyses did not detect larger changes that could be the precursors of chronic lung disease. It is not surprising that structural and biochemical parameters, which did change as a result of ozone exposure, were not significantly correlated with pulmonary function parameters, which did not change. When the authors correlated a single biochemical endpoint with three functional endpoints, only one of the correlations was statistically significant.

The second issue of concern is the combined analysis. The statistical significance of this analysis was very sensitive to the selection of the variables that entered into the analysis and to the mode of data presentation. The authors indicated that they could not find sufficient justification for including the content of two glycosaminoglycans in their analyses. However, this decision was post hoc and is without statistical justification (see the Health Review Committee Commentary in Research Report Number 65, Part X [Catalano et al. 1995]). It is unclear what level of significance can be attached to the post hoc analysis that excluded these glycosaminoglycans.

Thus, the investigators' conclusion that biochemical changes caused by exposure to ozone correlate with structural and functional indices of lung stiffness needs to be qualified; this conclusion appears to be based, to a large extent, on the statistically significant results obtained in the combined analysis, which involved post hoc deletion of certain endpoints and was very sensitive to their inclusion or exclusion.

What Is the Relation Between Changes in the Nose and Changes in the Rest of the Respiratory Tract? The authors examined changes in the amounts of stored intraepithelial secretory product in the nasal cavity, trachea, and the central, cranial, and caudal bronchi (representing different sizes, locations, and generation numbers of bronchi). After exposure to ozone, statistically significant increases were noted in stored secretory product in the nasal cavity, and in the caudal and cranial bronchi, and a statistically significant decrease was noted in the trachea (see Figure 20 in the Integrative Summary). The nose showed strong functional and structural responses to exposure to ozone. In contrast, structural changes in the lung were restricted to focal locations, and, the authors conclude, "appeared to have only minor functional consequences."

The authors conducted 12 correlative analyses to examine the relations between the levels of nasal mucus in four locations in the lung and the content of lung mucosubstances at each location (see Table 15 in the Integrative Summary). Four of twelve correlations were statistically significant: the amount of nasal mucus in the proximal and distal lateral meatuses correlated with increases in stored mucosubstances in the caudal and cranial bronchi.

Thus, the authors appropriately concluded that nasal changes induced in rats exposed to 0.5 or 1.0 ppm ozone correlated with changes in the distal pulmonary airways. However, it should be noted that these correlations were between related substances (mucosubstances) and that the correlations were found only in certain anatomic regions of the nose and tracheobronchial tree. Some support for the conclusion that nasal changes may be predictive of changes in the lower respiratory tract comes from the results of Graham and Koren (1990). They reported that people exposed to 0.4 ppm ozone for two hours, while exercising, showed qualitative, but not quantitative, increases in the levels of inflammatory cells in both nasal lavage fluid and bronchoalveolar lavage fluid compared with people that breathed clean air.

IMPLICATIONS FOR HUMAN HEALTH

The adverse health effects of inhaled ozone are of substantial public health and regulatory concern. Although the term "adverse health effect" is incorporated into the Clean Air Act, it is difficult to define. In 1985, the American Thoracic Society developed guidelines as to what constitutes an adverse respiratory health effect, especially in the context of epidemiologic studies of air pollution. The Society noted a spectrum of adverse respiratory health effects ranging from the most severe (increased mortality) to the least severe (odors). It also noted that it is difficult to distinguish between pathophysiologic changes of clinical significance and physiologic changes of uncertain significance. The guidelines defined an adverse respiratory health effect as "medically significant physiologic or pathologic changes generally evidenced by one of the following: (1) interference with the normal activity of the affected person or persons, (2) episodic respiratory illness, (3) incapacitating illness, (4) permanent respiratory injury, and/or (5) progressive respiratory dysfunction" (American Thoracic Society 1985).

Debate continues about whether the acute and chronic effects of ozone exposure constitute adverse health effects. The changes observed in FEV_1 after short-term exposures to ozone are transient and may represent an adaptation mechanism. However, because indicators of inflammation appear in the bronchoalveolar lavage fluid after short-term ozone exposures, it has been hypothesized that an inflammatory response, if repeated over time, could lead to permanent respiratory injury and the development of fibrosis. At the present time, animal studies provide our only insight into whether this does, indeed, occur. This section provides a perspective on the evidence for the development of certain respiratory diseases (rhinitis, asthma, pulmonary fibrosis, and bronchiolitis) in rats exposed to ozone under the conditions of the NTP/HEI Collaborative Ozone Project.

RHINITIS

Rhinitis (inflammation of the nasal mucous membrane) is a common condition and is responsible for widescale morbidity throughout the world. This is not surprising because the nasal cavity is the region of the respiratory tract that is most exposed to gaseous and particulate material in ambient air. A large fraction of inhaled materials are removed by trapping them in the mucous layer that covers the epithelial surface of the nasal cavity. Exposure to irritants or attachment of pathogenic microorganisms to the nasal epithelial cells can lead to hyperemia, increased mucus, edema, and cellular infiltration of the tissues.

There are different types of acute and chronic rhinitis. Irritants, infections, and allergic reactions all can lead to a syndrome of acute rhinitis. Allergic rhinitis is elicited by hypersensitivity to a variety of allergens. These responses are mediated by immune mechanisms leading to vasomotor responses that increase nasal mucous flow. Allergic rhinitis generally occurs in certain seasons due to exposure to airborne pollens, but can be chronic in an environment of continuous exposures. Rhinitis due to exposure to pollutants or infections tends to be sporadic, and is clinically characterized by sneezing, rhinorrhea, and obstruction of the nasal passages, all symptoms very similar to the common cold. Less widely recognized is that nasal irritation due to indoor irritants also exists in individuals who have irritation of the eyes and nose upon exposure to low levels of common volatile organic mixtures, such as perfume, cigarette smoke, and cleaning agents. If the injury from any cause is continuous, it can lead to chronic rhinitis, which is characterized by hyperplasia of tissues and fibrosis; fibrosis, then, may produce permanent thickening of the submucosa with fibrous tissue and infiltration of inflammatory cells.

Rhinitis, as identified by histologic evaluation and by functional studies that showed impaired mucous flow, was found in the NTP/HEI Collaborative Ozone Project (Harkema et al. 1994). Functional studies of the nose have not been done in humans exposed to high concentrations of ozone or other pollutants, so comparison between rats and humans of those endpoints is not possible. It is important, though, to consider whether the histologic lesions seen in the rat are similar to those found in humans with rhinitis. No systematic studies have been conducted on nasal changes in humans exposed to ozone. However, nasal changes have been reported in residents exposed to high concentrations of photochemical pollutants living in one section of Mexico City (Calderon-Garcidueñas et al. 1992, 1993). Ozone was the most important oxidant in this environment. Examination by light microscopy of the nasal mucosa isolated from Mexican subjects showed acute inflammatory intraepithelial infiltrates and marked proliferation of capillaries in the submucosa. The histologic findings in these human subjects are very similar to those found in the rats. The rats appeared to have more severe pathological changes because some showed atrophy of the mucosa, a finding not reported in the human population. In summary, the nasal changes in rats exposed for long periods of time to ozone are very similar in appearance to the lesions in humans who are exposed to high concentrations of ozone in the ambient atmosphere. Thus, the structural changes in rats exposed to ozone are probably closely related to the human disease.

ASTHMA

Asthma is a disorder that is characterized by (1) airway obstruction (or airway narrowing) that is generally reversible, either spontaneously or with treatment, (2) airway inflammation, and (3) airway hyperresponsiveness to a variety of stimuli. Air pollutants such as ozone can increase the risk of an asthma attack. It is not known, however, whether air pollution itself is one cause of asthma. The NTP/HEI Collaborative Ozone Project was not designed to address this issue. It would have been desirable to conduct tests of bronchial reactivity in vivo using methacholine or other bronchoactive stimuli. However, these experiments were not conducted because the investigators thought that drug treatment would interfere with the conduct of other tests on the same animals. The morphological studies did not provide any indication of airway inflammation; however, such changes, if present, could have disappeared during the one-week period of exposure to clean air. Therefore, no firm conclusions can be drawn from these experiments concerning the role of ozone in the etiology of asthma.

FIBROSIS

The physiologic and histologic findings do not indicate that the rats in the NTP/HEI Collaborative Ozone Project developed diffuse pulmonary fibrosis analogous to the human condition. This is an important point, because the term fibrosis is sometimes used indiscriminately in the literature, and can have distinct meanings in different scientific communities. In humans, pulmonary fibrosis may be the result of a number of insults, including the adult respiratory distress syndrome, prolonged occupational exposures to fibrogenic silicates such as asbestos, and response to certain drugs, or may be part of systemic diseases, among which are sarcoidosis, rheumatoid arthritis, and lupus erythematosus (Coultas 1993). Hereditary forms of pulmonary fibrosis also exist. Most often, however, diffuse pulmonary fibrosis occurs without any obvious cause, antecedent, or associated illness. In this form it is designated idiopathic pulmonary fibrosis.

Irrespective of whether the cause is known or whether it is idiopathic, human diffuse pulmonary fibrosis has characteristic clinical, physiologic, and pathologic features. Dominating the clinical picture is shortness of breath, which is usually progressive to a point of discomfort even at rest and even while breathing supplemental oxygen. From a physiologic standpoint, pulmonary fibrosis results in small, poorly compliant (stiff) lungs that are inefficient in alveolar gas exchange. Accordingly, pulmonary function testing reveals reduced lung volumes, decreased lung compliance, and a reduced DL_{CO}. The rats exposed for 20 months to ozone were normal in all of these parameters (Harkema and Mauderly 1994). Characteristically, the morphology of the lungs with human pulmonary fibrosis reveals loss of normal architecture with apparently thickened alveolar walls, excessive extracellular matrix (especially collagen), and an abundance of inflammatory cells consisting of neutrophils and mononuclear cells throughout the tissue (Figure 1). For comparison, the histopathologic lesions observed in rats exposed to clean air or 1.0 ppm ozone for 20 months (Figures 2A and 2B) illustrate the difference between diffuse pulmonary fibrosis in human and centriacinar fibrosis in rats. Studies in recent years, which have benefitted from techniques for identifying basement membranes, have emphasized the importance of alveolar collapse and intraalveolar fibrosis instead of older concepts of so-called interstitial fibrosis (Kuhn and Hogg 1993). The lungs of the NTP/HEI Collaborative Ozone Project animals, even those exposed to 1.0 ppm ozone for 20 months, did not show any of these histological features of human idiopathic pulmonary fibrosis, and not even the inflammatory lesions that might be forerunners of fibrotic lesions (Chang et al. 1995).

The NTP/HEI studies suggest that diffuse pulmonary fibrosis is not a result of prolonged ozone exposure at ozone concentrations of 1 ppm or less. However, one may still wonder whether ozone could act together with other pulmonary insults to induce or aggravate the development of pulmonary fibrosis. Because acute exposures to ozone elicit inflammation in the lungs of healthy human volunteers, as reflected by changes in bronchoalveolar lavage fluid and mucosal biopsies (Aris et al. 1993), it is possible that similar inflammatory responses could occur in people with pulmonary fibrosis who are exposed to ozone. Such inflammation, if it extended into the lung periphery, could promote or perpetuate the inflammation already present. However, the NTP/HEI Collaborative Ozone Project was not designed to provide insight into these issues.

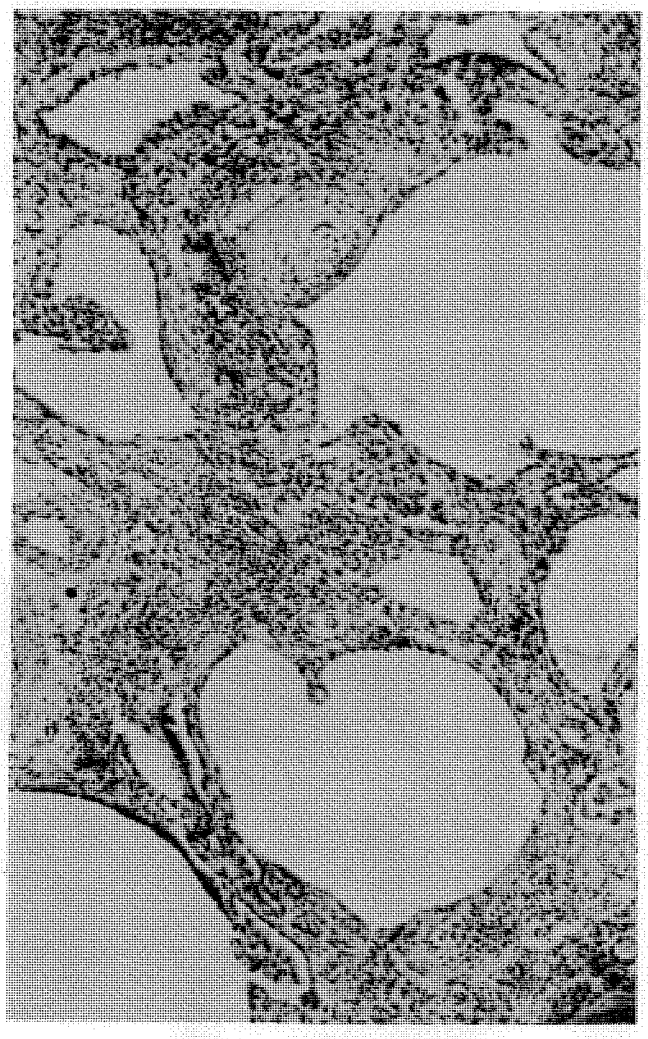

Figure 1. View of a biopsy from a patient with idiopathic pulmonary fibrosis. The architecture of the lung is severely deranged, producing enlarged, irregularly shaped airspaces in which the orderly pattern of alveoli and alveolar ducts is not recognizable. The walls separating the airspaces are markedly thickened by inflammatory cell infiltration and scarring. Hematoxylin-eosin stain. Magnification = ×37.

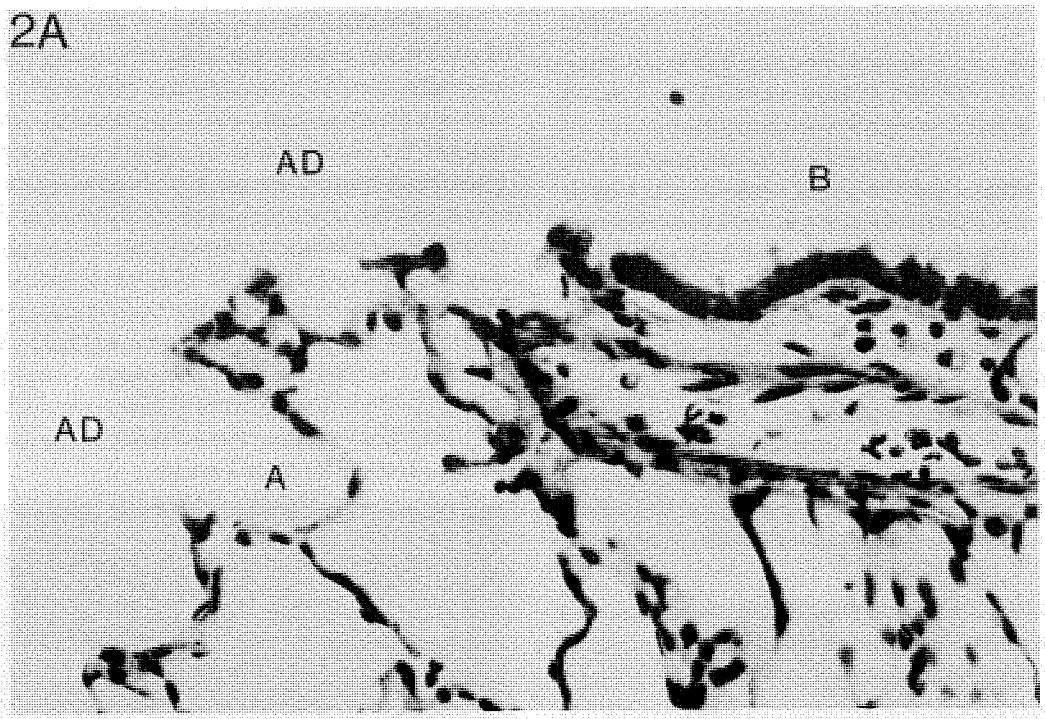

Figure 2A. Bronchiole-alveolar duct junction from a control rat. An abrupt transition is visible from the columnar epithelium of the bronchiole (B) to the alveolar outpocketings along the alveolar duct (AD) to the left. The alveoli (A) are lined by normal flat alveolar epithelium, which is too thin to resolve even at this relatively high magnification (× 1125). (Photo courtesy of Dr. Paul W. Mellick).

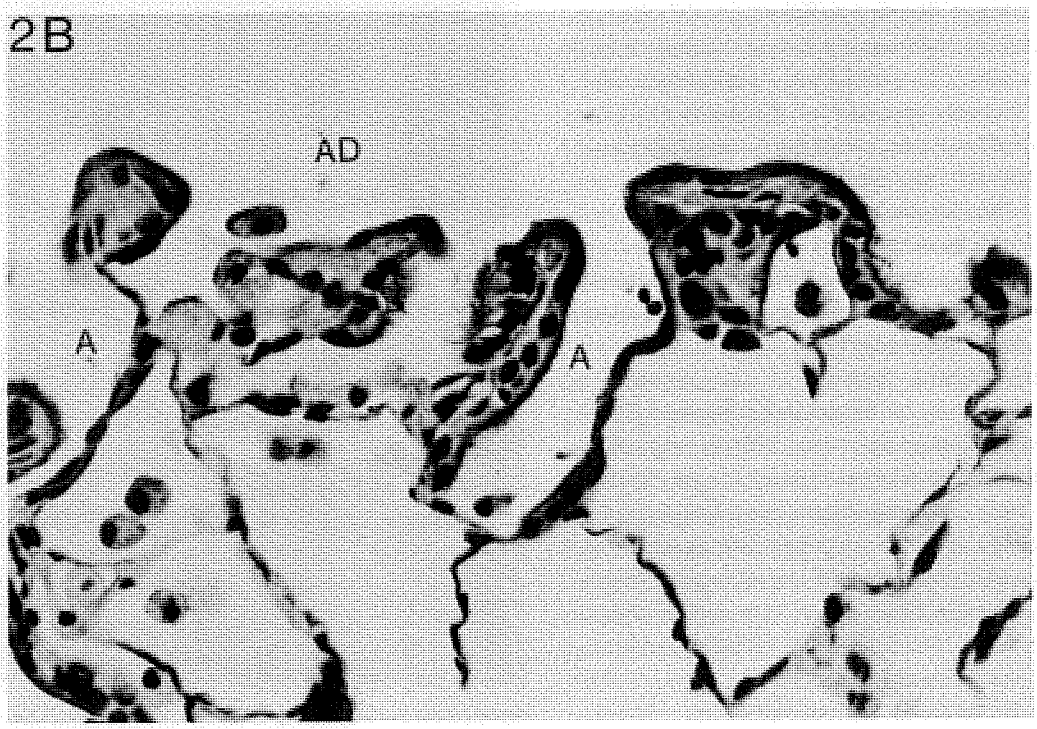

Figure 2B. Alveolar duct (AD) of a rat exposed to 1.0 ppm ozone. Bronchiolar epithelium has extended down the duct so that the alveolar septal tips are covered by columnar epithelium that extends into and partly lines the alveoli (A). The interstitium is slightly thickened at the septal tips, and the population alveolar macrophages has slightly increased. The change in rats exposed to 0.5 ppm ozone were qualitatively similar but less severe. Magnification = × 1125.

The progress of inflammation is influenced by the release of cytokines. For example, recent in vitro studies show the release of interleukin-6 and interleukin-8 from airway epithelial cells at ozone concentrations within the range used in this project (Devlin et al. 1994). At higher concentrations (2 ppm for three hours), rat macrophages were found to release elevated levels of fibronectin in response to transforming growth factor–β (Pendino et al. 1994), a protein that has been implicated in developing human pulmonary fibrosis (Broekelman et al. 1991). Although it is possible to envision a scenario in which ozone could both induce and enhance inflammatory and fibrogenic responses to factors such as transforming growth factor-β (factors that are present in the tissues already undergoing pulmonary fibrosis), more work needs to be done to connect these in vitro results to the situation in vivo where the effective dose of ozone that reaches the lower respiratory tract is only a fraction of the inhaled dose. In short, ozone may exert effects on the development of pulmonary fibrosis that could not have been determined from the NTP/HEI studies in which ozone was the only insult and in which the animals started with normal lungs.

RESPIRATORY BRONCHIOLITIS

If the rats exposed to ozone for 20 months did not have diffuse pulmonary fibrosis, are the lesions in the centriacinar region analogous to any respiratory disease in humans? Examining the physiology and histopathology of the lesions, and comparing these findings with those reported in humans leads to the conclusion that the respiratory injury observed in rats exposed to ozone is very similar to respiratory bronchiolitis in humans. These comparisons are illustrated in Table 2.

Respiratory bronchiolitis is a histologic lesion that is nearly universal in cigarette smokers but uncommon in nonsmokers (Niewoehner et al. 1974). Both respiratory bronchiolitis and the ozone-induced lesion are discretely localized in the proximal acinus at the termination of the conducting airways. Both are characterized by epithelial hyperplasia, mild thickening of the walls of airspaces by inflammation and fibrosis, and by increased numbers of macrophages in alveoli. In the lesion induced by ozone in the rat, epithelial hyperplasia predominates; in respiratory bronchiolitis, macrophage accumulation is the most conspicuous feature. These differences may relate to differences in the anatomy and biology of the two species, to the nature of the irritants involved, or both.

Respiratory bronchiolitis in humans is usually asymptomatic and associated with either no measured functional abnormalities or only mild functional abnormalities. It can persist after cessation of smoking (Wright et al. 1983). Although some have speculated that respiratory bronchiolitis may be a precursor of emphysema, little evidence has confirmed this to be the case. Nearly all smokers have respiratory bronchiolitis; however, only 10% to 20% develop clinical airflow obstruction. Therefore, additional factors must influence susceptibility to chronic obstructive pulmonary disease. The characteristics that identify susceptible subjects and the prevalence of those characteristics in the general population are not known. Because the duration of exposure in the NTP/HEI Collaborative Ozone Project extended for two-thirds of the rats' lifetimes, it seems unlikely that the proximal acinar lesions induced by ozone would progress to structurally significant changes.

The NTP/HEI Collaborative Ozone Project did not, however, address the issue of susceptible subpopulations. Although genetic differences in ozone sensitivity have been identified for different mouse strains (Kleeberger et al. 1990), rats have not been systematically examined. Also, as noted earlier, all possible precautions were taken to ensure that the animals used in this study were healthy, and especially that they were free of pathogens. The impact of ozone exposure on animals with underlying infections or genetic predisposition to oxidant injury may be quite different than the impact on healthy animals.

Finally, this study was designed to examine only one pollutant. People have varying susceptibility to inhaled agents and are exposed to multiple chemicals and pollutants, including cigarette smoke. Furthermore, most chronic lung diseases require a genetic susceptibility. Therefore, it may be a person's genetic makeup that determines if a specific exposure can lead to disease. These caveats need to be considered when extrapolating the results from animal studies to different populations of individuals.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

The NTP/HEI Collaborative Ozone Project provided new information on structural, functional, and biochemical changes in the respiratory tract of rats caused by prolonged exposure to ozone. This information provides a cornerstone for additional studies that can increase our understanding of ozone's effects on the respiratory system. Specifically, mechanisms, effects measured throughout the duration of exposure, and coincident insults by other pollutants are important areas for future investigations.

At present, little is known about the mechanisms by which ozone affects the structure of small airways. On the basis of current knowledge, we can assume that ozone causes oxidative damage to cells, which initiates protective

responses and repair processes that are characterized by cellular hyperplasia. Although it is known that ozone oxidizes cell membrane fatty acids and increases some antioxidant enzyme activities (Grose et al. 1989; Leikauf 1994; Pinkerton et al. 1995), the underlying mechanisms that connect these specific responses with the cellular and interstitial changes observed in the lung during prolonged exposure to ozone are not well understood. The NTP/HEI Collaborative Ozone Project raises the question, Why did the definitive structural changes not have major effects on pulmonary function?

Humans exposed to ozone for brief periods (one to six hours) on several consecutive days show a reduction in FEV_1; however this response to ozone is attenuated after three to five days of exposure (Hackney et al. 1977; U.S. Environmental Protection Agency 1986). The mechanisms for this "adaptation" are unknown. Because overall pulmonary function did not change in response to structural and biochemical changes, the authors suggest that the lungs of the animals exposed to ozone for 20 months became tolerant to this oxidant. However, these studies cannot answer the question of whether adaptation or tolerance to ozone occurred because a "snapshot" of the effects was taken only at the point of 20 months of exposure to ozone and adaptation can be measured only through a series of changes over time. Also, in these studies, little information was available concerning changes during the one-week recovery period, and no information was available on the effects of multiple exposure-recovery periods. Thus, conclusions about the implications for human health from the NTP/HEI Collaborative Ozone Project are limited because the results pertain to specific exposure conditions. Exposure conditions that differ in duration, periodicity, intensity, or recovery period may produce effects that differ from those found in the NTP/HEI Collaborative Ozone Project. A future direction of research might be to determine the exposure conditions that most closely simulate human exposure to ozone and conduct animal studies using those conditions.

Finally, the studies in the NTP/HEI Collaborative Ozone Project share the strength that ozone effects were investigated in detail in isolation from the effects of other pollutants. The design of the exposure protocol, which was mandated by the NTP, did not accommodate joint exposure to ozone and copollutants. However, any strategy for regulatory control must consider that the effects of environmental air pollutants often are affected by the presence of multiple copollutants. The NTP/HEI Collaborative Ozone Project provides a solid base of information on the effects of prolonged exposure to ozone. This base can be used as a starting point for investigating the effects of exposure to ozone together with cigarette smoke and other common air pollutants, including nitrogen and sulfur oxides.

SUMMARY AND CONCLUSIONS

The NTP/HEI Collaborative Ozone Project was carefully designed and well-executed. The selection of ozone expo-

Table 2. Comparison of Characteristics of Diffuse Pulmonary Fibrosis with Focal Proximal Acinar Fibrosis[a]

Characteristic	Diffuse Pulmonary Fibrosis	Focal Proximal Acinar Fibrosis	
		Ozone in Rats	Tobacco Smoke in Humans
Distribution	All regions of acinus	Limited to region just beyond bronchiole–alveolar duct junction	Limited to region just beyond bronchiole–alveolar duct junction
Severity	Varies; often severe and progressive	Mild	Mild
Functional Effects			
Total lung capacity	↓	NC	NC
Vital capacity	↓	NC	NC
Residual volume	↓	↓ (?)	NC
Lung compliance	↓	NC	NC
Carbon monoxide diffusing capacity	↓	NC	NC
Nitrogen washout	Varies	NC	↑ (Closing volume)

[a] ↓ = decrease; ↑ = increase; NC = no change; (?) = a statistically significant decrease was observed at one ozone concentration, but no concentration-response trend was found.

sure concentrations was based on a solid rationale, and the inclusion of four exposure groups provided an opportunity to make concentration-response comparisons across a range of ozone levels. The exposure and necropsy procedures were carried out under rigorously controlled conditions that included appropriate quality assurance and standard operating procedures. Moreover, the animal allocation scheme enhanced the study by providing an opportunity to integrate the results and to correlate the findings among the individual studies. Thus, the results of this Project provide insight into the question of whether prolonged exposure to ozone leads to permanent injury or to the development of chronic lung disease.

Eight groups of investigators examined the effects of prolonged ozone exposure on the respiratory tracts of F344/N rats. The endpoints included structural, biochemical, and functional measures in the nose, conducting airways, and the lungs. The animals were exposed to clean air or one of three concentrations of ozone (0.12, 0.5, or 1.0 ppm) for six hours per day, five days per week, for 20 months, thus allowing the evaluation of concentration-response relations. The investigators used standard analytical techniques and an innovative statistical procedure to integrate the diverse endpoints and to provide information about the effects of ozone on the respiratory system. The major findings of the studies are summarized in Table 1.

Exposure to ozone for 20 months, even at the highest concentration, had no effect on animal survival and only minimal effects on weight gain. Some of the most striking effects of prolonged exposure to ozone were on the structure and function of the epithelium of the nasal cavity. In the nose, functional changes included concentration-dependent reductions in mucous flow rates in rats exposed to 0.5 or 1.0 ppm ozone. Structural changes that were dependent on ozone concentration were documented at the same sites where mucous flow rates were impaired. Exposure to 0.12 ppm ozone had no significant effect on either nasal structure or function. The fact that both functional and structural assessments revealed injury from the same levels of ozone exposure strengthens the conclusion that the threshold of toxicity to the nasal mucosa of the F344/N rat lies between 0.12 and 0.5 ppm ozone under the exposure protocol described above. The nasal changes in rats exposed to ozone are very similar to the lesions observed in humans living in areas of high levels of ozone in the ambient atmosphere. Thus, the changes found in the animal studies are probably relevant to rhinitis in humans. However, because of anatomical differences between the structure of primate and rat nasal cavities, extrapolating these results to humans, in terms of exposure-response relations, is difficult.

Very few changes were observed in the conducting airways of rats exposed to ozone. The three most notable changes were variations in antioxidant enzyme levels, an increase in the area of the walls of the small airways, and an increase in the amount of smooth muscle in the small airways of rats exposed to 0.5 ppm ozone. The latter results are difficult to interpret because the in vitro responses to different contractile stimuli that occurred in the same exposure group decreased, a finding opposite to the expected increase in contractile response due to more smooth muscle. In addition, no response that was dependent on concentration was observed. Importantly, no decrease in luminal diameter, which might affect resistance to airflow, was found.

In the lungs, the major changes caused by 20 months of exposure to ozone were in the centriacinar region. These changes included structural changes in epithelial cells and increases in interstitial matrix components in the centriacinar region of rats exposed to 0.5 or 1.0 ppm ozone. Bronchial epithelial cell metaplasia was seen in the alveolar duct area. This process, called bronchiolarization, may protect the sensitive gas-exchange region of the lungs from damage by changing the structure of the epithelium to one that contains a greater number of cells that are resistant to ozone. It is not clear whether or not this process also occurred at the lowest ozone exposure concentration. In one study, no structural alterations were evident in the proximal alveolar region. In another study, in which the sampling strategy allowed systematic investigation of the regions closest to the junction of the terminal bronchiole and the alveolar duct, some small changes that were specific to site and gender were noted in the epithelial cells. Statistically significant changes in epithelial cells in the centriacinar region of animals exposed to 0.12 ppm ozone were observed only in the cranial lung lobe of male rats. They were not seen in the caudal lobe of male rats or in any region of the lungs of female rats. Given the fact that these small structural changes were the only statistically significant observations in animals exposed to 0.12 ppm ozone, they require further confirmation.

Biochemical studies of connective tissue components indicated a decrease in the level of glycosaminoglycans, components of proteoglycans that, together with collagen, confer structural support to the lung. Some trends were noted toward an increase in total collagen levels in female rats; however, this increase was statistically significant only in rats exposed to 1.0 ppm ozone. It is quite likely that analysis of total collagen is not sufficiently sensitive to detect small localized changes. Morphometric analyses and conventional light microscopic stains showed an increase in collagen fibrils in the interstitium in the centriacinar region of rats exposed to 0.5 or 1.0 ppm ozone. In situ hybridization techniques were used to determine whether

synthesis of connective tissue was stimulated by prolonged ozone exposure. No messenger RNAs coding for type I collagen or elastin were detectable in rats exposed to 1.0 ppm ozone. Thus, if collagen synthesis increased as a result of ozone exposure, it must have occurred earlier in the 20-month exposure period.

The results of an extensive battery of tests indicated that ozone exposure had little or no effect on overall pulmonary function. The only functional parameter that was affected by exposure to ozone was residual volume, which decreased in all exposure groups. However, this change was statistically significant only in the group exposed to 0.5 ppm ozone, and no concentration-response relation was evident. Thus, one can conclude that the structural and biochemical changes observed in the lungs of rats that were repeatedly exposed to ozone for 20 months did not significantly affect overall pulmonary function.

A total of 144 animals were available for the HEI studies. This limited the number of animals that could be assigned to each investigator. However, the investigators used three analytical approaches to integrate the results of the multiple studies. The integration of study findings produced an analysis of the results that is scientifically stronger than the results of individual studies standing alone.

One innovative technique involved combining data for a set of related parameters that had been measured in different animals; a statistical technique called median polish analysis was used to develop composite scores for the combined data. This technique, when applied to three disease surrogates (defined by the investigators as collections of endpoints "potentially related to different pathogenic processes"), produced some interesting insights into the relations among various endpoints. For the rhinitis disease surrogate, in which ozone exposure was clearly related to pathophysiologic processes, the composite scores showed highly significant differences between effects seen at 0.5 and 1.0 ppm ozone compared with those seen at 0 ppm ozone. Thus, the combined analysis agreed with the results of the qualitative analyses. However, for the other disease surrogates examined in this Integrative Summary, centriacinar fibrosis and chronic airway disease, the composite scores were heavily weighted by the structural changes in the centriacinar region and were sensitive to the inclusion or exclusion of some endpoints. Thus, for these models, the median polish analysis may have led to different conclusions than those derived from either qualitative or quantitative analyses that incorporate different sets of data.

One of the conclusions reached by the authors is that the results of the integrative analyses suggest that the lungs of the animals became tolerant to prolonged exposure to ozone. In contrast, the authors interpret the changes in nasal structure and function as indicating that the nose did not develop tolerance to ozone. The development of tolerance is a reasonable explanation for the authors' findings, because (1) lung structural and biochemical changes were subtle, (2) those changes that did take place were not accompanied by changes in pulmonary function, and (3) literature reports of inflammation in response to short-term exposures to ozone. However, the Project protocol did not allow for a true test of tolerance. Tolerance can only be demonstrated when repeated exposures to a stimulus, such as ozone, are shown to cause decreasing responses. The Project's design allowed the investigators to examine animals at only one time point, that is, after 20 months of exposure. Thus, the investigators could not determine whether changes, such as inflammation, developed early in the exposure period and then decreased with time. It is possible that adaptive changes took place in the lungs of rats exposed to ozone that enabled the development of tolerance. Adaptation implies changes that enable the respiratory epithelium to become more resistant to a stimulus. The results of the morphology studies, which indicated that cells that are more resistant to ozone replaced cells that are more sensitive in the centriacinar region of the lung and that the activity of antioxidant enzymes increased, suggest that such changes may have taken place. Alternative hypotheses are that the lungs of F344/N rats are less sensitive to ozone than the nose, or that inflammatory and necrotic changes resolved during the one-week recovery period in clean air. Therefore, additional experiments at multiple time points are necessary to determine conclusively if tolerance developed during prolonged exposure of F344/N rats to ozone.

The results of this project provide some insight into the question of whether prolonged exposure to ozone leads to permanent injury and the development of chronic lung disease. The centriacinar fibrosis that developed in rats exposed to 0.5 or 1.0 ppm ozone should not be considered as analogous to the disease entity known as diffuse pulmonary fibrosis. In humans, pulmonary fibrosis of either known or unknown etiology has characteristic clinical, physiologic, and pathologic features, including a diffuse inflammatory response and a reduction in some pulmonary function measurements. The rats exposed to ozone for 20 months, even at the highest ozone concentrations, did not show any of the characteristic features of human diffuse pulmonary fibrosis, and did not develop inflammatory lesions that might be forerunners of fibrotic lesions. The lesions that were observed in the centriacinar region were, however, very similar

to respiratory bronchiolitis in humans. Respiratory bronchiolitis involves histologic lesions that are nearly universal in cigarette smokers but uncommon in nonsmokers. These lesions in humans are usually asymptomatic and are usually associated with no measurable or mild functional abnormalities.

Some limitations must be considered when extrapolating the results of this study to humans. Despite the fact that both the exposure and experimental components of the study were conducted rigorously and thoroughly, the results are only applicable to healthy animals exposed to a single pollutant. Prolonged ozone exposure may have had a more severe effect in animals with compromised lungs or if the exposure had included other air pollutants. This study was not designed to provide insight into these issues. Ozone may exert effects on the development of chronic respiratory disease that could not have been determined from the NTP/HEI Collaborative Ozone Project, in which ozone was the only insult and in which the animals started with normal lungs and were housed under conditions that eliminated exposure to microorganisms.

REFERENCES

Abbey DE, Mills PK, Petersen FF, Beeson WL. 1991a. Long-term ambient concentrations of total suspended particulates and oxidants as related to incidence of chronic disease in California Seventh-Day Adventists. Environ Health Perspect 94:43–50.

Abbey DE, Moore J, Petersen FF, Beeson WL. 1991b. Estimating cumulative ambient concentrations of air pollutants: Description and precision of methods used for an epidemiological study. Arch Environ Health 46:281–287.

American Thoracic Society. 1985. Guidelines as to what constitutes an adverse respiratory health effect, with special reference to epidemiologic studies of air pollution. Am Rev Respir Dis 131:666–668.

Aris RM, Christian D, Hearnes PQ, Kerr K, Finkbeiner WE, Balmes J. 1993. Ozone-induced airway inflammation in human subjects as determined by airway lavage and biopsy. Am Rev Respir Dis 148:1363–1372.

Barr BC, Hyde DM, Plopper CG, Dungworth DL. 1988. Distal airways remodeling in rats chronically exposed to ozone. Am Rev Respir Dis 137:924–938.

Barry BE, Miller FJ, Crapo JD. 1985. Effects of inhalation of 0.12 and 0.25 parts per million ozone on the proximal alveolar region of juvenile and adult rats. Lab Invest 53:692–704.

Bjermer L, Lundgren R, Hallgren R. 1989. Hyaluronan and type III procollagen peptide concentrations in bronchoalveolar lavage fluid in idiopathic pulmonary fibrosis. Thorax 44:126–131.

Boorman GA, Catalano PJ, Jacobson B, Kaden DA, Nauss KM, Ryan LM. 1995a. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part VI, Background and Study Design. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Boorman GA, Hailey R, Grumbein S, Chou BJ, Herbert RA, Goehl T, Mellick PW, Roycroft JH, Haseman JK, Sills R. 1995b. Toxicology and carcinogenesis studies of ozone and ozone 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone in Fischer-344/N rats. Toxicol Pathol 22:545–554.

Boorman GA, Schwartz LW, Dungworth DL. 1980. Pulmonary effects of prolonged ozone insult in rats: Morphometric evaluation of the central acinus. Lab Invest 43:108–115.

Broekelman T, Limper AH, Colby TV, McDonald JA. 1991. Transforming growth factor-β1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. Proc Natl Acad Sci USA 88:6642–6646.

Brownstein DG. 1985. Lesions due to infection: Sendai virus infection, lung, mouse and rat. In: Respiratory System (Jones TC, Mohr U, Hunt RD, eds.) pp. 195–213. Springer-Verlag, New York, NY.

Calderon-Garcidueñas L, Osorno-Velazquez A, Bravo-Alvarez H, Delgado-Chavez R, Barrios-Marquez R. 1992. Histopathologic changes of the nasal mucosa in southwest metropolitan Mexico City inhabitants. Am J Pathol 140:225–232.

Calderon-Garcidueñas L, Roy-Ocotia G. 1993. Nasal cytology in southwest metropolitan Mexico City inhabitants: A pilot intervention study. Environ Health Perspect 101:138–144.

Catalano PJ, Rogus J, Ryan LM. 1995. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part X, Robust Composite Scores Based on Median Polish Analysis. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Chang L-Y, Huang Y, Stockstill BL, Graham JA, Grose EC, Ménache MG, Miller FJ, Costa DL, Crapo JD. 1992. Epithelial injury and interstitial fibrosis in the proximal alveolar regions of rats chronically exposed to a simulated pattern of urban ambient ozone. Toxicol Appl Pharmacol 115:241–252.

Chang L-Y, Stockstill BL, Ménache MG, Mercer RR, Crapo JD. 1995. Consequences of Prolonged Inhalation of Ozone on

F344/N Rats: Collaborative Studies, Part VIII, Morphometric Analysis of Structural Alterations in Alveolar Regions. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Costa DL, Hatch GE, Crapo JD. 1991. Does chronic ozone exposure lead to lung disease? In: Tropospheric Ozone and the Environment (Berglund RL, Lawson DR, McKee DJ, eds.) pp. 120–126. Air and Waste Management Association, Pittsburgh, PA.

Costa DL, Tepper JS, Stevens MA, Watkinson WP, Doerfler DL, Gelzleichter TR, Last JA. 1995. Functional evidence of restrictive lung disease in rats chronically exposed to a simulated urban profile of ozone. Am J Respir Crit Care Med (in press).

Coultas DB. 1993. Epidemiology of idiopathic pulmonary fibrosis. Semin Respir Med 14:181–196.

Crapo JD, Barry BE, Chang L-Y, Mercer RR. 1984. Alterations in lung structure caused by inhalation of oxidants. J Toxicol Environ Health 13:301–321.

Crystal RC, Ferrans VJ, Bassett F. 1991. Biologic basis of pulmonary fibrosis. In: The Lung: Scientific Foundations (Crystal RC, West JB, eds.) pp. 2031–2046. Raven Press, New York, NY.

DeLucia AJ, Adams WC. 1977. Effects of O_3 inhalation during exercise on pulmonary function and blood biochemistry. J Appl Physiol 43:75–81.

Devlin RB, McDonnell WF, Mann R, Becker S, House DE, Schreinemachers D, Koren HS. 1991. Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. Am J Respir Cell Mol Biol 4:72–81.

Devlin RB, McKinnon KP, Noah T, Becker S, Koren HS. 1994. Ozone-induced release of cytokines and fibronectin by alveolar macrophages and airway epithelial cells. Am J Physiol: Lung Cell Mol Physiol 10:L612–L619.

Dimitriadis VK. 1993. Tracheal epithelium of bonnet monkey (*Macaca radiaca*) and its response to ambient levels of ozone: A cytochemical study. J Submicrosc Cytol Pathol 25:53–61.

Euler GL, Abbey DE, Hodgkin JE, Magie AR. 1988. Chronic obstructive pulmonary disease symptom effects of long-term cumulative exposure to ambient levels of total oxidants and nitrogen dioxide in California Seventh-Day Adventist residents. Arch Environ Health 43:279–285.

Euler GL, Abbey DE, Magie AR, Hodgkin JE. 1987. Chronic obstructive pulmonary disease symptom effects of long-term cumulative exposure to ambient levels of total suspended particulates and sulfur dioxide in California Seventh-Day Adventist residents. Arch Environ Health 42:213–222.

Filipowicz C, McCauley R. 1986. The effects of chronic ozone exposure on pulmonary collagen content and collagen synthesis in rats. J Appl Toxicol 6:87–90.

Freeman G, Stephens RJ, Coffin DL, Stara JF. 1973. Changes in dogs' lungs after long-term exposure to ozone. Arch Environ Health 26:209–216.

Fujinaka LE, Hyde DM, Plopper CG, Tyler WS, Dungworth DL, Lollini LO. 1985. Respiratory bronchiolitis following long-term ozone exposure in bonnet monkeys: A morphometric study. Exp Lung Res 8:167–190.

Graham DE, Koren HS. 1990. Biomarkers of inflammation in ozone-exposed humans: Comparison of the nasal and bronchoalveolar lavage. Am Rev Respir Dis 142:152–156.

Grose EC, Stevens MA, Hatch GE, Jaskot RH, Selgrade MJK, Stead AG, Costa DL, Graham JA. 1989. The impact of a 12-month exposure to a diurnal pattern of ozone on pulmonary function, antioxidant biochemistry and immunology. In: Atmospheric Ozone Research and Its Policy Implications (Schneider T, Lee SD, Wolters GJR, Grant LD, eds.) pp. 535–544. Elsevier Science Publishing Co., New York, NY.

Gross KB, White HJ. 1987. Functional and pathological consequences of a 52-week exposure to 0.5 ppm ozone followed by a clean air recovery period. Lung 165:283–295.

Grotberg JB, Sheth BV, Mockros LF. 1990. An analysis of pollutant gas transport and absorption in pulmonary airways. J Biomed Eng 112:168–176.

Hackney JD, Linn WS, Mohler JG, Collier CR. 1977. Adaptation to short-term respiratory effects of ozone in men exposed repeatedly. J Appl Physiol: Respir Environ Exercise Physiol 43:82–85.

Harkema JR, Hotchkiss JA, Henderson RF. 1989. Effects of 0.12 and 0.80 ppm ozone on rat nasal and nasopharyngeal epithelial mucosubstances: Quantitative histochemistry. Toxicol Pathol 17:525–535.

Harkema JR, Mauderly JL. 1994. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part V, Effects on Pulmonary Function. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Harkema JR, Morgan KT, Gross EA, Catalano PJ, Griffith WC. 1994. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part VII, Effects on the Nasal Mucociliary Apparatus. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Harkema JR, Plopper CG, Hyde DM, St. George JA, Dungworth DL. 1987a. Effects of an ambient level of ozone on primate nasal epithelial mucosubstances: Quantitative histochemistry. Am J Pathol 127:90–96.

Harkema JR, Plopper CG, Hyde DM, St. George JA, Wilson DW, Dungworth DL. 1987b. Response of the Macaque nasal epithelium to ambient levels of ozone: A morphologic and morphometric study of the transitional and respiratory epithelium. Am J Pathol 128:29–44.

Hodgkin JE, Abbey DE, Euler GL, Magie AR. 1984. COPD prevalence in nonsmokers in high and low photochemical air pollution areas. Chest 86:830–838.

Horstman DH, Folinsbee LJ, Ives PJ, Abdul-Salam S, McDonnell WF. 1990. Ozone concentration and pulmonary response relationships for 6.6-hour exposures with five hours of moderate exercise to 0.08, 0.10, and 0.12 ppm. Am Rev Respir Dis 142:1158–1163.

Kleeberger SR, Bassett DJP, Jakab GJ, Levitt RC. 1990. A genetic model for evaluation of susceptibility to ozone-induced inflammation. Am J Physiol: Lung Cell Mol Physiol 258:L313–L320.

Koren HS, Devlin RB, Graham DE, Mann R, McGee MP, Horstman DH, Kozumbo WJ, Becker S, House DE, McDonnell WF, Bromberg PA. 1989. Ozone-induced inflammation in the lower airways of human subjects. Am Rev Respir Dis 139:407–415.

Kuhn C, Hogg JC. 1993. The pathogenesis of interstitial lung disease: Liebow updated. In: Frontiers in Pulmonary Radiology: State of the Art (Potchen EJ, Grainger R, Greene R, eds.) pp. 39–49. C.V. Mosby, Co., Markham, Canada.

Last JA, Gelzleichter, Harkema JH, Hawk S. 1994. Consequences of Prolonged Inhalation of Ozone on Fischer-344/N Rats: Collaborative Studies, Part I, Content and Cross-Linking of Lung Collagen. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Last JA, Greenberg DB, Castleman WL. 1979. Ozone-induced alterations in collagen metabolism of rat lungs. Toxicol Appl Pharmacol 51:247–258.

Last JA, Reiser KM, Tyler WS, Rucker RB. 1984. Long-term consequences of exposure to ozone: I. Lung collagen content. Toxicol Appl Pharmacol 72:111–118.

Leikauf GD, Zhao Q, Zhou S, Santrock J. 1994. Activation of Human Airway Epithelial Cells by Ozonolysis Products of Membrane Fatty Acids. Research Report 71. Health Effects Institute, Cambridge, MA (in press).

Lioy PJ, Dyba RV. 1989. The dynamics of human exposure to tropospheric ozone. In: Atmospheric Ozone Research and Its Policy Implications (Schneider T, Lee SD, Wolters GJR, Grant LD, eds.) pp. 711–721. Elsevier Science Publishing Co., New York, NY.

Lioy PJ, Volmuth TA, Lippmann M. 1985. Persistence of peak flow decrement in children following ozone exposures exceeding the National Ambient Air Quality Standard. J Air Pollut Control Assoc 35:1068–1071.

Lippmann M. 1992. Ozone. In: Environmental Toxicants: Human Exposures and Their Health Effects (Lippmann M, ed.) pp. 465–519. Van Nostrand Reinhold, New York, NY.

Lippmann M. 1993. Health effects of tropospheric ozone: Review of recent research findings and their implications to ambient air quality standards. J Expos Anal Environ Epidemiol 3:103–129.

McKee DJ. 1993a. Legislative requirements and ozone NAAQS review. In: Tropospheric Ozone: Human Health and Agricultural Impacts (McKee DJ, ed.) pp. 9–18. Lewis Publishers, Chelsea, MI.

McKee D. 1993b. Ozone Primary National Ambient Air Quality Standard: A proposed decision. In: Tropospheric Ozone: Non-attainment and Design Value Issues (Vostal JJ, ed.) pp. 32–48. Air and Waste Management Association, Pittsburgh, PA.

Mellick PW, Dungworth DL, Schwartz LW, Tyler WS. 1977. Short term morphological effects of high ambient levels of ozone on the lungs of rhesus monkeys. Lab Invest 36:82–90.

Miller FJ, Overton JH Jr, Jaskot RH, Menzel DB. 1985. A model of the regional uptake of gaseous pollutants in the lungs: I. The sensitivity of the uptake of ozone in the human to lower respiratory tract secretions and exercise. Toxicol Appl Pharmacol 79:11–27.

National Toxicology Program. 1995. Technical Report on the Toxicology and Carcinogenesis Studies of Ozone and Ozone/NNK in Fischer-344/N Rats and B6C3F$_1$ mice. NTP Technical Report No. 400. NIH Publication No. 93–3371. U.S. Department of Health and Human Services, National Toxicology Program Information Office, Research Triangle Park, NC.

Niewoehner DE, Kleinerman J, Rice DB. 1974. Pathologic changes in the peripheral airways of young cigarette smokers. N Engl J Med 291:755–758.

Nikula KJ, Wilson DW, Giri S, Plopper CG, Dungworth DL. 1988. The response of the rat tracheal epithelium to ozone exposure: Injury, adaptation, and repair. Am J Pathol 131:373–384.

Overton JH, Graham RC. 1989. Predictions of ozone absorption in humans from newborn to adult. Health Phys (Suppl) 57(1):29–36.

Parks WC, Roby JD. 1994. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part IV, Effects on Expression of Extracellular Matrix Genes. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Pendino K, Shuler RL, Laskin JD, Laskin DL. 1994. Enhanced production of interleukin-1, tumor necrosis factor-α and fibronectin by rat lung phagocytes following inhalation of a pulmonary irritant. Am J Respir Cell Mol Biol 11:279–286.

Phan SH. 1989. Diffuse interstitial fibrosis. In: Lung Cell Biology (Massaro D, ed.) pp. 907–980. Marcel Dekker, New York, NY.

Pinkerton KE, Dodge DE, Cederdahl-Demmler J, Wong VJ, Peake J, Haselton CJ, Mellick PW, Singh G, Plopper CG. 1993. Differentiated bronchiolar epithelium in alveolar ducts of rats exposed to ozone for 20 months. Am J Pathol 142:947–956.

Pinkerton KE, Ménache MG, Plopper CG. 1995. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part IX, Changes in the Tracheobronchial Epithelium, Pulmonary Acinus, and Lung Antioxidant Enzyme Activity. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Plopper CG, Harkema JR, Last JA, Pinkerton KE, Tyler WS, St. George JA, Wong VJ, Nishio SJ, Weir AS, Dungworth DL, Barry BE, Hyde DM. 1991. The respiratory system of nonhuman primates responds more to ambient ozone than does that of rats. In: Tropospheric Ozone and the Environment (Berglund RL, Lawson DR, McKee DJ, eds.) pp. 137–150. Air and Waste Management Association, Pittsburgh, PA.

Radhakrishnamurthy B. 1994. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part III, Effects on Complex Carbohydrates of Lung Connective Tissue. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Reiser KM, Last JA. 1981. Pulmonary fibrosis in experimental acute respiratory disease. Am Rev Respir Dis 123:58–63.

Reiser KM, Tyler WS, Hennessy SM, Dominguez JJ, Last JA. 1987. Long-term consequences of ozone exposure: II. Structural alterations in lung collagen of monkeys. Toxicol Appl Pharmacol 89:314–322.

Rombout PJ, Lioy PJ, Goldstein BD. 1986. Rationale for an eight-hour ozone standard. J Air Pollut Control Assoc 36:913–917.

Rombout PJA, van Bree L, Heisterkamp SH, Marra M. 1989. The need for an eight hour ozone standard. In: Atmospheric Ozone Research and Its Policy Implications (Schneider T, Lee SD, Wolters GJR, Grant LD, eds.) pp. 701–710. Elsevier Science Publishing Co., New York, NY.

Schlesinger RB, Gorczynski JE, Dennison J, Richards L, Kinney PL, Bosland MC. 1992. Long-term intermittent exposure to sulfuric acid aerosol, ozone, and their combination: Alterations in tracheobronchial mucociliary clearance and epithelial secretory cells. Exp Lung Res 18:505–534.

Schoeb TR, Lindsey JR. 1985. Lesions due to infection: Murine respiratory mycoplasmosis, lung, rat. In: Respiratory System (Jones TC, Mohr U, Hund RD, eds.) pp. 213–218. Springer-Verlag, New York, NY.

Seltzer J, Bigby BG, Stulbarg M, Holtzman MJ, Nadel JA, Ueki IF, Leikauf GD, Goetzl EJ, Boushey HA. 1986. O_3-induced change in bronchial reactivity to methacholine and airway inflammation in humans. J Appl Physiol 60:1321–1326.

Spector DM, Lippmann M, Lioy PJ, Thurston GD, Citak K, James DJ, Bock N, Speizer FE, Hayes C. 1988. Effects of ambient ozone on respiratory function in active, normal children. Am Rev Respir Dis 137:313–320.

Spector DM, Thurston GD, Mao J, He D, Hayes C, Lippmann M. 1991. Effects of single- and multiday ozone exposures on respiratory function in active normal children. Environ Res 55:107–122.

Stephens RF, Sloan MF, Evans MJ, Freeman G. 1974. Early response of lung to low levels of ozone. Am J Pathol 74:31–58.

Szarek JL. 1994. Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies, Part II, Mechanical Properties, Responses to Bronchoactive Stimuli, and Eicosanoid Release in Isolated Large and Small Airways. Research Report Number 65. Health Effects Institute, Cambridge, MA.

Tepper JS, Wiester MJ, Weber MF, Fitzgerald S, Costa DL. 1991. Chronic exposure to a simulated urban profile of ozone alters ventilatory responses to carbon dioxide challenge in rats. Fundam Appl Toxicol 17:52–60.

Tukey JW. 1977. Exploratory Data Analysis. Addison-Wesley Publishing Co., Reading, MA.

Tyler WS, Tyler NK, Last JA, Gillespie MJ, Barstow TJ. 1988. Comparison of daily and seasonal exposures of young monkeys to ozone. Toxicology 50:131–144.

U.S. Environmental Protection Agency. 1986. Air Quality Criteria for Ozone and Other Photochemical Oxidants. EPA-600/8-84-02aF-02eF. Environmental Criteria and Assessment Office, Research Triangle Park, NC.

U.S. Environmental Protection Agency. 1992. Summary of Selected New Information on Effects of Ozone on Health and Vegetation. EPA-600/8-88/105A. Environmental Criteria and Assessment Office, Research Triangle Park, NC.

Van Bree L, Lioy PJ, Rombout PJA, Lippmann M. 1990. A more stringent and longer-term standard for tropospheric ozone: Emerging new data on health effects and potential exposure. Toxicol Appl Pharmacol 103:377–382.

Weibel ER. 1963. Morphometry of the Human Lung. Springer-Verlag, New York, NY.

Wright ES, Kehrer JP, White DM, Smiler KL. 1988. Effects of chronic exposure to ozone on collagen in rat lung. Toxicol Appl Pharmacol 92:445–452.

Wright JL, Lawson LM, Pare PD, Wiggs BJ, Kennedy S, Hogg JC. 1983. Morphology of peripheral airways in current smokers and ex-smokers. Am Rev Respir Dis 127:474–477.

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