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The Role of Ozone in Tracheal Cell Transformation

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Includes the Commentary of the Institute's Health Review Committee

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HEI Statement

Synopsis of Research Report Number 50

Study of the Frequency of Precancerous Changes in Cells Exposed to Ozone

BACKGROUND

Ozone is a major component of urban smog. It is a highly reactive gas formed when emissions from mobile and industrial sources react in the presence of sunlight. The current National Ambient Air Quality Standard for ozone is 0.12 parts per million (ppm), and compliance requires that this level not be exceeded for more than one hour once per year. Presently, at least one-third of the population of the United States lives in areas that do not comply with the ozone standard.

In addition to concerns about the effects of ozone on lung function, researchers have questioned whether ozone has the potential to produce changes in airway cells that could result in cancer. The Health Effects Institute sponsored the present study to determine whether ozone exposure might influence a critical step in the development of lung cancer by increasing the frequency of early, precancerous changes in cell structure.

APPROACH

Epithelial cells line the airways of the lung. Because most human cancers arise from epithelial cells, researchers frequently study the changes induced when these cells are treated with carcinogens (cancer-causing agents). Dr. David Thomassen and coworkers examined the ability of ozone to alter the structure and growth characteristics of epithelial cells from rat tracheas in ways consistent with precancerous changes. The frequency of such alterations was counted in rat tracheal epithelial cells exposed in culture under various conditions: that is, a single 40-minute exposure to ozone (0.7 or 10 ppm), multiple ozone exposures (0.7 ppm, twice weekly for five weeks), or exposure to ozone (0.7 ppm) either before or after treatment with the known chemical carcinogen MNNG. Such alterations were also counted in tracheal epithelial cells removed from rats exposed to ozone (0.12, 0.5, or 1 ppm) for six hours per day, five days per week, for one to four weeks.

RESULTS AND IMPLICATIONS

Rat tracheal epithelial cells exposed once to ozone in culture exhibited the same frequency of precancerous changes as did control cells exposed to clean air. Tracheal epithelial cells cultured from rats that breathed ozone also showed similar frequencies of altered cells as those from rats exposed to clean air. There are questions about whether the test procedure used in these comparisons was sufficiently sensitive to detect small changes in the number of altered cells. In contrast, the frequency of altered cells increased twofold after multiple exposures of cultured cells to ozone. There was also the suggestion of an increased frequency of such cells in cultures treated first with MNNG and then with multiple exposures to ozone. Interpretation of the increase in altered cells after multiple ozone exposures is complicated by the fact that the results may have been influenced by the cell culture conditions. A single exposure of cells to ozone before MNNG treatment either decreased the frequency of altered cells or had no effect, depending on the carcinogen concentration.

The negative results for cells exposed in rats and the variable findings for cells exposed in culture do not allow firm conclusions to be drawn regarding the effects of ozone. Rather, they illustrate the strong influence of experimental variables, such as the timing, duration, and conditions of exposure, on the outcome of studies of the effects of ozone on the frequency of precancerous changes.

This Statement is a summary, prepared by the Health Effects Institute (HEI) and approved by the Board of Directors, of a research project sponsored by HEI from 1988 to 1990. This study was conducted by Dr. David G. Thomassen and associates at the Lovelace Biomedical and Environmental Research Institute. The complete report (The Role of Ozone in Tracheal Cell Transformation, Research Report Number 50) can be obtained from the Health Effects Institute, 141 Portland Street, Suite 7300, Cambridge, MA 02139 (Telephone: 617-621-0266). The Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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ABSTRACT

This project examined the potential role of ozone as a respiratory carcinogen by characterizing its ability to induce or modulate the preneoplastic transformation of rat tracheal epithelial cells. The chemical reactivity of ozone and the types of damage it can cause suggest that it may have a role in environmental carcinogenesis. Previous reports have described an increase in the incidence and number of lung tumors per animal in strain A mice exposed to ozone. However, the role of ozone in the development of the tumors has not been clear. Ozone also has been reported to act alone and synergistically with ionizing radiation to induce changes related to neoplasia in primary hamster embryo cells and in the mouse C3H/10T1/2 cell line in culture. Few other studies have examined the direct cytotoxic or transforming effects of ozone after in vivo or in vitro exposure of cells, and no studies have been reported on the comparative effects of ozone on respiratory cells exposed in vivo or in vitro.

The induction of early preneoplastic changes in populations of rat tracheal epithelial cells by carcinogens can be detected and quantified in vitro after exposures in vivo or in vitro of tracheal epithelial cells. This cell culture and transformation system was used to characterize the transforming potency of ozone.

Tracheal epithelial cells were isolated from Fischer-344/N rats that had been exposed for six hours per day, five days per week for one, two, or four weeks to 0, 0.12, 0.5, or 1.0 parts per million (ppm)* ozone (sea-level equivalents). Cell populations were examined in culture for increases in the frequency of preneoplastic variants. Rats exposed to ozone did not exhibit an increase in the frequency of preneoplastic tracheal cells, although exposed tracheas did exhibit dose-dependent morphological changes.

Rat tracheal epithelial cells were given single, 40-minute in vitro exposures to concentrations of ozone that did not result in any detectable decrease in colony-forming efficiency (approximately 0.7 ppm) and to concentrations that resulted in approximately a 40% decrease (approximately 10 ppm). Exposed cultures were examined for increases in the frequency of preneoplastic variants. The results of these experiments, like those for the in vivo experiments described above, suggest that a single ozone exposure does not induce preneoplastic variants of rat tracheal epithelial cells. In contrast, cultures of rat tracheal cells exposed to 0.7 ppm ozone twice weekly for about five weeks exhibited approximately a twofold increase in the frequency of preneoplastic variants compared with control cultures.

Rat tracheal epithelial cells also were exposed to highly- or weakly-transforming doses of the direct-acting chemical carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and to 0.7 to 0.8 ppm ozone and examined for ozone-modulated alterations in the frequency of transformation. When a single ozone exposure preceded the high level of carcinogen exposures, the transforming potency of the subsequent carcinogen exposure was reduced. When single carcinogen exposures preceded multiple ozone exposures, there was no statistically significant interaction between the effects of ozone and the carcinogen on transformation.

These results demonstrate that under some conditions ozone can induce preneoplastic variants of rat tracheal epithelial cells. In addition, depending on the sequence or combinations of exposures, ozone can reduce, or possibly increase, the transforming potency of the carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for rat tracheal cells in culture.

INTRODUCTION

Ozone, a reactive form of oxygen, is a major constituent of photochemical smog and creates a potential health hazard for humans. Upon inhalation, ozone is capable of triggering a large array of biological effects in the lung, the primary target organ (Lippmann 1989). In the acute phase of exposure, ozone increases pulmonary airway resistance, injures bronchiolar ciliated epithelia and Type I alveolar epithelia, and induces inflammation. Short-term exposures to ozone also impair mechanisms responsible for inactivating and physically clearing infectious agents. With pro-

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* A list of abbreviations appears at the end of this report for your reference.

longed exposure, pulmonary airway resistance may remain elevated, the epithelium undergoes repopulation with cells (Clara cells in airways and Type II cells in alveoli) that are more resistant to ozone injury, some inflammation persists, and fibrogenic activity begins in the mucosal connective tissue.

Determining the carcinogenic potential of an environmental pollutant such as ozone is of critical importance to human health. The chemical reactivity of ozone and the types of damage it causes both in vivo and to cells in vitro provide a basis for suggesting a role for ozone as an initiator or promoter of carcinogenesis. Ozone is able to interfere directly with the plasma membrane by oxidizing sulfhydryl or amine groups on proteins protruding from the membrane (Mudd and Freeman 1977). Ozone also can oxidize intracellular enzymes and susceptible groups of low molecular weight molecules, such as glutathione. Reactions of ozone with lipids can generate hydrogen peroxide and a chain of free radicals leading to the formation of lipid peroxides (Menzel 1984). These radical intermediates and peroxides are highly reactive and potentially toxic. The oxidative stress of an ozone exposure can be enhanced by a concomitant inflammatory response leading to the generation of activated oxygen-species and products of arachidonic acid breakdown (Fantone and Ward 1982). These molecules are also genotoxic and can interact with and damage DNA (Freeman and Crapo 1982; O'Brien 1985).

Few studies have examined the direct genotoxic effects of ozone on cells in vivo or in vitro. The inductions of chromosomal aberrations by ozone in vivo in peripheral blood lymphocytes (Zelac et al. 1971a,b), and in vitro in cultured fibroblasts (Guerrero et al. 1979), have been reported as measures of ozone-induced genotoxicity. Mutational effects of ozone also have been demonstrated in *Escherichia coli* (Hamelin and Chung 1975a,b), *Drosophila virilis* (Erdman and Hernandez 1982), and *Saccharomyces cerevisiae* (Dubeau and Chung 1979, 1982). However, in each of these studies the relevance of the endpoints and the cells that were used with respect to respiratory carcinogenesis are not clear.

Two studies with A/J mice, a strain that develops spontaneous lung adenomas in up to 50% of animals, resulted in an increase in the incidence and the number of lung adenomas per animal after exposure to ozone (Hassett et al. 1985; Last et al. 1987). In contrast, no ozone-induced effects were seen in Swiss Webster mice, which do not display as high an incidence of spontaneous lung tumors as A/J mice (Last et al. 1987). Both of these reports also described the modulating effects of ozone on urethan-induced lung tumors that appeared to differ depending on the treatment protocol. Hassett and coworkers (1985) described an increase in the average number of lung tumors per animal when mice were given urethan repeatedly after weekly exposures to ozone. However, when animals were given a single dose of urethan and exposed to ozone every other week, no effect was seen. Last and colleagues (1987) observed a decrease in the number of lung tumors in mice given daily exposures to ozone after a single, large dose of urethan. Differences in the timing of the ozone and urethan treatments may have been responsible for the apparently contradictory effects of ozone in these studies.

Witschi (1988) recently reviewed the data from these two reports and concluded that there was little evidence to implicate ozone directly as a pulmonary carcinogen. However, it was noted that these and other studies provide evidence suggesting that ozone may act to enhance or inhibit lung tumor development, depending on the ozone and carcinogen exposure sequence.

Finally, ozone (5 ppm for five minutes) has been shown to act alone and synergistically with ionizing radiation to induce changes in culture related to neoplasia in primary hamster embryo cells and in the preneoplastic mouse cell line, C3H/10T1/2 (Borek et al. 1986, 1989). The animal studies discussed above may not support a role for ozone as a complete pulmonary carcinogen; however, this in vitro study, despite its use of fibroblastic cells in culture, suggests that ozone is capable of inducing some changes that may be involved in multistage carcinogenesis.

As indicated by the studies of Borek and associates (1986, 1989), cell culture systems and studies with isolated cells potentially are very useful experimental models. The most relevant cell culture systems employ cells from the target tissues for the carcinogen or carcinogens of interest. The rat tracheal epithelial cell culture and transformation system provides a useful model for studying early, preneoplastic events characterizing the role of potential respiratory epithelial carcinogens, such as ozone, in multistage carcinogenesis.

The rat tracheal epithelial cell transformation system is a useful model for studies of neoplastic progression in vivo or in vitro and serves as a model for the development of bronchial tumors in humans. More than any other segment of the respiratory tract of rodents, the rat trachea resembles the human bronchus (Kendrick et al. 1974). Both contain cartilage, compound mucous glands, and goblet cells. These components are either sparse or nonexistent in the bronchi of inbred rats, mice, and hamsters, but are typical of the human bronchus.

Carcinogenesis studies of rat tracheal epithelial cells can be carried out totally in vitro using culture conditions that permit the proliferation of normal rat tracheal epithelial cells (Thomassen et al. 1983, 1986). The first detectable

stage in the neoplastic progression of rat tracheal epithelial cells in culture is the formation of large colonies of altered cells, termed enhanced growth variants, which are resistant to serum-mediated squamous differentiation. Normal rat tracheal epithelial cells are exposed to carcinogens in vitro using culture conditions that allow their proliferation. After allowing for the carcinogen-induced changes, the culture medium is replaced with a medium that permits the proliferation of enhanced growth variants, but not normal cells. The frequency of variants induced in these experiments can be quantified by dividing the number of variants by the number of colony-forming cells at risk. In these studies, culture conditions that support the proliferation of carcinogen-induced preneoplastic variants, but not normal cells, are used to select variants after carcinogen treatment.

The induction of preneoplastic variants of rat tracheal epithelial cells can also be quantified in vitro after cells have been exposed to known or suspected carcinogens either in vivo (Marchok et al. 1977, 1978; Thomassen et al. 1989) or in vitro (Thomassen et al. 1986). After in vivo exposures, rat tracheal epithelial cells are isolated and plated in vitro by the method described above. Altered cells are found specifically in sections of tracheas with moderate to severe atypia, as measured by conventional cytological and histopathological criteria (Marchok et al. 1982). These cells become malignant after further proliferation in vitro and form squamous cell carcinomas when injected into suitable hosts (Pai et al. 1983).

The in vivo studies described above, examining the potential role of ozone in respiratory carcinogenesis (Hassett et al. 1985; Last et al. 1987; Witschi 1988), suggest that although ozone is not a complete respiratory carcinogen, it may play a role in modulating respiratory carcinogenesis. The in vitro studies described above (Borek et al. 1986) also suggest that ozone may be capable of inducing some changes involved in multistage carcinogenesis. The present study was designed for the following purposes: (1) to characterize the transforming potency of ozone for normal respiratory epithelial cells by quantifying the frequency of ozone-induced preneoplastic variants of rat tracheal epithelial cells exposed to ozone in vivo or in vitro; and (2) to determine whether ozone can modify the transforming potency of a known chemical carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), for the preneoplastic transformation of normal rat tracheal epithelial cells.

SPECIFIC AIMS

This project examined the cytotoxic effects and the transforming potency of ozone on respiratory epithelial cells in vivo and in vitro, either alone or in combination with the known chemical carcinogen, MNNG. These two hypotheses were tested: (1) Ozone can induce preneoplastic transformation of rat tracheal epithelial cells exposed to ozone in vivo or in vitro; and (2) Ozone can interact synergistically with MNNG, resulting in a more-than-additive induction of preneoplastic transformation of rat tracheal epithelial cells.

The project was initiated in response to the Health Effects Institute's Request for Applications 86–1, "Genotoxic, Carcinogenic, and Cocarcinogenic Effects of Ozone."

A goal of this project was to expose normal rat tracheal epithelial cells to ozone, either in vivo or in vitro, and to quantify the frequency of preneoplastic variants in the exposed populations using an in vitro assay. An additional goal was to quantify the frequency of preneoplastic variants induced by combined in vitro exposures to ozone and MNNG, using two different treatment regimens. These regimens included a single, brief ozone exposure followed by a single MNNG exposure and a single MNNG exposure followed by multiple ozone exposures.

METHODS

EXPERIMENTAL DESIGN

Ozone-Induced Rat Tracheal Epithelial Cell Transformation in Vivo

Male Fischer-344/N rats were exposed by inhalation to 0, 0.24, 1.0, or 2.0 mg/m^3 ozone (0, 0.12, 0.5, or 1.0 ppm sea-level equivalents), for six hours per day, five days per week, for one, two, or four weeks. (The Institute is located at an elevation of 1,728 m, where the average barometric pressure is 625 mm Hg, or 82% of sea-level atmosphere.) Concentrations of ozone were chosen to correspond with those used in a National Toxicology Program carcinogenesis study with rats and mice. After exposure, rat tracheal epithelial cells were isolated and examined in culture for ozone-induced increases in the frequency of preneoplastic variants. Tracheas from each exposure also were examined histologically for morphological changes. The experimental groups and the numbers of animals in each group are outlined in Table 1.

Ozone-Induced Rat Tracheal Epithelial Cell Transformation in Vitro

Rat tracheal epithelial cells were isolated from male Fischer-344/N rats and plated in a serum-free medium that allowed normal rat tracheal epithelial cell proliferation.

Table 1. Experimental Groups Used to Examine Transforming Potency of Inhaled Ozone

Ozone Concentration[a] (ppm)	Exposure Duration (weeks)	Number of Rats Used	
		Cell Transformation	Histology
0	1	4	2
	2	4	2
	4	4	2
0.12	1	4	2
	2	4	2
	4	4	2
0.50	1	4	2
	2	4	2
	4	4	2
1.00	1	4	2
	2	4	2
	4	4	2

[a] Sea-level equivalents.

Approximately 20 hours after plating, cultures were exposed to ozone and examined for increases in the frequency of preneoplastic variants. Two concentrations of ozone were used in these studies: (1) concentrations that overlapped with peak levels of environmental exposure (0.6 to 0.8 ppm [= 0.5 to 0.7 ppm sea-level equivalents] for 40 minutes); and (2) a concentration that resulted in approximately 50% cytotoxicity (10.3 ppm [= 8.6 ppm sea-level equivalents] for 40 minutes). These concentrations of ozone will be referred to as 0.7 and 10 ppm in the remainder of the text. Actual concentrations used in individual experiments will be indicated in data tables.

A 50% cytotoxic dose of MNNG (approximately 0.045 μg/mL) was used as a positive control for the induction of preneoplastic variants. Each experiment was conducted at least twice. A complete listing of the experiments involving in vitro exposures of rat tracheal epithelial cells to ozone described in this report is shown in Table 2.

Combined Exposures of Rat Tracheal Epithelial Cells to Ozone and MNNG in Vitro

Rat tracheal epithelial cells were isolated from male Fischer-344/N rats and plated in a serum-free medium permitting normal rat tracheal epithelial cell proliferation. Approximately 20 hours after plating, cultures were entered into one of two treatment regimens. In some experiments, cultures were exposed to a "low" concentration of ozone (0.7 ppm for 40 minutes) and then exposed to either highly or weakly transforming doses of MNNG (approximately 0.2 μg/mL or approximately 0.045 μg/mL, respectively). The frequency of preneoplastic variants in these cultures was determined after a five-week selection period. The timing of exposures in these experiments represents the standard use of the rat tracheal epithelial cell transformation assay (Thomassen et al. 1986). In other experiments, cultures were exposed to weakly transforming doses of MNNG (0.045 μg/mL) and were exposed subsequently to a low concentration of ozone twice weekly for five weeks (total of nine exposures). The multiple ozone exposures were given concurrently with the normal selection for preneoplastic variants. This multiple exposure regimen is similar to that pre-

Table 2. In Vitro Exposures of Rat Tracheal Epithelial Cells to Ozone and MNNG

		Ozone Alone			MNNG Alone		Combined Ozone and MNNG		
Experiment	Control	0.7 ppm	10 ppm	9 Exposures 0.7 ppm	Approximately 50% Cytotoxic Dose	Approximately 90% Cytotoxic Dose	0.7 ppm Ozone + Approximately 90% Cytotoxic Dose MNNG	0.7 ppm Ozone + Approximately 50% Cytotoxic Dose MNNG	Approximately 50% Cytotoxic Dose MNNG + 9 Exposures 0.7 ppm Ozone
OZT1	x		x						
VT3	x		x		x				
VT4	x	x				x	x		
VT7	x	x							
VT11A	x		x		x				
VT12	x	x			x			x	
VT14A	x		x		x				
VT14B	x		x		x				
VT15	x	x				x	x		
VT16	x				x	x	x	x	
VT17	x			x	x[a]				x
VT18	x			x	x[a]				x

[a] Data from VT17 and VT18 are not included in Table 6 because all cultures exposed to MNNG also were given nine 40-minute exposures to buffer during the five weeks of selection for preneoplastic variants as a control for the ozone exposures.

viously described for initiation-promotion studies that have been conducted with both rat tracheal epithelial cells and other cells in culture (Nettesheim et al. 1984). Although rat tracheal epithelial cells given multiple exposures to ozone may respond differently than cells given only a single exposure, the multiple exposure regimen is not, in itself, transforming (Nettesheim et al. 1984). At the end of each experiment, cultures were examined for ozone-mediated increases or decreases in the frequency of MNNG-induced preneoplastic transformation. Each experiment was repeated a minimum of two times. A complete listing of the experiments involving in vitro exposures of rat tracheal epithelial cells to MNNG or ozone and MNNG described in this report is shown in Table 2.

PROCEDURES

Animals and Maintenance

Male Fischer-344/N rats were obtained from the Institute's specific pathogen-free breeding colony. All rats were seven to nine weeks old, and those used for inhalation studies were randomized by weight. All rats were housed in barrier-maintained colony housing until being killed for in vitro experiments, or until being acclimatized in exposure chambers. Animals were housed in polycarbonate cages with filter tops and sterilized hardwood bedding. Feed (Wayne Lab Blox, Allied Mills, Chicago, IL) and tap water were provided ad libitum. Rooms were maintained at 20°C to 22°C, with a relative humidity of 40% to 60%, and a 12-hour light-dark cycle.

Rats being exposed to ozone by inhalation were housed continuously in individual wire cages, within glass and stainless steel chambers (Hazelton HC-1000, Hazelton Systems, Aberdeen, MD) beginning one week before the start of ozone exposures. Feed and water were provided ad libitum. Chambers were maintained at 19°C to 26°C, with 40% to 60% relative humidity and 5 cfm airflow, and on a 12-hour light-dark cycle. Bacteriostatic cageboard was changed daily and chambers were washed weekly.

Cells and Cell Culture

Rat tracheal epithelial cells were isolated by a procedure described by Thomassen and associates (1986). Cells for in vitro exposures were isolated from several animals and pooled. Cells isolated from animals exposed to ozone in vivo were isolated from individual animals and were not pooled. Cells were isolated from four animals in each exposure group to determine cytotoxicity and transformation. Animals were killed by carbon dioxide asphyxiation; their tracheas were removed, filled with 1% Pronase solution (protease type XIV, Sigma Chemical Co., St. Louis, MO) and incubated for 16 to 20 hours at 4°C. Rat tracheal epithelial cells were rinsed from the tracheas through sterile gauze with cold Ham's F12 medium (GIBCO, Grand Island, NY) containing 15 mM *N*-2-hydroxyethel-piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (GIBCO), penicillin (100 units/mL) and streptomycin (100 μg/mL) (Hazelton Research Products, Lenexa, KS). Each trachea typically yielded 3 to 6×10^5 cells, of which more than 95% were viable, as indicated by Trypan dye exclusion (Gray et al. 1983). After centrifugation, cells were resuspended in a serum-free medium, plated in 100-mm-diameter tissue culture dishes (Corning Glass Works, Corning, NY), and incubated at 37°C in a humid atmosphere containing 5% carbon dioxide and 95% air.

The serum-free medium used for primary cultures of rat tracheal epithelial cells, which was described by Thomassen and colleagues (1986), is composed of Ham's F12 medium supplemented with 15 mM HEPES buffer (pH 7.3), 0.8 mM calcium chloride (final concentration) (ultrapure, Alpha Products, Danvers, MA), 1% (v:v) bovine pituitary extract prepared from whole pituitaries (Pel Freeze, Rogers, AK), as previously described by Bertolero and associates (1984), 3 mg/mL bovine serum albumin (fraction V, Sigma A 4503), 1 nM cholera toxin (Sigma), 5 ng/mL epidermal growth factor (Collaborative Research, Waltham, MA), 50 μM ethanolamine (Sigma), 0.3 μM hydrocortisone (Sigma), 5 μg/mL insulin (Calbiochem, La Jolla, CA), 50 μM phosphoethanolamine (Sigma), and 5 μg/mL transferrin (Sigma).

Exposures and Measurements of Ozone Exposure Atmospheres

Ozone was generated for inhalation exposures from compressed air using OREC (Ozone Research and Equipment Corp., Phoenix, AZ) and Sander (model IV) ozone generators. An OREC generator was used to generate ozone for in vitro exposures from house air filtered through a type H ultrafilter (Mine Safety and Appliances Co., Pittsburgh, PA). Ozone concentrations were monitored using factory-calibrated Mast (model 727-3, Mast Development Co., Davenport, IA) and Dasibi (model 1003-AH, Dasibi Environmental Corp., Glendale, CA) monitors. At the end of the study, the calibration of all monitors was rechecked against a factory-calibrated Dasibi monitor (model 1003-AH), kindly provided by Dasibi for that purpose, and all monitors were found to read within 10% of the calibrated monitor. Ozone concentrations were recorded by a Linear 0141 Strip Chart Recorder (Linear Instrument Corp., Reno, NV).

Rat tracheal epithelial cell cultures were exposed to ozone by rocking the cultures in an atmosphere containing ozone so that attached cells were alternately covered by the medium or exposed to air. For exposures, the serum-free

culture medium was replaced with 5 mL per dish of calcium- and magnesium-free phosphate-buffered saline (GIBCO). Culture dishes were placed without their lids at room temperature in a polycarbonate modular incubator chamber (Billups-Rothenberg, Del Mar, CA) whose interior had been siliconized with Surfa Sil (Pierce, Rockford, IL). The chamber was rocked at approximately 10 cycles per minute during exposures. Cultures were exposed for designated intervals (10, 20, or 40 minutes) plus an additional five minutes before timing began to allow the ozone concentration in the exposure chamber to reach approximately 75% of its maximal level. Control cultures were rocked at room temperature with saline for the same amounts of time as for corresponding ozone-exposed cultures.

Ozone entered the chamber from the ozonizer through a port at the top of the chamber, which had been modified by the insertion of a plexiglass deflector plate. The ozone was exhausted to the ozone monitor through a port in the side of the chamber. Teflon tubing was used to conduct ozone into and out of the exposure chamber. A 4-L dilution flask with an adjustable air inlet was placed in-line between the ozonizer and the exposure chamber and provided an extra degree of control for generating low concentrations of ozone with a generator designed to produce high (defined as greater than 10 ppm) concentrations.

Histopathology

The toxic effects of inhalation exposure to air or ozone were determined histologically by sampling tracheas from each exposure group one day after the final exposure. Morphological assessment of the tracheal epithelium in vivo was conducted on two rats from each exposure group. Tracheas were excised immediately after death and fixed in 10% neutral buffered formalin. After fixation, a cross-section of the proximal trachea and another from the mid-trachea were embedded in paraffin, sectioned at 4 to 6 microns, and stained with hematoxylin, eosin, and alcian blue (pH 2.7) for light microscopic examination.

Cytotoxicity Assays

The cytotoxic response of rat tracheal epithelial cells to ozone was determined in preliminary experiments using colony-forming assays to identify exposure levels that did and did not reduce the colony-forming efficiency of rat tracheal epithelial cells for use in subsequent cell transformation assays. In these initial experiments, cultures were exposed to one or more combinations of different ozone concentrations and exposure times. The colony-forming efficiencies of cells in these cultures were compared to those in unexposed, concurrent controls. Approximately 5,000 cells were plated in each 100-mm diameter culture dish, and cultures were exposed to ozone after 20 hours. After 10 days, cultures were fixed with 70% ethanol and stained with 10% aqueous Giemsa, and the number of colonies with at least 30 cells was determined (the majority of colonies contained at least 200 cells) (see Figure 1). Colony-forming efficiencies (the fraction of plated cells that survived and formed colonies) ranged from 1% to 8% for untreated controls (4.3 ± 0.4% [mean ± SE] for controls for the 24 transformation experiments reported here). The data were used to calculate the relative colony-forming efficiency of cells in exposed versus control cultures.

Transformation Assays

Rat tracheal epithelial cells were plated and treated in the method described above using a number of cells sufficient to produce 150 to 250 colonies per dish after treatment. For example, in experiments that involved exposures to highly toxic concentrations of MNNG, the number of cells plated

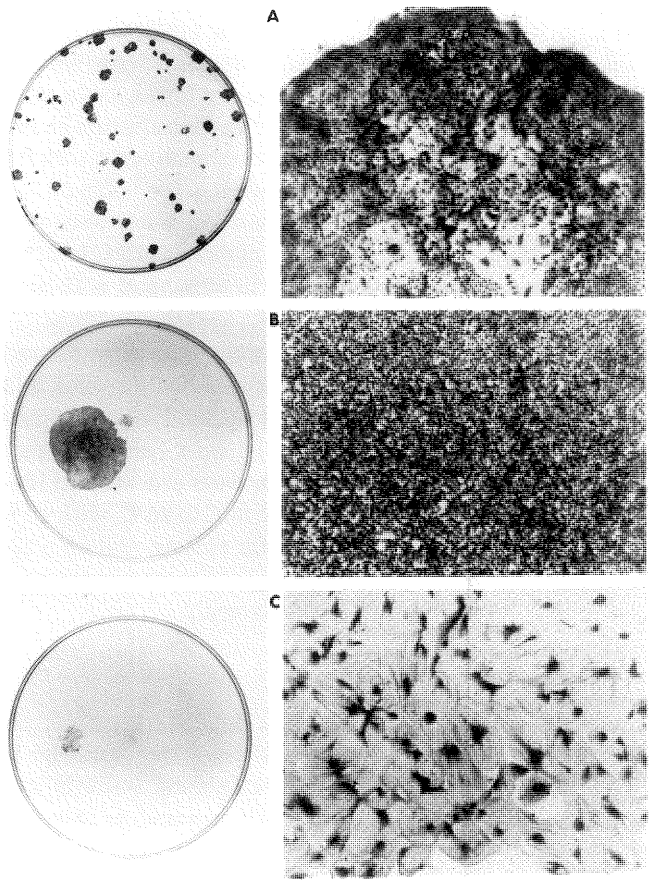

Figure 1. Photomicrographs of (A) rat tracheal epithelial cell colonies 10 days after plating 3,000 primary tracheal cells in a 60-mm-diameter dish with serum-free medium; **(B) preneoplastic variants of rat tracheal epithelial cells** 35 days after exposure of primary culture to MNNG and selection for preneoplastic variants; and **(C) nontransformed rat tracheal epithelial cell colony** remaining 35 days after exposure of primary culture to MNNG and selection for preneoplastic variants. Panels in the right column are magnified × 120.

per dish was increased by up to 10-fold to adjust for MNNG-induced cell killing. Four days after plating, selection for preneoplastic variants was begun by replacing the serum-free medium with a selective medium containing serum (Ham's F12 medium containing 5% fetal bovine serum [lot number 97F-0501, Sigma], 1 μg/mL insulin, 0.3 μM hydrocortisone and antibiotics) (Thomassen et al. 1983, 1986). The culture medium was changed twice each week for five weeks. The cultures were fixed and stained by the method described above and scored for variants by the method previously described (Thomassen et al. 1983).

Under these culture conditions, normal rat tracheal epithelial cells enlarge and appear squamous-like as their rate of proliferation decreases. The cells either detach from their dishes after one to two weeks or persist without proliferation. Colonies previously described as Type III or IV transformants (Kitamura et al. 1986) were scored as transformed colonies (see Figure 1). These colonies were large, basophilic (Giemsa stain), had a high cell density, and consisted primarily of closely packed, small round cells with high nuclear:cytoplasmic ratios. In addition, many of these colonies revealed two to four stratified layers of poorly differentiated cells and cells with features of keratinocyte differentiation, as evidenced by abundant tonofilament bundles, well developed desmosomes, and occasional keratohyaline granules. Frequencies of transformation were calculated by dividing the total number of variants by the total number of colony-forming cells at risk. The total number of colony-forming cells at risk was equal to the average number of colonies per dish, determined from parallel cytotoxicity assays, multiplied by the total number of dishes in the transformation assay (Thomassen et al. 1983). Frequencies of transformation were calculated using data from a minimum of two experiments per condition.

Statistical Analyses

Transformation data, such as that obtained in this study, usually involves counting the number of transformed colonies for a known number of colony-forming cells in a dish. Because the total number of transformed colonies is often small, it is necessary that a statistical analysis take into account the nature of the statistical distribution of the numbers of transformed colonies. The Poisson distribution is an appropriate way to describe such variations in this study because there were relatively few transformed colonies, and the occurrence of a transformed colony was a relatively rare event among the colony-forming cells in each dish. Furthermore, the distribution of transformants in rat tracheal epithelial cell transformation assays has been shown to be Poisson (Thomassen et al. 1983).

If we consider the number of transformed colonies to be Poisson-distributed, then the probability of observing X colonies is $e^{-\lambda}\lambda^X/X!$, where $X = 0, 1, 2, \ldots$, for a known number of colony-forming cells in a dish, and λ is the mean of the Poisson distribution. We take into account the Poisson distribution through a technique known as log-linear modeling, which is a special case of generalized linear models (McCullagh and Nelder 1983). This technique allows us to analyze Poisson-distributed data using methods analogous to analysis of variance and linear regression. Usual analysis of variance and linear regression techniques are actually just examples of generalized linear models used when data are considered to be normally distributed. Another type of generalized linear model is probit analysis, which is used for binomially distributed data.

In log-linear models, the log of the mean of the Poisson distribution, λ, is modeled as a linear function of the factors and covariates of the experimental design, as in analysis of variance or linear regression. The covariates included in the model in the studies described here were the type of exposure (ozone, MNNG, or ozone and MNNG) and individual experiments. Log-linear models differ from the usual linear models in that the variance of the estimated mean values depends on the estimated mean value. This leads to a weighted analysis of the data, with the weights depending on the variance of the estimated means. Because of their relatively larger variance, experiments with low numbers of transformants have less influence on the final estimated mean value than experiments with high numbers of transformants. Computations of mean values in log-linear models must be made using an iterative technique, in which the observations are reweighted at each step, depending on the currently estimated mean value. This contrasts to computations in analysis of variance and linear regression, which require a single step. In our application of log-linear models it is also necessary to take into account the number of colony-forming cells on a dish in order to model the transformation frequency, rather than the number of transformants. This is done by calculating the log of the estimated mean divided by the number of colony-forming cells, and is a standard technique in log-linear models that include an offset in the model. Because the log of the transformation frequencies is being calculated, the differences between groups is naturally expressed as a relative risk of one group in relation to another group.

Each experiment had one control group and up to three exposure groups. Because experiments were conducted at different times, the model was adjusted for changes in the control incidence among experiments. After all the above parameters were estimated, a likelihood ratio test was conducted to determine whether the residual variation was larger than would be estimated for Poisson-distributed data. Such extra-Poisson variation affects the parameter estimates for the model and often, to a larger degree, the estimates of

the standard errors of the parameters. It was assumed that this additional error was due to variation between experiments, and that its variance should be added to the Poisson variance in the log-linear model. If X is a Poisson random variable with extra variation, where $E[X] = \lambda$, then $E[\log X] = \log \lambda$ and $\text{var}[\log X] = \sigma^2 + 1/\lambda$. It was assumed that σ^2 was the same for all groups in this study. Methods for estimating σ^2 have been described by Breslow (1984).

To determine statistical significance, each treated group was compared to its own control, and a relative risk was estimated. The relative risks were multiplied by the concurrent control frequencies of transformation to obtain the values for the corresponding treated cultures. Statistical significance was determined from the 95% confidence limits for each relative risk. Relative risks were considered statistically significant if the 95% confidence interval did not overlap with 1.0.

RESULTS

CYTOTOXIC AND TRANSFORMING POTENCY OF INHALED OZONE FOR RAT TRACHEAL EPITHELIAL CELLS

Exposure of rats to 0, 0.12, 0.5, or 1.0 ppm ozone (sea-level equivalents) for one, two, or four weeks did not result in systematic concentration- or time-dependent alterations in the frequency of preneoplastic cells in the isolated rat tracheal epithelial cell populations (Table 3). The data were analyzed as a two-factor log-linear model, and neither of the main effects, ozone nor duration of exposure, nor the interaction between the two, were significant ($p > 0.05$). The overall mean frequency of transformation observed in these experiments (0.1 ± 0.01%) was similar to the average background frequency of transformation observed in our laboratory in over 90 experiments over a period of more than three years (0.14 ± 0.02%; unpublished data). The colony-forming efficiency data in Table 3 were analyzed using two-way analysis of variance. The duration of the exposure was statistically significant ($p = 0.016$), but the effect of ozone and the interaction between ozone and the duration of exposure were not statistically significant ($p > 0.05$). Although the duration of exposure was significant, it is not important to the outcome of this study for two reasons. First, the variation was small, with an average colony-forming efficiency of 4.5% for week one of exposure, 5.5% for two weeks of exposure, 5.9% for four weeks of exposure, and with a standard error of 0.36% on these averages. Second, experiments were corrected for any variation in colony-forming efficiency because that factor was measured in each experiment.

No exposure-related alterations were evident in the proximal or midtracheal sections from rats exposed for one, two, or four weeks to 0 or 0.12 ppm ozone.

Ozone-induced alterations were restricted to the surface

Table 3. Cytotoxic and Transforming Potency of Inhaled Ozone for Rat Tracheal Epithelial Cells[a]

Ozone Concentration[b] (ppm)	Length of Exposure[c] (weeks)	Colony-Forming Efficiency[d] (%)	Total Colony-Forming Cells at Risk	Total Variants	Frequency of Transformation[d,e] ($\times 10^3$)
0	1	4.4 ± 0.6	14,500	19	1.4 ± 0.3
0.12		3.7 ± 0.4	13,200	8	0.8 ± 0.4
0.5		5.3 ± 0.4	20,200	14	0.8 ± 0.4
1.0		4.4 ± 0.4	17,800	15	0.9 ± 0.3
0	2	5.2 ± 0.6	21,000	13	0.6 ± 0.2
0.12		5.5 ± 1.0	21,600	24	1.0 ± 0.1
0.5		4.9 ± 1.1	19,500	16	0.9 ± 0.1
1.0		6.2 ± 0.7	24,900	20	0.7 ± 0.4
0	4	6.6 ± 0.8	26,900	29	1.3 ± 0.3
0.12		4.9 ± 0.7	26,400	30	1.3 ± 0.3
0.5		6.4 ± 0.6	35,000	36	1.0 ± 0.4
1.0		5.6 ± 0.5	30,400	33	1.2 ± 0.4

[a] Cells were isolated from animals exposed to ozone by inhalation and examined in culture for colony formation and preneoplastic transformation. Twenty-three dishes with 5,000 cells each were plated from each animal.

[b] Sea-level equivalents.

[c] Six hours per day, five days per week.

[d] Mean ± SE of individual experiments, with cells from four rats in each group.

[e] Total variants/total colony-forming cells at risk.

epithelium lining the proximal and midtracheal sections from rats exposed to 0.5 or 1.0 ppm ozone. The most severe morphologic changes took place in rats exposed for two or four weeks to 1.0 ppm ozone. The ozone-induced lesions in the tracheal epithelium in these rats consisted of focal areas of marked attenuation (thinning of the epithelium) and loss of cilia, occasional necrosis and exfoliation of individual epithelial cells, and a few intraepithelial and luminal neutrophils. Lesions in the tracheal epithelium were randomly distributed across cartilaginous and noncartilaginous portions of the trachea and involved approximately 50% to 75% of the luminal surface epithelium. Similar lesions were also evident in the rats exposed to 1.2 ppm for one week, except for a noticeable absence of epithelial cell necrosis and exfoliation.

Rats exposed to 0.5 ppm ozone for one, two, or four weeks had a few widely scattered, focal areas of attenuation and loss of cilia on the surface epithelium lining the proximal and midsections of the trachea. No epithelial cell necrosis, exfoliation, or inflammation was microscopically evident in the sections examined from these animals. In addition, less than a third of the surface of the epithelium was altered.

CYTOTOXIC RESPONSE OF RAT TRACHEAL EPITHELIAL CELLS TO OZONE EXPOSURE IN VITRO

The cytotoxic response of rat tracheal epithelial cells exposed to ozone in culture was determined to identify exposures that did or did not reduce the colony-forming efficiency of cultured cells used in cell transformation experiments. Compared with concurrent controls, rat tracheal epithelial cells exposed to 10 ppm ozone for 40 minutes exhibited significant reductions in colony-forming efficiency ($p<0.05$, Student's t test). However, rat tracheal epithelial cells exposed to 0.7 ppm ozone did not exhibit any time-dependent alterations in colony-forming efficiency (Table 4). As a result, 40-minute exposures to 10 ppm and 0.7 ppm ozone were used in all subsequent experiments and are defined as "high" and "low" exposures, respectively.

Table 4. Cytotoxic Response of Rat Tracheal Epithelial Cells to a Single Ozone Exposure in Vitro

Exposure[a]	Relative Colony-Forming Efficiency[b]
Control	1.00
10 minutes, air	0.89 ± 0.01
20 minutes, air	1.00 ± 0.03
40 minutes, air	1.00 ± 0.01
10 minutes, 0.7 ppm O_3	1.06
20 minutes, 0.7 ppm O_3	1.08
40 minutes, 0.7 ppm O_3	1.02 ± 0.16
10 minutes, 10 ppm O_3	0.81 ± 0.27
20 minutes, 10 ppm O_3	0.80 ± 0.08
40 minutes, 10 ppm O_3	0.63 ± 0.13

[a] Rat tracheal epithelial cells were exposed to ozone or air approximately 20 hours after plating. Ozone concentrations were 0.7 ± 0.1 ppm and 10.3 ± 0.1 ppm (mean ± SE).

[b] Mean colony-forming efficiency (± SD) relative to untreated controls. No SD shown indicates a single determination, 10- and 20-minute groups had two determinations performed, and 40-minute groups had five to seven determinations performed.

PRENEOPLASTIC TRANSFORMATION OF RAT TRACHEAL EPITHELIAL CELLS BY SINGLE OZONE EXPOSURES IN VITRO

Single exposures of rat tracheal epithelial cells to ozone in vitro did not induce preneoplastic variants. Cultures of rat tracheal epithelial cells exposed to high levels of ozone (10 ppm for 40 minutes) had no higher frequency of preneoplastic variants than concurrent controls exposed to air (relative risk = 0.9 to 1.4, 95% confidence interval) (Table 5). To ensure that carcinogen-induced transformation could be detected after exposure to concentrations of a carcinogen that reduced the colony-forming efficiency of rat tracheal epithelial cells by approximately 40%, rat tracheal epithelial cells were exposed to a 40% cytotoxic dose of MNNG, a carcinogen whose transforming potency for rat tracheal epithelial cells has been well characterized (Thomassen et al. 1983, 1986). This exposure induced statistically significant increases in the frequency of preneoplastic variants compared with concurrent controls (relative risk = 2.2 to 3.9, 95% confidence interval) (Table 6). It should be noted that although the absolute frequencies of transformation in both treated and control cultures varied considerably between experiments, the frequencies of transformation in MNNG-treated cultures were greater than in their concurrent controls in every case. Exposure to low doses of ozone (0.7 ppm) also were not transforming for rat tracheal epithelial cells in vitro (relative risk = 0.7 to 1.1, 95% confidence interval) (Table 7).

Very little extra-Poisson variation was observed in these experiments. The largest amount of variation observed was for experiments involving the transformation of rat tracheal epithelial cells by MNNG. However, even for these experiments, extra-Poisson variation did not change the significance of any of the reported results. Differences between cultures treated with MNNG and control cultures did not decrease as a result of extra-Poisson variation, and variances decreased only slightly.

Table 5. Preneoplastic Transformation of Rat Tracheal Epithelial Cells by a Single Exposure to 10 ppm Ozone

Experiment[a]	Total Dishes	Colony-Forming Efficiency (%)	Total Colony-Forming Cells at Risk	Total Variants	Dishes Without Variants	Frequency of Transformation[b] ($\times 10^3$)	Relative Risk[c]
Ozone Exposures							
OZT1	54	0.83	890	1	53	1.1	
VT3	93	2.85	5,530	33	65	6.0	
VT11A	42	0.70	3,530	1	41	0.3	
VT14A	42	2.10	10,600	16	27	1.5	
VT14B	42	2.10	12,300	25	27	2.0	
							1.1 (1.0, 1.3)
Control Groups							
OZT1	59	0.82	2,370	2	57	0.8	
VT3	98	4.75	29,170	156	33	5.3	
VT11A	62	0.94	2,240	0	40	0.0	
VT14A	82	3.10	15,300	28	64	1.8	
VT14B	80	3.45	16,630	26	60	1.6	

[a] Cells were exposed to ozone (mean concentration ± SE was 10.3 ± 0.1 ppm) or air for 40 minutes approximately 20 hours after plating. The mean relative survival ± SE of ozone-exposed rat tracheal epithelial cells was 0.63 ± 0.05.

[b] Total variants/total colony-forming cells at risk.

[c] Estimate of risk relative to concurrent controls, as indicated by matching experiment designations. Values in parentheses are 95% confidence interval. Group exposed to ozone not significantly different from control groups ($p > 0.05$).

PRENEOPLASTIC TRANSFORMATION OF RAT TRACHEAL EPITHELIAL CELLS BY SINGLE EXPOSURES TO OZONE ALONE AND IN COMBINATION WITH MNNG

Single exposures of rat tracheal epithelial cells to ozone in vitro reduced their susceptibility to subsequent MNNG-induced preneoplastic transformation (Table 8). Rat tracheal epithelial cells were exposed to low concentrations of ozone (0.7 ppm for 40 minutes), followed immediately by exposure to highly or weakly transforming doses of MNNG in experiments designed to detect ozone-modulated changes in MNNG-induced transformation. Cultures of rat tracheal

Table 6. Preneoplastic Transformation of Rat Tracheal Epithelial Cells by a 50% Cytotoxic Dose of MNNG

Experiment[a]	Total Dishes	Colony-Forming Efficiency (%)	Total Colony-Forming Cells at Risk	Total Variants	Dishes Without Variants	Frequency of Transformation[b] ($\times 10^3$)	Relative Risk[c]
MNNG Exposures							
VT3	50	0.79	24,650	189	2	7.7	
VT11A	39	0.67	3,140	11	29	3.5	
VT12	37	1.34	6,730	14	24	2.1	
VT14A	40	1.80	8,640	21	25	2.4	
VT14B	41	1.93	9,500	29	24	3.1	
VT16	38	3.57	16,280	6	34	0.4	
							2.9 (2.2, 3.9)
Control Groups							
VT3	98	4.75	29,170	156	33	5.3	
VT11A	40	0.94	2,240	0	40	0.0	
VT12	35	2.20	5,390	1	34	0.2	
VT14A	82	3.10	15,300	28	64	1.8	
VT14B	80	3.45	16,630	26	60	1.6	
VT16	40	4.96	11,900	4	37	0.3	

[a] Cells were exposed to MNNG or buffer for four hours approximately 20 hours after plating (mean relative survival ± SE was 0.6 ± 0.04).

[b] Total variants/total colony-forming cells at risk.

[c] Estimate of relative risk to concurrent controls, as indicated by matching experiment designations. Values in parentheses are 95% confidence interval. Group exposed to MNNG significantly higher than control groups ($p < 0.05$).

Table 7. Preneoplastic Transformation of Rat Tracheal Epithelial Cells by a Single Exposure to 0.7 ppm Ozone

Experiment[a]	Total Dishes	Colony-Forming Efficiency (%)	Total Colony-Forming Cells at Risk	Total Variants	Dishes Without Variants	Frequency of Transformation[b] ($\times 10^3$)	Relative Risk[c]
Ozone Exposures							
VT4	30	6.00	9,000	5	26	0.6	
VT7	43	3.06	6,580	8	36	1.2	
VT12	39	2.30	6,280	6	33	1.0	
VT15	39	2.90	6,970	8	33	1.2	
							0.9 (0.7, 1.1)
Control Groups							
VT4	36	5.76	10,370	10	31	1.0	
VT7	41	2.68	5,490	9	32	1.6	
VT12	35	2.20	5,390	1	34	0.2	
VT15	42	3.80	9,580	19	26	2.0	

[a] Cells were exposed to ozone (mean concentration ± SE was 0.7 ± 0.1 ppm) or air for 40 minutes approximately 20 hours after plating (mean relative survival ± SE was 1.02 ± 0.07).

[b] Total variants/total colony-forming cells at risk.

[c] Estimate of risk relative to concurrent controls, as indicated by matching experiment designations. Values in parentheses are 95% confidence interval. Group exposed to ozone not significantly different from control groups ($p > 0.05$).

epithelial cells exposed to doses of MNNG that reduced colony-forming efficiency by approximately 90% exhibited large increases in the frequency of preneoplastic variants compared with concurrent controls (relative risk = 13 to 19, 95% confidence interval) (Table 8). In contrast, cultures exposed to ozone immediately before exposure to MNNG had a statistically significant reduction in their frequency of MNNG-induced transformation (relative risk = 0.6 to 0.8, 95% confidence interval) (Table 8). No extra-Poisson variation was observed in these experiments. The data in Table

Table 8. Preneoplastic Transformation of Rat Tracheal Epithelial Cells by Combined Exposure to 0.7 ppm Ozone Followed by a 90% Cytotoxic Dose of MNNG

Experiment[a]	Total Dishes	Colony-Forming Efficiency (%)	Total Colony-Forming Cells at Risk	Total Variants	Dishes Without Variants	Frequency of Transformation[b] ($\times 10^3$)	Relative Risk[c]
Ozone + MNNG Exposures							
VT4	36	1.12	20,160	111	2	5.5	
VT15	40	0.09	2,160	69	14	31.9	
VT16	41	1.06	26,080	42	19	1.6	
							0.7 (0.6, 0.8)[d]
MNNG Exposures							
VT4	37	0.86	15,910	124	0	7.8	
VT15	42	0.07	1,760	73	13	41.5	
VT16	40	1.01	24,240	61	9	2.5	
							15 (13, 19)[e]
Control Groups							
VT4	36	5.76	10,370	10	31	1.0	
VT15	42	3.80	9,580	19	26	2.0	
VT16	40	4.96	11,900	4	37	0.3	

[a] Approximately 20 hours after plating, cells were exposed either to ozone (mean concentration ± SE was 0.7 ± 0.2 ppm) for 40 minutes and immediately to MNNG for four hours (mean relative survival ± SE was 0.12 ± 0.05), or to air and then MNNG, or to air and then buffer.

[b] Total variants/total colony-forming cells at risk.

[c] Estimate of risk relative to concurrent controls, as indicated by matching experiment designations. Values in parentheses are 95% confidence intervals.

[d] Group exposed to ozone and MNNG was significantly lower than group exposed to MNNG alone ($p < 0.05$).

[e] Group exposed to MNNG was significantly higher than control group ($p < 0.05$).

8 also were analyzed as a two-factor experiment by adding the data for ozone exposure for experiments VT-4 and VT-15 (found in Table 7) to the data in Table 8. The interaction term for ozone and MNNG was not significant ($p = 0.5$), whereas the main effects of ozone and MNNG were significant ($p < 0.001$).

Preexposure of rat tracheal epithelial cells to ozone had little effect on the transforming potency of subsequent exposures to doses of MNNG that reduced colony-forming efficiency by approximately 40%. As with the results described above and in Table 6, this level of MNNG resulted in a significantly higher frequency of transformation in exposed cultures, compared with concurrent controls (relative risk = 1.9 to 5.1, 95% confidence interval) (Table 9). Preexposure of cultures to ozone did not change the frequency of MNNG-induced transformation (relative risk = 0.8 to 1.2, 95% confidence interval) (Table 9); however, the overall relative risk of 0.9 is consistent with the ozone-mediated decrease in MNNG-induced transformation noted above. Very little extra-Poisson variation was observed in these experiments. The data in Table 9 were analyzed as a two-factor experiment by adding the data for ozone exposure for experiment VT-12 (found in Table 7) to the data in Table 9. There was no significant interaction ($p = 0.9$) between ozone and MNNG, and the main effect of ozone was not significant ($p = 0.7$), although the main effect of MNNG was significant ($p = 0.005$).

PRENEOPLASTIC TRANSFORMATION OF RAT TRACHEAL EPITHELIAL CELLS BY MULTIPLE EXPOSURES TO OZONE ALONE OR IN COMBINATION WITH MNNG

Multiple exposures of rat tracheal epithelial cells to ozone in vitro induced preneoplastic variants. Rat tracheal epithelial cell cultures exposed to ozone (approximately 0.7 ± 0.01 ppm, mean ± SE) twice each week for five weeks (nine 40-minute exposures) had approximately twofold increases in their frequencies of preneoplastic variants compared with concurrent controls (relative risk = 1.5 to 2.1, 95% confidence interval) (Table 10). Some cultures were exposed to a single weakly transforming dose of MNNG (approximately 40% cytotoxicity) 20 hours after plating, before initiating ozone exposures, to determine whether multiple ozone exposures would promote MNNG-induced transformation. This treatment regimen resulted in frequencies of transformation that were consistent with independent, rather than synergistic, effects of ozone and MNNG on transformation (Table 10). Very little extra-Poisson variation was observed in these experiments.

The data in Table 10 also were analyzed as a two-factor experiment. The interaction between MNNG and ozone was not significant ($p = 0.2$), whereas the main effects were both significant ($p < 0.001$). This suggests that MNNG and ozone are acting independently, which for a log-linear model

Table 9. Preneoplastic Transformation of Rat Tracheal Epithelial Cells by Combined Exposure to 0.7 ppm Ozone Followed by a 50% Cytotoxic Dose of MNNG

Experiment[a]	Total Dishes	Colony-Forming Efficiency (%)	Total Colony-Forming Cells at Risk	Total Variants	Dishes Without Variants	Frequency of Transformation[b] ($\times 10^3$)	Relative Risk[c]
Ozone + MNNG Exposures							
VT12	33	1.60	7,390	10	25	1.4	
VT16	42	3.57	17,990	10	33	0.6	
							0.9 (0.7, 1.2)[d]
MNNG Exposures							
VT12	37	1.30	6,730	14	24	2.1	
VT16	38	3.57	16,280	6	34	0.4	
							3.1 (1.9, 5.1)[e]
Control Groups							
VT12	35	2.20	5,390	1	34	0.2	
VT16	40	4.96	11,900	4	37	0.3	

[a] Approximately 20 hours after plating, cells were exposed either to ozone (mean concentration ± SE was 0.8 ± 0.25 ppm) for 40 minutes and immediately to MNNG for four hours (relative survival ± SE was 0.66 ± 0.04), or to air and then MNNG, or to air and then buffer.

[b] Total variants/total colony-forming cells at risk.

[c] Estimate of risk relative to concurrent controls, as indicated by matching experiment designations. Values in parentheses are 95% confidence intervals.

[d] Group exposed to ozone and MNNG was not significantly different from group exposed to MNNG alone ($p > 0.05$).

[e] Group exposed to MNNG alone was significantly higher than control group ($p < 0.05$).

Table 10. Preneoplastic Transformation of Rat Tracheal Epithelial Cells by Combined Exposure to a 50% Cytotoxic Dose of MNNG Followed by Multiple Exposures to 0.7 ppm Ozone

Experiment[a]	Total Dishes	Colony-Forming Efficiency (%)	Total Colony-Forming Cells at Risk	Total Variants	Dishes Without Variants	Frequency of Transformation[b] ($\times 10^3$)	Relative Risk[c]
MNNG + Ozone Exposures							
VT17	41	1.00	4,930	39	18	7.9	4.4 (3.2, 6.1)
VT18	35	2.26	11,095	17	21	1.5	
Buffer + Ozone Exposures							
VT17	41	3.45	8,490	34	22	4.0	2.5 (1.8, 3.4)
VT18	42	3.33	9,790	9	37	0.9	
MNNG + Air Exposures							
VT17	42	1.12	5,630	17	29	3.0	2.9 (2.1, 4.1)
VT18	45	1.89	11,880	24	28	2.0	
Buffer + Air Exposures (Control Groups)							
VT17	41	2.65	6,520	10	33	1.5	
VT18	43	5.36	4,520	2	41	0.4	

[a] Cells were exposed to MNNG (relative survival ± SD was 0.39 ± 0.05) or buffer for four hours approximately 20 hours after plating. Cultures then were exposed to ozone (mean concentration ± SE was 0.7 ± 0.01) or air for 40 minutes, twice each week, beginning three days after exposure to MNNG, for a total of nine exposures.

[b] Total variants/total colony-forming cells at risk.

[c] Estimate of risk relative to concurrent controls, as indicated by matching experiment designations. Values in parentheses are 95% confidence intervals. Groups exposed to MNNG and ozone, ozone alone, and MNNG alone were each significantly different from the buffer control groups ($p < 0.05$).

means that the relative risks estimated for ozone and MNNG are multiplied together to estimate the relative risk of combined exposure ($2.5 \times 2.9 = 7.3$). The estimated relative risk for combined exposure was 4.4, which is lower than, but not statistically different from, the product of the relative risks. Another way of combining the main factors is to add the excess relative risk (relative risk − 1) of ozone and MNNG to estimate the excess relative risk of combined exposure. The sum of the excess relative risks for MNNG and ozone was 3.39, and an excess relative risk of 3.44 was estimated for the combined exposure. The similarity of these numbers indicates that this estimate is also consistent with the data.

SUMMARY OF RESULTS

The major findings from both in vivo and in vitro experiments are as follows:

1. Single exposures of rat tracheal epithelial cells to ozone in vitro (0.7 or 10.0 ppm ozone for 40 minutes) did not increase the frequency of preneoplastic variants in the exposed populations.
2. Single exposures of rat tracheal epithelial cells to ozone in vitro (0.7 ppm for 40 minutes) reduced the transforming potency of a subsequent MNNG exposure by 30%.
3. Multiple exposures of rat tracheal epithelial cells to ozone in vitro (0.7 ppm twice each week for five weeks) increased the frequency of preneoplastic variants in the exposed populations.
4. Multiple exposures of rat tracheal epithelial cells to ozone in vitro (0.7 ppm twice each week for five weeks) had an additive or, possibly, multiplicative effect on the transforming potency of a single MNNG exposure given before the ozone exposures.
5. Multiple exposures of rats to ozone by inhalation (0.12, 0.5, or 1.0 ppm for six hours per day, five days per week for one, two, or four weeks) did not increase the frequency of preneoplastic variants in populations of rat tracheal epithelial cells isolated from exposed animals.

DISCUSSION

These studies demonstrated that exposure of normal rat tracheal epithelial cells to ozone in vitro can either increase or decrease the frequency of preneoplastic variants in the exposed populations, depending on the timing, duration, and conditions of exposure. In contrast, exposing rats to ozone by inhalation, using concentrations of ozone that were transforming after multiple exposures in vitro, did not

affect the frequency of preneoplastic variants in populations of tracheal cells isolated from exposed animals.

A major goal of these studies was to provide information that would be useful in determining the carcinogenic risk associated with exposure to ozone. The results presented here provide information that (1) can be used to evaluate better the role of ozone in respiratory carcinogenesis, and (2) will be useful in the design of additional experiments to characterize better that role. However, the results also illustrate the complexity of clearly defining the risk from exposure to an environmental pollutant such as ozone, for which human exposures are constantly varying in duration, concentration, and coexposure with other potential carcinogens.

Brief exposures to ozone (5 ppm for five minutes) have been reported to induce preneoplastic or neoplastic changes in hamster embryo and mouse C3H/10T1/2 cells, respectively (Borek et al. 1986). In contrast, in the experiments reported here, single exposures to ozone (0.7 or 10 ppm for 40 minutes) did not induce preneoplastic variants in primary cultures of rat tracheal epithelial cells. Although an increase in the frequency of transformation was not observed after rat tracheal epithelial cells were exposed to ozone, the exposures were not completely without effect. Exposure to 10 ppm ozone for 40 minutes reduced the colony-forming efficiency of exposed cells by approximately 40%, whereas exposure to 0.7 ppm ozone for 40 minutes had no detectable effect on the colony-forming efficiency.

There are several differences between the experiments reported here and those of Borek and colleagues (1986) that may explain the differences observed in the responsiveness of the cells exposed to ozone. The two principle exposure differences, the rocking of rat tracheal epithelial cell cultures during exposure in this study versus stationary cultures in Borek's study, and the ozone concentration and exposure periods used probably were not responsible for the differences in response. The conditions used in the experiments described here should result in similar (0.7 ppm ozone for 40 minutes) or substantially greater (10 ppm ozone for 40 minutes) total exposures than those described by Borek and associates (1986) (5 ppm ozone for five minutes).

However, differences in the types of cells used in these two studies may have been responsible for the observed differences in response. Borek and colleagues (1986) used early passage (hamster embryo) or preneoplastic (C3H/10T1/2) fibroblastic cells, whereas the present study used primary epithelial cells. Basic differences in the responsiveness of fibroblastic and epithelial cells to ozone could exist. The types of changes required to transform fibroblastic cells also may be very different (and inducible by ozone) from those required to transform epithelial cells (which may not be inducible by ozone). However, the transformation of rat tracheal epithelial cells by multiple ozone exposures reported here makes these varied requirements for change an unlikely explanation for the observed differences.

Another possible explanation of the noted differences is the in vitro "age" of the cells used in the different studies. The results reported here involved exposures of cells to ozone within 24 hours after their being isolated from animals. In contrast, the cells exposed to ozone in the studies by Borek and associates (1986) were isolated from animals either days (hamster embryo cells) or years (C3H/10T1/2 cells) before their exposure. Isolated cells may rapidly lose in vivo functions or capacities (for example, antioxidant capabilities) that protect them from environmental insults such as ozone exposure. Primary cultures of rat tracheal epithelial cells still may possess near-normal levels of their in vivo defense capabilities 20 hours after isolation. However, hamster embryo cells, C3H/10T1/2 cells, and rat tracheal epithelial cells exposed to ozone over a period of five weeks may have partially or entirely diminished levels of these defense capabilities. These defense mechanisms may have been responsible for the lack of responsiveness to ozone exhibited by tracheal cells exposed in vivo. Additional studies comparing the antioxidant capabilities of primary rat tracheal epithelial cells with those of hamster embryo cells, C3H/10T1/2 cells, or preneoplastic rat tracheal epithelial cells would be interesting and informative.

The observation that multiple, but not single, ozone exposures resulted in preneoplastic transformation of rat tracheal epithelial cells suggests that these cells may require a much greater total exposure to ozone to induce preneoplastic variants than the cells used by Borek and colleagues (1986). However, the experiments reported here do not fully define the number or timing of exposures required. Additional studies investigating the transforming potency of either single or multiple ozone exposures of rat tracheal epithelial cells at different times after their isolation are required to define more precisely the number of exposures required for transformation and the importance of the timing of those exposures.

In addition to these variables, the target size of transformation-susceptible cells in cultures given multiple ozone exposures over the five-week course of the multiple exposure experiments is likely to change. Thus, quantification of transformation frequencies based on the initial number of colony-forming cells at risk may not accurately represent the true transformation frequencies. Studies also are needed to determine the effect on transformation of time-dependent changes in the number of cells at risk. The multiple expo-

sure protocol itself was not responsible for the increase in transformation frequency observed in cultures given multiple ozone exposures. The frequencies of transformation in the multiple buffer controls in these experiments were the same as those for single exposure controls in other experiments. Previous studies (Nettesheim et al. 1984) also have demonstrated that multiple exposures of rat tracheal epithelial cells to the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate, during the five weeks of selection for preneoplastic variants after carcinogen exposure did not affect the frequency of preneoplastic variants, although these exposures did affect the frequency of cells with phenotypes thought to be associated with later stages of neoplastic progression.

Finally, it should be noted that although single exposures of rat tracheal epithelial cells in culture did not transform the cells, these exposures were not without effect. Single exposures to high concentrations of ozone reduced the colony-forming efficiency of exposed cells, whereas single exposures to low concentrations reduced the transforming potency of subsequent MNNG exposures.

Although this discussion has focused on many possible differences between the results of Borek and coworkers (1986) and those from the present study, it should be emphasized that ozone can induce neoplasia-related changes in normal rat tracheal epithelial cells, hamster embryo cells, and C3H/10T1/2 cells, despite differences in the number or nature of required ozone exposures. These results suggest that exposure to environmental ozone should be considered a risk factor for the development of respiratory cancer.

There are few other published studies that have examined the direct genotoxic effects of ozone on cells in vitro. Guerrero and associates (1979) reported that ozone (no more than 1.0 ppm for one hour) induced dose-related increases in chromosomal aberrations in cultured human diploid fetal lung fibroblasts. As discussed above, these cells had been in culture for months (passage 37 cells were used) before their exposure to ozone. Mutational effects of ozone have been demonstrated in *Escherichia coli* (Hamelin and Chung 1975a,b), *Drosophila virilis* (Erdman and Hernandez 1982), and *Saccharomyces cerevisiae* (Dubeau and Chung 1979, 1982). However, the relevance of the cells used in these other studies or of the relationship of the exposures given (for example, 30 ppm ozone for three hours, 50 ppm ozone in water for 30 to 90 minutes, 0.1 ppm ozone in water for 60 minutes) to those used in this study cannot be determined.

Although single exposures of rat tracheal epithelial cells to ozone in vitro did not transform these cells, and multiple exposures did result in increases in the frequency of preneoplastic cells, the same exposures given under different exposure conditions had dramatically different effects. The lack of a transformation response in populations of tracheal epithelial cells isolated from rats exposed to ozone for one, two, or four weeks (maximum total exposure was 1.2 ppm of ozone for six hours per day, five days per week, for four weeks) was surprising in view of the increased frequency of transformation in cultures of rat tracheal epithelial cells exposed only nine times over a period of five weeks (total exposure was 0.7 ppm of ozone for 40 minutes, for nine exposures). In spite of the ozone-induced morphological changes seen in exposed tracheas, intracellular targets for ozone-induced cell killing and cell transformation still may have been protected from critical ozone-induced damage. Alternatively, ozone-induced cytotoxic and transforming damage could have been repaired more efficiently in vivo, rather than in vitro. Chronic exposure of the respiratory epithelium to ozone can result in an adaptive or tolerance response in which the epithelium may exhibit an increased resistance to ozone-induced transformation (Dungworth 1989).

The results of our in vivo studies, in contrast with the results of our in vitro transformation experiments, do not suggest that exposure to environmental ozone is a significant risk factor in the development of respiratory cancer. It is important to note, however, that rat tracheal epithelial cells can be transformed by inhalation exposures to carcinogens such as cigarette smoke (five weeks of exposure; Thomassen et al. 1989) or radon progeny (single exposures; Thomassen et al. 1990). In addition, the morphology of tracheas isolated from animals exposed to ozone by inhalation indicates that the ozone exposures did affect the tracheal epithelium. Thus, the lack of a transformation response in tracheal cells isolated from animals exposed to ozone is not due simply to the inability of inhaled carcinogens to transform tracheal cells or to a lack of exposure of the tracheal epithelium in these studies to inhaled ozone. Additional studies characterizing the intracellular effects and long-term biological consequences of inhaled ozone are needed to clarify these results.

The interactive effects of ozone and other agents in causing preneoplastic or neoplastic transformation also appear to be complicated. Although single ozone exposures in vitro did not increase the frequency of preneoplastic rat tracheal epithelial cells in the population, single exposures of rat tracheal epithelial cells to ozone (0.7 ppm ozone for 40 minutes) reduced the transforming potency of the MNNG exposures that followed these ozone exposures by approximately 30%. In contrast, exposing C3H/10T1/2 cells to ozone before exposure to gamma-rays neither enhanced nor reduced the gamma-ray-induced transformation response (Borek et al. 1989). Although the reason for this effect is not

known, exposing rat tracheal epithelial cells to ozone may induce enzymes involved in the repair of MNNG-induced damage. This does not seem likely, however, because the cytotoxic response of rat tracheal epithelial cells to MNNG in these studies was the same in cultures preexposed to ozone or air. Alternatively, the preexposure of rat tracheal epithelial cells to ozone could alter the progression of cells through the cell cycle, which could, in turn, alter the cells' responsiveness to MNNG. The cytotoxic and transformation effects of MNNG for C3H/10T1/2 cells have been shown to be cell cycle–dependent (Grisham et al. 1980). It is not known whether MNNG-induced transformation of rat tracheal epithelial cells exhibits a similar cell cycle dependence.

Although single exposures of rat tracheal epithelial cells to 10 ppm ozone did not induce preneoplastic transformants, they did reduce the colony-forming efficiency of exposed cells. Differences in the time-dependent colony-forming efficiency and transformation responses of cells to chemical carcinogens have been described for BALB/3T3 cells in culture (Saffiotti et al. 1984). These findings suggest that carcinogen-induced damage responsible for reducing colony-forming efficiency and for transforming cells may be different, and, thus, have different susceptibilities to repair. Characterizing the effect of ozone exposures on DNA repair and on inducing repair enzymes in rat tracheal epithelial cells may provide an explanation for the inhibitory effect of ozone on MNNG-induced transformation.

Single exposures of hamster embryo cells or C3H/10T1/2 cells to ozone after exposure to gamma rays resulted in an increased frequency of transformation, which was consistent with a synergistic interaction between the agents (Borek et al. 1986). In contrast, the interaction from exposing rat tracheal epithelial cells to MNNG and then to ozone for multiple exposures was not statistically significant. Thus, depending on the cells being used, the sequence of exposures, or the combination of exposures, ozone can potentially have a positive effect, a negative effect, or no effect on the transforming potencies of other carcinogens.

Similar results have been described for the modulating effects of ozone on tumor development in laboratory animals. Strain A/J mice given injections of urethan after weekly exposures to ozone developed more lung tumors than mice in ozone-only or urethan-only control groups (Hassett et al. 1985). These results were consistent with a synergistic interaction between ozone and urethan. In contrast, when mice were exposed to a single, small dose of urethan before biweekly exposures to ozone, no increase in the incidence of tumors was observed when compared with mice exposed to urethan only (Hassett et al 1985). Furthermore, mice exposed to a single, large dose of urethan before daily exposures to ozone had a lower incidence of lung tumors than animals exposed only to urethan (Last et al. 1987). Thus, as with the effects of ozone on the preneoplastic transformation of rat tracheal epithelial cells in vitro, in vivo exposures to ozone potentially can have a positive effect, a negative effect, or no effect on the development of carcinogen-induced lung tumors in laboratory animals.

SIGNIFICANCE OF FINDINGS

The findings of this study suggest that environmental exposure to ozone may present a significant health risk with respect to the development of lung cancer in humans. The observation that brief, biweekly ozone exposures can induce preneoplastic variants of rat tracheal epithelial cells in culture suggests that ozone-induced damage can initiate the multistep progression of respiratory epithelial cells to neoplasia. In contrast, the observation that protracted, daily exposures of rats to ozone did not increase the frequency of preneoplastic tracheal cells, although similar ozone concentrations were transforming in vitro, suggests that ozone may not be capable of initiating neoplastic progression in vivo. These apparently contradictory results have several possible interpretations that need to be considered when extrapolating the effects of ozone in experimental systems to effects on humans: (1) cells may respond differently to ozone in vitro and in vivo; (2) the effective concentrations of ozone reaching the tracheal cells may be different in vitro and in vivo; and (3) the nature of ozone-induced damage may depend on the duration and frequency of ozone exposures. The experiments reported here and information available in the literature cannot distinguish between these possibilities. However, it is interesting to speculate on these possibilities and their potential ramifications in estimating human risk from exposure to ozone.

Differences in the responsiveness or total exposure of tracheal epithelial cells to ozone in vitro and in vivo would not be surprising given the presence of the mucous layer over the tracheal epithelium in vivo and the very different cell geometries in vivo and in vitro. However, as previously discussed, preneoplastic variants of tracheal epithelial cells can be induced in vivo by exposing rats to cigarette smoke (Thomassen et al. 1989) or radon progeny (Thomassen et al. 1990). Thus, the in vivo environment of the rat tracheal epithelium does not preclude the induction of preneoplastic variants.

In the experiments reported here, differences in the effects of ozone exposure on the transformation of tracheal epithelial cells in culture (i.e., increased or decreased frequencies of transformation) depended on the timing and frequency of exposure. Similar effects of treatment fre-

quency were described when characterizing the role of ozone in the induction of mouse lung tumors (Hassett et al. 1985; Last et al. 1987). Most human exposures to high concentrations of ozone are both sporadic and of relatively short duration. If human responsiveness to ozone is determined not only by dose but also by the duration and frequency of exposure, the risk of transformation of tracheal cells from biweekly exposures to ozone in culture may predict more accurately the human risk from exposure to ozone than the results of the chronic in vivo exposures. Chronic exposure of the respiratory epithelium to ozone can result in an adaptive or tolerance response in which the epithelium has an increased resistance to ozone-induced damage and, potentially, a resultant decrease in ozone-induced transformation (Dungworth 1989). Thus, preneoplastic variants of the tracheal epithelium might have been induced by in vivo ozone exposures if they had been less frequent and of shorter duration. To interpret accurately these data and determine the human risk from exposure to ozone, we must understand the differences in responsiveness of respiratory epithelium and respiratory epithelial cells to ozone as a function of the frequency and duration of ozone exposures.

The findings of this study and other studies on the role of ozone in neoplastic and preneoplastic transformation in vivo and in vitro demonstrate that there is a critical need for additional information on the effect of variables of the exposure protocol, the biological systems used, and the biological endpoints with respect to the projected risk from exposure to ozone. Although the data provided by this study and the findings of Witschi (1988) suggest a possible cancer risk for humans from exposure to ozone, these data certainly are not conclusive. The neoplastic or preneoplastic responses of cells or tissues to ozone depended not only on the species or cell types used but also on the timing, duration, and conditions of exposure.

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PUBLICATIONS RESULTING FROM THIS RESEARCH

Thomassen ĐG, Harkema JR, Stephens ND, Griffith WC. 1991. Preneoplastic transformation of rat tracheal epithelial cells by ozone. Toxicol Appl Pharmacol 109:137–148.

ABBREVIATIONS

HEPES	*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid
MNNG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
O_3	ozone
ppm	parts per million

INTRODUCTION

In the summer of 1986, the Health Effects Institute (HEI) issued a Request for Applications (RFA 86–1) to solicit proposals on "Genotoxic, Carcinogenic, and Cocarcinogenic Effects of Ozone." David G. Thomassen, of the Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute in Albuquerque, New Mexico, submitted a proposal entitled "The Role of Ozone in Tracheal Cell Transformation" in response to this RFA. The experiments he proposed were designed to determine whether ozone produced early, precancerous cellular changes. HEI approved a two-year study in March 1987, and the project began in March 1988. Total expenditures were $230,681. The Investigators' Report for the project was submitted for review in October 1990. The revised report was received in March 1991 and was accepted by the Health Review Committee in April 1991.

During the review of the Investigators' Report, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective, as an aid to the sponsors of the HEI and to the public.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Section 202(a), (b)(1), (g), and (h) and Section 207(c)(4), (5), and (6) impose specific requirements for reductions in motor vehicle emissions of certain oxidants (and other pollutants) and, in some cases, provide the EPA with limited discretion to modify those requirements.

In addition, Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards (NAAQS) to protect the public health. The current primary and secondary NAAQS for ozone is 0.12 parts per million (ppm)*. This standard is met when the number of days per year with maximum hourly average concentrations above 0.12 ppm is equal to or less than one. Section 181 of the Act classifies the 1989 nonattainment areas according to the degree that they exceed the NAAQS and assigns a primary standard attainment date for each classification.

Because determining the appropriate standards for emissions of oxidants and their precursors depends, in part, on an assessment of the health risks that they present, research into the health effects of ozone in studies like this one is essential to the informed regulatory decision-making required by the Clean Air Act.

SCIENTIFIC BACKGROUND

The protective ozone layer in the upper atmosphere shields the earth from the sun's harmful ultraviolet radiation. However, at lower elevations, ozone is a pollutant; it is the major component of photochemical smog. At ground level, ozone is a major health concern because it is highly reactive, and, at high concentrations, can injure tissues and cells (U.S. Environmental Protection Agency 1986, 1991b).

Ozone is not emitted directly into the atmosphere, but is formed by complex photochemical reactions between nitrogen oxides and certain species of hydrocarbons, termed "volatile organic compounds," in the presence of sunlight. Both volatile organic compounds and nitric oxide are present in motor vehicle and industrial emissions (U.S. Environmental Protection Agency 1991b). Another source of volatile organic compounds is evaporative emissions from fuel lines and gasoline tanks. Because the reactions that produce ozone are stimulated by both sunlight and high temperature, peak ozone concentrations occur during the warmer times of the year.

The current NAAQS for ozone is 0.12 ppm, a level not to be exceeded for more than one hour once per year. In 1990, daily one-hour maximum ozone levels ranged from 0.06 ppm in the cleaner sites of the United States to 0.3 ppm in Los Angeles and San Bernadino counties in California (U.S. Environmental Protection Agency 1991a). Ozone is the most pervasive air pollutant in the United States, with an estimated 66.7 million people living in counties that do not meet the ozone standard (U.S. Environmental Protection Agency 1991b). Broad peaks of ozone, reaching 0.1 ppm and higher, sometimes lasting for 8 to 12 hours, have been detected both in the United States and in the Netherlands. Exposures like these can continue for several consecutive days

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

during a summer air pollution episode (Rombout et al. 1986; Van Bree et al. 1990).

After a short description of some of ozone's effects on respiratory function in humans and injury to the lungs of laboratory animals, this section will focus on (1) the mechanisms by which ozone can cause cell damage; (2) ozone's ability to induce benign lung tumors in mice; (3) ozone's ability to produce cell changes associated with cancer development; and (4) changes in tracheal epithelial cells in culture that reflect their conversion to precancerous and cancerous cells.

Because of its high reactivity, ozone has the potential to produce cellular changes that could result in cancer. However, there is presently no firm evidence linking ozone exposure with the development of cancer (Witschi 1988, 1991).

EFFECTS OF OZONE EXPOSURE ON HUMANS AND LABORATORY ANIMALS

People exposed to ambient air containing ozone at the level of the current standard can experience transient decreases in lung function during outdoor exercise or recreation (McDonnell et al. 1983; Lippmann 1989; Horstman et al. 1990). Because ethical constraints limit human ozone exposures in clinical studies to levels under 0.5 ppm, laboratory animals are used to evaluate the effects of prolonged exposure to higher ozone concentrations. Such studies show that ciliated epithelial cells at the important junction between conductive airways and the respiratory region of the lung are injured in rats exposed to either 0.12 or 0.25 ppm ozone for 12 hours per day for six weeks (Barry et al. 1985, 1988). In vitro studies show that exposure to approximately 1 ppm ozone results in cell damage and a loss of ciliated cells in rat tracheal epithelial tissue (Nikula and Wilson 1990), and decreased production of proteins that confer the characteristic viscosity to mucus (Last and Kaizu 1980). Injury to ciliated cells and changes in the properties of mucus can decrease the lung's ability to remove foreign particles.

CELL DAMAGE CAUSED BY OZONE

Ozone is one of the most powerful oxidants. Because of its high reactivity, ozone has the potential to damage cells by several mechanisms (summarized by Mustafa 1990). These include the formation of free radicals (compounds containing one or more unpaired electrons) and reactive intermediates; the initiation of lipid peroxidation chain reactions; the induction of inflammation; and the alteration of membrane permeability.

Ozone itself is not a free radical. When dissolved in the aqueous environment of cells, ozone decomposes to produce hydrogen peroxide and the superoxide (O_2^-) and hydroxyl (OH) radicals, which can initiate reactions that damage cells. Ozone's reaction with the lipid component of cell membranes produces lipid peroxides that may also induce cell damage. Because highly reactive radicals are short-lived and susceptible to cellular defense mechanisms, Goldstein (1979) and Pryor (1991) proposed that if ozone exposure results in damage to genetic material, it is the products of oxygen radical attack on cell membranes that are responsible for damage at more distant sites, such as the cell nucleus.

Inhaling ozone can cause inflammation of lung tissue. As a result, the inflammatory cells that migrate to the affected tissue add to the damage initiated by ozone by releasing additional oxygen free radicals and destructive enzymes (Esterline et al. 1989; Mustafa 1990; Weitzman and Gordon 1990). Ozone also can attack the protein component of cell membranes, increasing cell permeability and weakening the barrier that prevents the passage of molecules across the membrane (Mustafa 1990).

DOES OZONE PLAY A ROLE IN TUMOR DEVELOPMENT AND CARCINOGENESIS?

The only evidence that ozone might actually play a role in tumor development is that, under some experimental conditions, it induces benign lung tumors in mice. In early studies on ozone's toxicity to laboratory animals, neoplasia and inflammation were seen in mice exposed to pollutant mixtures containing relatively high concentrations of ozone (estimated at 1 to 4 ppm) generated from artificial smog (Kotin and Falk 1956; Kotin et al. 1958; Nettesheim et al. 1970). In contrast, lifetime exposures of hamsters to synthetic smog did not result in respiratory tract tumors (Nettesheim et al. 1975).

Swiss Webster mice exposed intermittently to 2.5 to 4.5 ppm ozone in air for 75 or 120 days developed tracheal epithelial lesions and a statistically significant increase in the number of benign lung adenomas (Werthamer et al. 1970; Penha and Werthamer 1974). However, the high concentrations of ozone used in these studies make it difficult to extrapolate the results to human exposures.

More recently, ozone has been shown to induce benign lung tumors in A/J mice, a strain with a high spontaneous incidence of lung tumors. The proportion of mice with lung tumors was significantly higher in the A/J mice exposed to 0.5 ppm ozone for six months than in the control animals exposed to air (Hassett et al. 1985). Last and coworkers (1987) exposed both A/J and Swiss Webster mice to 0.4 and 0.8 ppm ozone intermittently for 18 weeks. Compared with unexposed control animals, ozone exposure did not change

the frequency of tumors in Swiss Webster mice. However, A/J mice displayed an increase in both the number of animals with adenomas and the number of tumors per lung after exposure to 0.8 ppm ozone. Interpreting the importance of the lung tumors in mice poses a dilemma because it is difficult to evaluate ozone's potential to induce tumors from observations made primarily in the most susceptible strain of one species. In addition, Witschi (1991) has suggested that the positive results may not have been due entirely to a substantial increase in lung adenomas in mice exposed to ozone, but rather to abnormally low tumor incidences in control mice not exposed to ozone.

Both groups of investigators (Hassett et al. 1985; Last et al. 1987) examined the effect of chronic ozone exposure on the development of chemically-induced lung tumors. Mice exposed to 0.5 ppm ozone intermittently for six months and injected with the carcinogenic chemical urethan after each exposure developed an increased number of lung adenomas compared to mice treated with urethan but not ozone. The investigators suggested that ozone acted as a cocarcinogen with urethan. When the investigators changed their experimental protocol they obtained different results. Mice injected only once with urethan, before intermittent exposure to 0.3 ppm ozone, did not show an increased tumor frequency, compared to animals treated only with urethan (Hassett et al. 1985). Last and colleagues (1987) injected Swiss Webster and A/J mice with urethane once, and one day later exposed them to 0.4 or 0.8 ppm ozone for 18 weeks. Under these conditions, both strains of mice displayed dose-dependent decreases in the number of tumors per lung; however the decrease in Swiss Webster mice was not statistically significant. The results of Hassett and colleagues (1985) and Last and coworkers (1987) suggest that the experimental design used to study the effects of ozone exposure on tumor development plays a large role in the results that are obtained (Witschi 1991).

Current theories postulate that carcinogenesis is a multistage process (reviewed by Harris 1991). Initiation, the first step in tumor formation, is characterized by alterations in DNA, the genetic material of the cell. At the later stage of tumor promotion, proliferation and clonal expansion of initiated cells take place. Increased cell proliferation raises the probability of additional genetic damage by enlarging the number of cells with altered DNA (Harris 1991). A cascade of ozone-initiated reactions may damage DNA, induce cell proliferation, or both.

EFFECTS OF OZONE ON DNA

DNA can be damaged by alterations in chromosomes (which contain DNA) and by the crosslinking of DNA. These reactions have been demonstrated in vitro and in cells isolated from humans and laboratory animals that inhaled ozone.

Damaged chromosomes were found in peripheral blood lymphocytes isolated from hamsters exposed to 0.2 ppm ozone for five hours (Zelac et al. 1971a,b). Merz and associates (1975) observed single-strand DNA breaks in lymphocytes isolated from human subjects exposed to 0.5 ppm ozone for six or ten hours. However, these results were not confirmed by McKenzie and coworkers (1977), who exposed human subjects to a slightly lower ozone concentration (0.4 ppm) for a shorter time (four hours). Rithidech and coworkers (1990) isolated alveolar macrophages with damaged chromosomes after exposing rats for six hours to ozone levels of 0.12 to 0.80 ppm. The investigators proposed that the damage resulted from breaks in single-stranded DNA. Although airway epithelial cells, rather than alveolar macrophages, are the major cells at risk for the induction of lung cancer, the damage to macrophages may be predictive of epithelial cell damage.

Chromosomal damage also can occur by breaks in double stranded DNA (Wolff et al. 1976). Although Guerrero and colleagues (1979) reported that lymphocytes isolated from humans exposed to 0.5 ppm ozone for two hours showed no chromosomal abnormalities, they did observe a dose-dependent increase in this type of DNA damage in human fetal lung cells exposed in vitro for one hour to ozone concentrations ranging from 0.25 to 1 ppm. Studies such as those summarized above suggest that exposure to ozone may result in altered DNA and cause genetic damage by producing breaks in either single- or double-stranded DNA.

Chromosomal alterations induced by exposure to ozone may reflect an interaction between nuclear components and the products of ozone's reaction with cell membranes, such as malonaldehyde (Goldstein 1979; Steinberg et al. 1990; Pryor 1991). This reactive compound can form crosslinks in vitro between DNA molecules (Reiss et al. 1972) and between DNA and histones, a class of proteins found in the cell nucleus (Goldstein 1979). If these crosslinks formed in vivo, DNA replication might be abnormal. Malonaldehyde has also been implicated as an initiator of carcinogenesis in mouse skin (Shamberger et al. 1974).

In addition to DNA damage produced by the products, such as malonaldehyde, of ozone's reaction with cell membranes, hydroxyl free radicals may be a causal factor in DNA strand breaks in target cells exposed to hydrogen peroxide in vitro (reviewed by Weitzman and Gordon 1990). Hydrogen peroxide is produced when ozone dissolves in water, and Goldstein (1973) has shown that rats and mice exposed to ozone produce hydrogen peroxide in their red blood cells. Hydrogen peroxide, formed extracellularly, penetrates efficiently into target cells, and may generate destructive

hydroxyl radicals within the cell near the DNA. Whether this reaction occurs in vivo is not known; high levels of endogenous antioxidant defenses may prevent the reaction.

EFFECTS OF OZONE ON CELL PROLIFERATION

Cancer research has focused increasingly on chemicals that do not act directly on DNA, but which might induce carcinogenesis by producing increased cell proliferation at high doses; a cell undergoing division is much more at risk for mutations than a quiescent cell (Ames and Gold 1990; Cohen and Ellwein 1990; Preston-Martin et al. 1990; Harris 1991). Both in vivo (Slaga et al. 1981; Goldstein et al. 1983) and in vitro (Gindhart et al. 1985) studies have implicated oxygen free radicals in tumor promotion, a later stage of carcinogenesis characterized by increased cell proliferation. Free radicals produced by ozone's reaction with respiratory epithelium and released by infiltrating inflammatory cells may be responsible for the enhanced cell proliferation in the lungs of laboratory animals that inhaled ozone (Bils and Christie 1980). Changes in the proliferative properties of cells and their subsequent development of a tumor-forming capacity are collectively referred to as malignant transformation, or simply, transformation (Darnell et al. 1986).

EFFECTS OF OZONE ON TRANSFORMATION IN VITRO

Because transformation can be carried out entirely in cell culture, it is widely used as an analogue of cancer induction in animals (Darnell et al. 1986). Transformation proceeds through a succession of changes that convert a normal cell into one with altered properties. The cellular changes most germane to Dr. Thomassen's study are morphological changes (changes in a cell's shape and structure) and an increased proliferative capacity.

Borek and colleagues were the first to study the transforming properties of ozone (Borek et al. 1986, 1989a,b). They exposed primary hamster embryo cells and mouse fibroblasts in culture to two classical transforming agents, gamma radiation and ultraviolet radiation, and to ozone (5 or 6 ppm for five or ten minutes). The exposed cells exhibited morphological changes, grew in multiple layers, and did not undergo the characteristic loss of proliferative capacity common to normal cells as they form intercellular contacts.

Combined exposures to gamma radiation and ozone revealed that the experimental protocol affected the eventual outcome. Exposing cells to ozone after treatment with gamma rays produced a greater-than-additive enhancement of radiation-induced transformation. In contrast, an additive level of transformation was seen in cells exposed to ozone before radiation (Borek et al. 1986, 1989b). Additive effects on transformation also were obtained in cells exposed to ultraviolet light before ozone (Borek et al. 1989a).

Transformation produced by ultraviolet light and ozone, alone or together, was inhibited by vitamin E, an antioxidant that can protect cells from the harmful effects of free radicals. Thus, the investigators proposed that transformation by ozone and ultraviolet light was mediated, at least in part, by free radicals (Borek et al. 1989a).

The mechanism by which transformation took place was explored next by Borek and associates (1989b). They transferred DNA from ozone-transformed cells to unexposed cells and observed morphologic evidence of neoplastic transformation. The investigators proposed that DNA was a target in ozone-induced transformation, and that this transformation involved the activation of dominant transforming genes that were expressed when transferred into unexposed cells.

TRANSFORMATION OF TRACHEAL EPITHELIAL CELLS IN VITRO

Because most human cancers arise from epithelial cells (Higginson and Muir 1973; Cairns 1975), researchers have used these cells grown in culture as a model system for studying genetic and other cellular changes caused by exposure to carcinogens.

Invasive cancer is preceded by early, or preneoplastic, changes in cells (Nettesheim and Barrett 1984). Marchok and colleagues (1977, 1978) cultured epithelial cells isolated from rat tracheas that had been implanted into the backs of rats of the same species and exposed in vivo to a carcinogen. (This technique is called heterotopic tracheal transplantation and is described in the next section.) Marchok and associates (1977, 1978) observed a marked increase in the cells' ability to proliferate in culture after in vivo exposure to the carcinogen. After 400 days in culture, the cells produced carcinomas when they were injected into rats. The investigators proposed that exposing the cells in vivo caused initiation, and these processes then proceeded slowly, in vitro, to the stage where they were able to induce tumor formation. They also suggested that the increased ability of the cells to proliferate in culture may be an important early change associated with transformation. Nettesheim and Barrett (1984) referred to the cells with an increased growth capacity as enhanced-growth variants. The variant cells differed in shape from normal cells and continued to divide after normal cells ceased proliferation.

Thomassen and associates (1983, 1985) reported that enhanced growth variants appeared in culture after they ex-

posed normal rat tracheal epithelial cells in vitro to the chemical carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Because cells can acquire enhanced-growth properties before they acquire the ability to produce tumors, these cells were also referred to as preneoplastic variants. Steele and colleagues (1989) also noted the appearance of morphological changes characteristic of preneoplastic transformation after treating rat tracheal epithelial cells in culture with a series of chemical carcinogens. Thus, the use of tracheal epithelial cells in culture has allowed the identification of cellular changes that occur after in vivo or in vitro exposure to carcinogens.

The present study by Dr. Thomassen and colleagues is a continuation of their investigations of preneoplastic transformation in rat tracheal epithelial cells and represents the first attempt to study ozone's effects on tracheal epithelial cells exposed in vivo and in vitro.

JUSTIFICATION FOR THE STUDY

Ozone inhalation can trigger a cascade of events in the epithelial cells that line the respiratory tract. Several of these events potentially can cause cellular changes leading to carcinogenesis. Therefore, it is crucial to determine the carcinogenic potential of ozone in order to help resolve uncertainties regarding its possible effects at ambient levels of exposure. Under RFA 86–1, "Genotoxic, Carcinogenic, and Cocarcinogenic Effects of Ozone," HEI solicited applications for laboratory studies addressing the question of whether exposure to ozone affects the risk of cancer. Applications were requested to explore whether prolonged exposure to ozone in concentration ranges relevant to ambient conditions leads to preneoplastic or neoplastic changes, and to identify which of the diverse effects associated with low-level (less than 1 ppm) exposures may play prominent roles in processes leading to cancer. The HEI Health Research Committee identified free radicals, inflammation, genotoxicity, injury repair, and immunosuppression as candidates of interest for mechanistic studies of carcinogenesis. Ozone's potential interaction with other pollutants was also of interest.

Three studies were supported by the Institute under RFA 86–1. Two of the studies (Drs. Carmia Borek and David Thomassen) tested the capacity of ozone to transform cells in vivo and in vitro. A third study (Donaldson et al. 1991) investigated the ability of inflammatory leukocytes from ozone-exposed rats to injure pulmonary epithelial cells. Through support of these research projects, the Institute intended to address uncertainties regarding the possible carcinogenicity of ozone at ambient concentrations.

Dr. Thomassen and his colleagues proposed to use a rat trachea model to study the transforming potency of ozone and two known carcinogens on tracheal epithelial cells. Cells were to be exposed in vivo and in vitro, either to ozone or carcinogens alone, or to ozone in combination with carcinogens. Originally, the in vivo exposures were to be performed using heterotopic tracheal transplants, whereby tracheas from donor rats are implanted subcutaneously into the backs of recipient rats of the same strain. This technique allows easier access to tracheal epithelial cells for subsequent exposure to ozone, carcinogens, or both. This technique also allows the same cells exposed to ozone in vivo to be studied in vitro. After either in vivo or in vitro exposures, cell cultures would be examined for the presence of preneoplastic cell variants by an in vitro transformation assay.

The tracheobronchial epithelium is a primary interface with the environment, and the vast majority of lung cancers arise from the cells lining the large airway passages (Galofre et al. 1964; Mass and Kaufman 1984). An advantage of the model proposed by Dr. Thomassen and colleagues is that the epithelial lining of the rat trachea consists of the same cell types present in human airways, down to the level of bronchioles (Reznik-Schuller 1983). Therefore, preneoplastic developments in the upper airways of the rat may be comparable to those preceding lung tumor development in humans. Dr. Thomassen was experienced in the study of neoplastic transformation of tracheal epithelial cells in culture (Thomassen et al. 1983, 1985; Thomassen 1986), as well as with the tracheal transplant procedure, having worked with Dr. Paul Nettesheim, in whose laboratory the technique was developed.

OBJECTIVES AND STUDY DESIGN

The overall objective of the study was to determine whether ozone induces preneoplastic changes in rat tracheal epithelial cells, either as a direct carcinogen, or by acting as a cocarcinogen in concert with the known carcinogens, MNNG and benzo[*a*]pyrene.

The investigators proposed to expose rat tracheal epithelial cells to two or three levels of each of the three agents, both in vivo (in the heterotopic transplant model) and in vitro, and to examine cells in vitro for the presence of preneoplastic variants. They also proposed to expose rat tracheal epithelial cells to the combined effects of ozone and carcinogens, both in the in vivo transplant model and in vitro.

The original study design was modified as the research progressed. The tracheal transplant procedure was labor in-

tensive, the surgery was complicated, and the exposures required extensive technician time. In addition, it was difficult to obtain a reproducible release of carcinogen in the transplants, and it was uncertain whether the entire surface of tracheal epithelium was exposed to consistent levels of ozone. During this study, Dr. Thomassen and colleagues obtained tracheal epithelial cells from normal rats exposed to ozone (which were part of another project at the Institute). Because of preliminary results obtained on cell transformation with these cells, Dr. Thomassen suggested that he expose additional rats that had not been operated on to a range of ozone levels. The HEI Health Research Committee agreed, and recommended that Dr. Thomassen substitute the inhalation of ozone by this group of animals in place of the tracheal transplant exposures. The Research Committee also agreed with the investigator's proposal to discontinue testing benzo[*a*]pyrene and to focus on MNNG.

IN VITRO EXPOSURE AND PRENEOPLASTIC TRANSFORMATION OF RAT TRACHEAL EPITHELIAL CELLS

Single Exposures to Ozone

Rat tracheal epithelial cell preneoplastic transformation was studied after a single exposure to ozone alone. Cells were exposed for 40 minutes to either 0.7 or 10 ppm ozone. The latter concentration produced approximately 50% cytotoxicity.

The ability of ozone to transform rat tracheal epithelial cells was unknown. Therefore, the cells were treated with MNNG (whose transforming potency for these cells is well established) at a concentration that also produced 50% cytotoxicity. Thus, MNNG served as a positive control for the induction of preneoplastic variants. The investigators reasoned that, if preneoplastic transformation could be identified with a known carcinogen at a concentration that produced 50% cytoxicity, the possible transforming activity of a compound of unknown potency also might be detected at a similar level of cytotoxicity.

Cytotoxicity was measured by determining the rat tracheal epithelial cells' reduced ability to form colonies in culture. Proliferating rat tracheal epithelial cells form aggregates, called colonies, that can be seen by light microscopy. Cells that do not survive treatment with an agent, such as ozone or MNNG, cannot divide repeatedly to form a growing colony of cells in culture. Therefore, determining the number of cell colonies in a culture dish provides a reliable indication of the number of cells that survive a particular treatment (Nettesheim and Barrett 1984).

After determining the cytotoxicity levels of the ozone concentrations to be used, preneoplastic transformation was measured using the assay system described below.

Multiple and Combined Exposures Using Ozone and MNNG

Rat tracheal epithelial cell preneoplastic transformation was studied after (1) a single exposure to ozone followed by MNNG; (2) exposure to MNNG followed by multiple exposures to ozone; and (3) multiple exposures to ozone alone. Rat tracheal epithelial cells in culture were exposed to ozone and MNNG using two different experimental conditions. In the first series of experiments, cells were exposed to ozone (0.7 or 0.8 ppm) for 40 minutes, and then to either strongly or weakly cytotoxic doses of MNNG (approximately 0.2 and 0.045 μg/ml of culture medium, respectively). Control cultures received either no additions or MNNG alone. In a second series of experiments, cells were first exposed to the lower concentration of MNNG, and then to 0.7 ppm ozone for 40 minutes, twice weekly for five weeks. In this series of experiments, cells also were exposed to 0.7 ppm ozone alone, for 40 minutes, twice weekly for five weeks. Control cell cultures received either no additions or MNNG alone. At the conclusion of each experimental period, cell cultures were examined for changes in the frequency of preneoplastic variants using the assay system described below.

IN VIVO EXPOSURE FOLLOWED BY IN VITRO PRENEOPLASTIC TRANSFORMATION OF RAT TRACHEAL EPITHELIAL CELLS

Groups of rats inhaled 0.12, 0.50, or 1.0 ppm ozone for six hours per day, five days per week for one, two, or four weeks. A control group of rats inhaled only filtered air. Tracheal epithelial cells were isolated after each exposure interval and examined in vitro for the number of preneoplastic variants. Tracheas from each group also were examined for changes in morphology using standard histological methods.

ASSAY PROCEDURE FOR DETECTION OF PRENEOPLASTIC VARIANTS

The first detectable change in the progression of rat tracheal epithelial cells in culture to the preneoplastic state is the formation of large colonies of altered cells with an enhanced ability to proliferate. Normal tracheal epithelial cells do not proliferate well in a culture medium that is selective for the growth of preneoplastic variants and eventually either detach from the surface of culture dishes or persist without proliferation. After five weeks in a selective medium, the cultures were examined, and the numbers of large colonies of small, proliferating cells were counted. These new cells, which continued to proliferate, were inter-

preted as having undergone preneoplastic alterations (Kitamura et al. 1986).

The frequency of preneoplastic cell transformation was obtained by dividing the total number of variants by the number of colony-forming cells considered to be "at risk of transformation," or simply "at risk." The number of colony-forming cells at risk was obtained by multiplying the average number of colonies per dish by the number of dishes in the transformation assay.

The in vitro experiments in which rat tracheal epithelial cells received a single exposure to ozone were conducted in a culture medium permissive for the proliferation of normal cells. The cells remained in this medium for four days to allow the fixation and expression of ozone-induced changes in the initiated cells. The culture medium then was replaced with a medium that was selective for the proliferation of those cells that had begun to undergo preneoplastic changes; the cell cultures remained in this medium for five weeks. At the end of this period, the investigators determined the number of preneoplastic variants in both control and treated cultures.

In the in vitro experiments that examined the effect of multiple ozone exposures, the initial exposure of rat tracheal epithelial cells to ozone was carried out in a medium permissive for the growth of normal cells. After four days, the culture medium was replaced with one that was selective for the proliferation of preneoplastic variants. The additional exposures to ozone were conducted in this selective medium over a five-week period.

In the in vivo exposures, the preneoplastic transformation of rat tracheal epithelial cells isolated from tracheas of rats that had inhaled ozone was assayed by first culturing cells in a medium permissive for the growth of normal cells. The medium then was replaced with one that was selective for the growth of preneoplastic variants; this culture was continued for five weeks.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The overall goals of the study were to assess ozone's ability to induce preneoplastic transformation of rat tracheal epithelial cells in vitro, either alone or in combination with MNNG, and to determine whether the frequency of preneoplastic transformation of rat tracheal epithelial cells increased after rats inhaled ozone.

The investigators successfully completed a series of in vitro experiments to determine the frequency of preneoplastic transformation of rat tracheal epithelial cells after single exposures to two concentrations of ozone, or after a single exposure to ozone before the addition of MNNG. The following aspects of the originally proposed protocol were not completed: First, extensive dose-response data relating ozone exposure and cytotoxicity were not presented because the investigators found that only high concentrations of ozone (10 ppm) produced cytotoxicity. Second, ozone was not tested together with a second carcinogen, benzo[*a*]pyrene. Third, rat tracheal epithelial cells were not exposed in vitro to high and low levels of ozone and MNNG in different sequences. At the suggestion of the HEI Research Committee, the originally proposed exposures of heterotopic tracheal transplants were replaced with in vivo inhalation studies in Fisher-344 rats.

ASSESSMENT OF METHODS AND STUDY DESIGN

The experiments were generally well conceived and performed. However, several methodological issues hamper interpretation of the results. These include sensitivity of the in vitro transformation assay, variability of the assay, changes in the conditions of cell culture used for multiple ozone exposures, and the lack of a concurrent positive control in the in vivo studies.

Because ozone's potential as a transforming agent was unknown, the investigators exposed rat tracheal epithelial cells in vitro to either ozone or MNNG at concentrations producing 50% cytotoxicity. They chose this approach by reasoning that preneoplastic transformation by ozone might be detectable if the dose had the same cytotoxic potential as a compound (MNNG) whose transforming ability was known. However, even at the 50% cytotoxicity level, MNNG induced a relatively low (but statistically significant) frequency of preneoplastic variants. This raises concerns about the sensitivity of the in vitro assay, and whether it accurately detects changes in the frequency of preneoplastic transformation of rat tracheal epithelial cells.

Sensitivity of the in vitro assay system is also an issue in the experiments assessing ozone's ability to induce preneoplastic transformation after exposure of rats to the gas in vivo. Because the rats were not exposed to an agent that enhanced transformation (a positive control), we do not know whether the assay system was able to detect low levels of variants that might have been induced in vivo.

The variability of the assay is also a potential concern because variations in transformation frequency were often greater within a group receiving the same treatment than between groups receiving different treatments. The basis for this variability is not known; however, the investigators speculate that it may have been due to variability in the cell populations they isolated from different groups of animals.

The investigators dealt with this problem by comparing only control and treated groups within individual experiments, and by basing their conclusions on an overall pattern of responses resulting from multiple experiments.

During the course of the study, rat tracheal epithelial cells were exposed to ozone under different culture conditions. During exposure of rat tracheal epithelial cells to ozone over a five-week period in a medium selective for the growth of preneoplastic variants (in place of a single exposure in medium permissive for growth of normal cells), normal cells could have continued to proliferate for a relatively finite period of time. Therefore, the actual number of transformation-susceptible cells could have increased, so that the calculation of preneoplastic transformation frequencies based on the initial number of colony-forming cells at risk prior to ozone exposure may be inaccurate. Changing the culture conditions for the multiple exposure experiments added a technical variable that is difficult to interpret.

STATISTICAL METHODS

The experimental protocols were carefully organized and thoroughly controlled. To determine the statistical significance, each treatment group was compared to its own control; most of these results are expressed in terms of the "relative risk" of preneoplastic transformation, a comprehensible, standardized ratio index for comparing one exposure group to another. To calculate relative risk, the investigators used the method of log-linear modeling, which is discussed in great detail in their report. Log-linear modeling produces tests of main effects (dose, duration) and statistical interaction (dose × duration) based on likelihood ratios, rather than on variance ratios. Because the logarithm of the transformation frequencies is modeled, the differences between groups are expressed as a relative risk for one group in relation to another.

The results of experiments in which tracheal epithelial cells were exposed to ozone before being treated with either high (Table 8) or low (Table 9) cytotoxic doses of MNNG were analyzed by two-way log-linear modeling. In the experiment using high doses of MNNG, the interaction between ozone and MNNG was not significant; however, the main effects of MNNG (an increase in the frequency of preneoplastic variants) and ozone (a reduction in the frequency of MNNG-induced transformation) were significant. In the experiment using low doses of MNNG, both the interaction between ozone and MNNG, and the effect of ozone itself (a reduction in MNNG-induced transformation) were not significant; however, the main effect of MNNG (an increase in the frequency of preneoplastic variants) was significant. The experiments in which multiple ozone exposures followed treatment with MNNG utilized a two-way analysis that indicated no statistical interaction between ozone and MNNG, indicating that their effects were independent, rather than synergistic.

RESULTS AND INTERPRETATIONS

The investigators used a well-characterized cell culture model for their initial studies of preneoplastic transformation. Dr. Thomassen and colleagues (1983, 1985) and other investigators (Marchok et al. 1977, 1978; Nettesheim and and Barrett 1984; Steele et a. 1989) have characterized changes in cell morphology and growth characteristics that occur during the progression of rat tracheal epithelial cells from normal to preneoplastic and neoplastic states. Dr. Thomassen is the first to attempt to use this model as a quantitative method for assessing ozone's potential for inducing preneoplastic changes in rat tracheal epithelial cells. However, the results were heavily dependent on factors such as the timing, duration, and conditions of exposure.

Single Exposure to Ozone in Vitro

When the researchers exposed rat tracheal epithelial cells once to 0.7 or 10 ppm ozone for 40 minutes in vitro, they found no difference in the frequency of preneoplastic transformation, compared to control cells that were not exposed to ozone. The failure to detect changes in the frequency of preneoplastic transformation after a single exposure to 10 ppm ozone may have been due to ozone's inability to induce transformation under these experimental conditions. Alternatively, failure to detect changes in preneoplastic transformation at an ozone concentration that killed 50% of the rat tracheal epithelial also may have been due to the assay's inability to detect low levels of variants, and to its variability.

Exposing rat tracheal epithelial cells to ozone before treating them with a highly cytotoxic dose of MNNG decreased the frequency of preneoplastic transformation compared to cells treated with MNNG alone. Ozone may have altered the cells' progression through their cycle of division and quiescence. If this occurred, rat tracheal epithelial cells may have been less responsive to the carcinogen if it was added at a period of the cell cycle different from that for control cells treated with MNNG alone. The investigators did not detect a lower frequency of preneoplastic transformation when the cells were exposed to 0.8 ppm ozone before treatment with a lower dose of MNNG. This may have been due to a decreased frequency of preneoplastic transformation in the presence of the lower dose of MNNG.

Multiple Exposures to Ozone in Vitro

Two experimental protocols produced an apparent increase in the frequency of preneoplastic variants over that of controls: multiple exposures of rat tracheal epithelial cells to 0.7 ppm ozone and multiple exposures to 0.7 ppm ozone after rat tracheal epithelial cells were treated with MNNG.

A characteristic feature of these positive experiments was that the highest levels of preneoplastic transformation appeared in cell cultures with a relatively small number of cells at risk. The investigators interpreted the preneoplastic transformation in the individual cell colonies as independent events. However, the initial area of preneoplastic transformation within a small cell population in a culture dish may have increased the probability of a second transformation event in that dish. Terzaghi and Little (1976) and Borek (1980) observed that the number of cells in a culture dish affected the transformation of another type of cell in culture (human skin or embryonic mouse fibroblasts). These investigators reported that transformation decreased when the number of surviving cells exceeded a certain level. Farber and Rubin (1991) summarized the results of studies showing that transformation frequency is inversely proportional to the number of cells in culture. Therefore, the number of cells at risk may have played a role in the frequency of preneoplastic transformation.

The cell culture conditions may also have affected the frequency of preneoplastic transformation attained after multiple ozone exposures. The investigators have observed that preneoplastic variants are relatively resistant to ozone's cytotoxic effects (D.G. Thomassen, unpublished data). Therefore, increased preneoplastic transformation after multiple exposures may have reflected an enhanced survival of spontaneously occurring variants (variants that appear in the absence of ozone). The survival of variants may have been favored because the cells were exposed to ozone in a medium that was selective for the growth of preneoplastic variants. Spontaneous transformation in this system can depend heavily on cell culture conditions and when the selection conditions for variants are imposed. The dynamics of spontaneous variant induction and variant expression for this system are poorly understood.

There is an additional problem in interpreting the results of experiments that measure the effect of multiple exposures to ozone after treating rat tracheal epithelial cells with MNNG. The investigators compared the frequency of preneoplastic transformation in two experiments using this experimental protocol with such transformation induced by MNNG alone. Although one experiment with MNNG and ozone produced an enhanced frequency of preneoplastic transformation, the other showed either no change or a slight decrease. When the results of the two experiments were combined, the relative risk of preneoplastic transformation was greater in the ozone plus MNNG protocol because a large positive result more than offset a smaller negative result. These results may be due to variability of the assay procedure, and do not fully support the conclusion that multiple exposures to ozone increased MNNG-induced preneoplastic transformation.

Multiple Exposures to Ozone in Vivo

Dr. Thomassen and his colleagues found no difference in the frequency of preneoplastic transformation between rat tracheal epithelial cells isolated from control rats breathing clean air and rats that inhaled ozone. However, some cell damage occurred because the investigators observed severe morphological changes in specific areas of tracheas from rats that had been exposed to 1 ppm ozone for two or four weeks.

There are several possible reasons for ozone's apparent inability to induce an increased frequency of preneoplastic transformation. First, ozone indeed may not transform tracheal epithelial cells in vivo. Second, the inability to detect an increased frequency of preneoplastic transformation after rats inhaled ozone may have been due to assay insensitivity. Third, after being exposed to ozone for three to five days, humans and animals develop a tolerance or adaptation to its effects (Lippmann 1989). Adaptation may have produced new epithelial cells resistant to ozone (Ospital 1985). An additional possibility is that the protective layer of mucus lining the airways may have prevented the gas from reaching the epithelial cells.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

Conditions of in vitro ozone exposure and cell culture should be more thoroughly investigated. Although multiple 40-minute exposures of rat tracheal epithelial cells to ozone in a medium selective for the growth of preneoplastic variants appeared to increase preneoplastic transformation frequency, a single 40-minute exposure of rat tracheal epithelial cells in the medium permissive for the growth of normal cells did not. Additional experiments might examine the effect of longer single exposure periods, several ozone concentrations, and multiple exposures to ozone in the permissive medium. Dr. Thomassen and his colleagues obtained evidence suggesting that the frequency of preneoplastic transformation increased when multiple exposures of rat tracheal epithelial cells to ozone in vitro followed

treatment with MNNG. However, because the investigators did not test the effect of a single exposure to ozone on cells pretreated with MNNG, it is uncertain whether multiple exposures are required.

Additional research is needed to determine the appropriate conditions for multiple exposures of animals to ozone in vivo. The possible development of tolerance to ozone over the one- to four-week exposure period may have affected the results obtained in the subsequent in vitro transformation assay. Therefore, acute exposures in vivo should be carried out for four days or less, as well as for longer periods. In addition, animals also should be exposed by inhalation to a compound that is known to induce rat tracheal epithelial cell preneoplastic transformation. The inclusion of such a positive control would validate the assay used to detect the formation of preneoplastic variants.

Although the use of heterotopic tracheal transplants was abandoned because of technical difficulties, this model provides an attractive method for exposing cells to oxidants and carcinogens in vivo, and should be investigated further. The model provides easy access to tracheal epithelial cells, and the cells that are exposed in vivo can be studied in vitro.

CONCLUSIONS

Dr. Thomassen and his colleagues investigated ozone's potential to induce preneoplastic transformation of rat tracheal epithelial cells in vivo and in vitro. Ozone's potential cocarcinogenic activity was determined by investigating its ability to modulate preneoplastic cell transformation produced by MNNG in vitro.

Under the conditions of the in vivo studies, ozone did not induce preneoplastic transformation of rat tracheal epithelial cells. The possible limitations of the assay procedure suggest that a firm conclusion be reserved until additional information on assay sensitivity, or results derived from a different assay method, become available.

Some of the in vitro results suggest that ozone might increase the incidence of preneoplastic transformation under appropriate conditions. It is not surprising that a reactive gas such as ozone would affect epithelial cells exposed repeatedly to the gas in culture. However, these experiments need confirmation because the results may have been affected by the cell culture method used during ozone exposure. Because other in vitro results showed either no effect or decreased preneoplastic transformation, another conclusion from the in vitro studies is that the experimental protocol plays a major role in determining ozone's ability to induce preneoplastic transformation.

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