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Failure of Ozone and Nitrogen Dioxide to Enhance Lung Tumor Development in Hamsters

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

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HEI Statement

Synopsis of Research Report Number 60

Oxidant Air Pollutants and Lung Cancer: An Animal Model

BACKGROUND

Ozone and nitrogen dioxide are highly reactive oxidant gases that are derived from the combustion of fossil fuels and the atmospheric transformation of these combustion products. The U.S. Environmental Protection Agency sets National Ambient Air Quality Standards (NAAQS) for these and other pollutants largely on the basis of scientific data documenting their effects on lung structure, function, and response to infectious agents. A major unanswered question is whether or not exposure to oxidant air pollutants contributes to lung cancer.

Small cell lung cancer, a highly malignant form of cancer, represents 25% of human lung cancers. Small cell lung tumors are composed primarily of cells with neuroendocrine characteristics. In the past it has been difficult to investigate the role of environmental factors in the pathogenesis of small cell lung cancer because of the lack of a suitable animal model. However, an animal model for neuroendocrine lung cancer appeared to have been developed when Drs. Schuller, Witschi, and colleagues reported that hamsters developed neuroendocrine cell tumors when they were treated with the chemical carcinogen, *N*-diethylnitrosamine (DEN), while inhaling 70% oxygen. The investigators proposed that exposure to high oxygen concentrations (hyperoxia) caused neuroendocrine cells to proliferate and that the dividing cells were susceptible to tumor induction by DEN. Because exposure to both ozone and nitrogen dioxide also causes proliferation of lung cells, including neuroendocrine cells, the researchers suggested that these pollutants could mimic the effects of high oxygen exposures in tumor development. The Health Effects Institute sponsored the present study to examine whether exposure to ozone or nitrogen dioxide enhances the development of tumors induced by DEN, particularly neuroendocrine tumors, in the respiratory tract of hamsters.

APPROACH

Dr. Witschi exposed hamsters continuously to 0.8 ppm ozone for 16 weeks, or 15 ppm nitrogen dioxide for 16 or 24 weeks, or filtered air. The concentrations of the oxidant gases were much higher than those found in ambient air. However, the objective of the study was to determine whether the pollutants had any effects on lung tumor development under extreme conditions before testing ambient levels. Animals also received injections of DEN or saline twice each week. Hamsters exposed to 65% oxygen and injected with DEN were to serve as positive controls. Animals were killed after 16, 24, or 32 weeks, and various tissues were examined by the pathologists, Drs. Schuller and Breider, for neoplastic lesions (carcinomas, adenomas, papillary polyps) and nonneoplastic lesions (hyperplasia and necrosis).

RESULTS AND IMPLICATIONS

Neither ozone nor nitrogen dioxide caused the occurrence of lung tumors in DEN-treated hamsters to be greater than that in control animals. Also, the investigators were unable to verify their earlier findings that exposing hamsters to high oxygen concentrations and DEN produces neuroendocrine cell tumors in hamster lungs. The experimental protocol proposed by the investigators for this study did differ somewhat from their previous work. However, the reason for the discrepant findings in the two studies is not known. Possible explanations include: unexpected variations in the susceptibility of the test animals; the fact that the tissue samples were kept in fixative too long, resulting in the loss of markers on the neuroendocrine cells; the possibility that neuroendocrine lung tumors may have been present at earlier time periods and subsequently regressed, or that they may not have been fully developed at the time of necropsy; or the results of the earlier research may have been misinterpreted.

There was some suggestion that instead of increasing the occurrence of tumors, the oxidant exposures actually delayed or inhibited the development of some types of respiratory tract tumors. However, these findings need to be interpreted cautiously because of differences in the health status and body weights of the hamsters in the different treatment groups and the small number of tumor-bearing animals. In summary, this study did not clarify whether ozone or nitrogen dioxide affects the development of lung cancer in this animal model.

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 1989 to 1991. This study was conducted by Dr. Hanspeter Witschi of the University of California at Davis and Drs. Hildegard Schuller and Michael Breider of the University of Tennessee. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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INVESTIGATORS' REPORT

Failure of Ozone and Nitrogen Dioxide to Enhance Lung Tumor Development in Hamsters

Hanspeter Witschi, Michael A. Breider, and Hildegard M. Schuller

ABSTRACT

We tested the hypothesis that the two common oxidant air pollutants, ozone and nitrogen dioxide, modulate the development of respiratory tract tumors in Syrian golden hamsters. The animals received subcutaneous injections of the carcinogen diethylnitrosamine (20 mg/kg) twice a week while being exposed continuously to an atmosphere of 0.8 parts per million (ppm)* of ozone or 15 ppm of nitrogen dioxide. Animals were killed 16 weeks or 24 to 32 weeks after the beginning of the treatment. Ozone delayed the appearance of tracheal tumors and reduced the incidence of tumors in the lung periphery. A suspected neuroendocrine differentiation of those lung tumors could not be established by immunocytochemistry due to overfixation of tissues. On the other hand, ozone seemed to mitigate development of hepatotoxic lesions mediated by diethylnitrosamine. In animals treated with diethylnitrosamine and exposed to nitrogen dioxide, fewer tracheal tumors and no lung tumors were found. Only a few lung tumors were produced in animals treated with diethylnitrosamine and kept in an atmosphere of 65% oxygen. The previously observed neuroendocrine nature of tumors induced by simultaneous exposure to diethylnitrosamine and hyperoxia could not be established because the long fixation of tissues precluded immunocytochemical stains. Animals treated with diethylnitrosamine and kept in filtered air while being housed in wire-mesh cages developed fewer lung tumors than animals given the same treatment and kept on conventional bedding in shoebox cages. Although all inhalants tested are known to produce substantial cell proliferation in the respiratory tract, it was not possible to document whether this would enhance lung tumor development. The role of the two common air pollutants, ozone and nitrogen dioxide, as possible additional risks in the pathogenesis of lung cancer in animals continues to remain uncertain.

INTRODUCTION

Lung cancer is the leading cause of cancer deaths both in the United States and worldwide (Doll and Peto 1981; Silverberg et al. 1990). The active smoking of tobacco is by far the biggest single risk factor encountered by the general population. The role of active smoking in the pathogenesis of lung cancer is undisputed (International Agency for Research on Cancer 1985). The role of passive smoking, i.e., exposure to and inhalation of environmental tobacco smoke, remains, for the time being, somewhat less certain (Samet et al. 1987, 1988). An increased incidence of lung cancer has been found among workers in certain occupations such as uranium mining, asbestos, and foundry work (Frank 1978; Committee on the Biological Effects of Ionizing Radiation 1988). The question of whether air pollution, originating from mobile and stationary sources, constitutes an additional risk factor in the development of cancer in the respiratory tract has been raised repeatedly. Although epidemiological studies occasionally have suggested such a link, they have failed to demonstrate unequivocally that air pollutants are directly involved in the development of lung cancer in humans (Carnow and Meier 1973; Menck et al. 1974; Vena 1983; Jacobson 1984; National Institute of Environmental Health Sciences Workshop 1986). Nevertheless, ozone (O_3) has been listed as a potential contributor to lung carcinogenesis in humans (Sawicki 1977). Animal studies remain inconclusive. A more recent analysis of the relatively small experimental data base found little evidence that would implicate directly two of the most abundant air pollutants, O_3 and nitrogen dioxide (NO_2), as pulmonary carcinogens. However, the two air pollutants seem to have the potential to modulate pulmonary carcinogenesis in laboratory animals (Witschi 1988).

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

This Investigators' Report is one part of Health Effects Institute Research Report Number 60, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Hanspeter Witschi, Institute of Toxicology and Environmental Health, University of California at Davis, Old Davis Road, Davis, CA 95616.

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

The suggestion that O_3 and NO_2 might modulate pulmonary carcinogenesis originates from several experimental observations. Ozone is a highly reactive chemical and interacts readily with multiple biological targets (Pryor 1991). Studies with isolated cell systems in vitro found that O_3 increases chromosome breakage and chromatid-type deletions and enhances cell transformation (Fetner 1962; Gooch et al. 1976; Borek et al. 1986, 1988). Multiple in vitro exposures of rat tracheal epithelial cells to O_3 increased the frequency of preneoplastic variants and, in addition, enhanced the transforming potency of the chemical carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. However, in tracheal cells harvested from animals exposed to the oxidant in vivo, no evidence for increased transformation was found (Thomassen et al. 1991). When inhaled over prolonged time periods (6 to 120 weeks), O_3 produces morphologic alterations in the respiratory tracts of mice. The lesions suggest that irreversible proliferative changes occurred (Werthamer et al. 1970; Penha and Werthamer 1974). On occasion, an increase in the incidence of pulmonary adenomas was found (Stokinger 1963; Hassett et al. 1985; Last et al. 1987).

It also has been suggested that NO_2 may increase the development of spontaneously occurring or chemically induced lung tumors in mice and rats (Adkins et al. 1986; Ichinose et al. 1991). Nitrogen dioxide also facilitates the formation of metastatic tumors in the lung (Richters and Kuraitis 1983; Richters 1988). These studies are offset by others that have shown no effect of NO_2 or even inhibition of lung tumor development in mice (Henschler and Ross 1966; Ide and Otsu 1973; Otsu and Ide 1975). Experimental data on a potentially carcinogenic or cocarcinogenic action of the two air pollutants thus remain ambiguous.

In humans, lung cancers can be subdivided into two major forms: non–small cell cancers (adenocarcinomas, squamous cell carcinomas, large cell carcinomas, and bronchoalveolar carcinomas) and tumors that express neuroendocrine markers (Bunn 1988). Adenocarcinomas represent the major leading type of non–small cell lung cancer today; the incidence of squamous cell carcinoma has decreased (Wynder et al. 1985). It has been speculated that this shift in incidence is related to the production of cigarettes containing less tar, and the fact that the predominant carcinogens in tobacco smoke now may be nitrosamines (Schuller 1988). The different forms of neuroendocrine lung cancer range from the relatively benign carcinoids to the most malignant form, small cell lung cancer. The putative cell of origin is the Kultchitsky (amine precursor uptake decarboxylase [APUD] cell) of the epithelium lining the airways. Small cell cancer of the lung accounts for approximately 30% of all pulmonary cancers (Iannuzzi and Scoggins 1986). It is found almost exclusively in smokers.

In the lungs of laboratory animals, polycyclic aromatic hydrocarbons have produced squamous cell carcinomas, whereas nitrosamines have induced adenomas and adenocarcinomas (Reznik-Schuller 1983). The different types of non–small cell cancers of the lung are readily induced by various procedures such as intratracheal instillation of polycyclic aromatic hydrocarbons, exposure to toxic inhalants, or systemic administration of certain nitroso-compounds. Several well studied animal models are available (Nettesheim and Griesemer 1978). On the other hand, a model for lung cancer expressing neuroendocrine markers has only recently been described (Schuller et al. 1988). In hamsters kept in 70% oxygen (O_2) and simultaneously given injections of diethylnitrosamine (DEN) twice a week for eight weeks, tumors developed in the lungs of most animals within eight to 12 weeks. Histopathological analysis, electron microscopy, and specific immune stains showed that the tumors expressed several neuroendocrine markers. The tumors contained the typical electron-dense secretory granules and stained positive for bombesin, calcitonin, and neuron-specific enolase. Although their histological appearance differed from that of human small cell lung cancer, without a classification of neuroendocrine rodent lung tumors, these neoplasms were diagnosed as "neuroendocrine carcinomas" because of the markers they contained. In a subsequent publication that described identical lung tumors induced in this model system by hyperoxia and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), these tumors were further subclassified as "atypical carcinoids" (Schuller et al. 1990b).

Although small cell lung cancer in humans is believed to arise from preexisting pulmonary neuroendocrine cells (Gould et al. 1983), in the hamster model the atypical carcinoids appear to be derived from alveolar type II cells (Nylen et al. 1990; Schuller et al. 1990b). Neuroendocrine cells are rarely found in the lungs of adult animals, but occur in abundance in the lungs of fetal and newborn animals. The cells are believed to play a role in helping the lung to adjust to changes in the oxygen content of the inspired air. Histochemical evidence shows that the cells contain vasoactive peptides such as bombesin, a growth factor for neuroendocrine cells, as well as serotonin and calcitonin, which act as broncho- or vaso-dilators or -constrictors. Neuroendocrine cells act as chemoreceptors; they respond to hyperoxia and hypoxia in the lung (Schuller 1988; Johnson and Georgieff 1989). Acute or chronic hypoxia dramatically increases the number of neuroendocrine cells in the airways of rats and rabbits (Moosavi et al. 1973; Taylor 1977; Keith and Will 1981; Pack et al. 1986). Daily exposure to smoke produced from a single standard research cigarette (seven minutes of exposure) for 90 days produced a measurable in-

crease in the number of pulmonary neuroendocrine cells (Tabassian et al. 1989). Neuroendocrine cell hyperplasia may also be involved in the development of lung disease in humans (Miller 1989). In patients suffering from chronic obstructive lung disease, the number of pulmonary neuroendocrine cells is increased. Greater cell numbers also are found in people living at high altitudes (Becker 1984). The role of pulmonary neuroendocrine cells in the pathogenesis of chronic lung disease and lung cancer thus deserves attention.

SPECIFIC AIMS

The specific aim of the work presented in this report was to examine whether the two common oxidant air pollutants, O_3 and NO_2, would modulate the development of chemically induced lung tumors in hamsters. Both pollutants are known to produce sustained proliferation of the epithelium lining the respiratory tract (Bils and Christie 1980). Given the putative role of cell proliferation in tumor promotion (Cohen and Ellwein 1990), it was anticipated that such a general and nonspecific response might enhance respiratory tract tumor development. We also wanted to test the hypothesis that exposure to either pollutant would produce lung tumors expressing neuroendocrine markers. This particular hypothesis was based on previous work in which we had shown that concomitant exposure to O_2 and DEN produced these tumors in the hamster lung (Schuller et al. 1988). A key element in this model system appeared to be the transformation of preexisting alveolar type II cells into cells with neuroendocrine function which, under sustained exposure to hyperoxia and nitrosamine, then gave rise to lung tumors with neuroendocrine differentiation (Schuller et al. 1990b). Hyperplasia of the alveolar type II cell population has been described regularly in the lungs of laboratory animals exposed to O_3 or NO_2 (Bils and Christie 1980). In addition, hyperplasia of pulmonary neuroendocrine cells has been described in monkeys exposed to O_3 (Castleman et al. 1980). The effects of NO_2 on the pulmonary neuroendocrine cell population are less consistent. In rats exposed to NO_2, an increased number of these cells was found in the airways (Kleinerman et al. 1981; Marchevski and Kleinerman 1982). In hamsters, NO_2 inhalation decreased the number of APUD cells in the airways (Palisano and Kleinerman 1980). In view of these observations, it was reasonable to postulate that exposure to the two air pollutants would modulate DEN-induced lung tumor development in hamsters.

To test the hypothesis, two pilot studies were conducted. In one experiment, Syrian golden hamsters were treated with DEN while being exposed continuously to 0.8 ppm O_3. In the second experiment, animals treated with DEN were kept in an atmosphere of 15 ppm NO_2.

MATERIALS AND METHODS

CHEMICALS

Diethylnitrosamine was purchased from Sigma Chemical Co. (St. Louis, MO); purity was greater than 95% when checked upon delivery. Solutions for injection were prepared every two weeks. Two milliliters of DEN stock solution were diluted to 200 mL with injectable saline (0.9% sodium chloride [NaCl]), producing a 1% solution of DEN. The diluted DEN solution was aliquoted into 25-mL glass bottles and kept in the dark until use. Medical-grade oxygen was delivered to the inhalation facility by Moore Brothers (Sacramento, CA). Dinitrogen tetroxide (N_2O_4) was purchased form Matheson Gas Products (Newark, CA).

ANIMALS

Hamsters were purchased from Charles River Canada (St. Constant, Quebec, Canada). The originating colony was Lak:LVG (SYR) Golden Syrian, descended from two original colonies acquired in 1949 and 1951 and operated as a closed colony since that time. The animals were six to eight weeks old upon arrival. According to the supplier, the health status of the animals was as follows: serology (Sendaivirus, Rheovirus type 3, Pneumonia virus of mice, lymphocytic Choriomeningitis virus, and *Encephalitozoon cuniculi*), bacteriology (*Mycoplasma pulmonis*, *Salmonella* spp, *Bordetella bronchiseptica*), and parasitology (ectoparasites, endoparasites), all negative. There were no histopathological lesions consistent with active infectious disease. Upon arrival, three animals from each shipment were given to the Comparative Pathology Laboratory at the University of California at Davis for health tests. The results of health screens are summarized in Table 1. All animals were housed in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. All protocols were approved by the Animal Use and Care Administrative Committee of the University of California at Davis.

After a suitable quarantine period, the animals were assigned at random to the different treatment groups so that the body weights in the different groups were as homogeneously distributed as possible. The animals exposed to O_3, NO_2, or filtered air were kept at the inhalation facility of the California Regional Primate Research Center (CRPRC), University of California at Davis. The animals were con-

Table 1. Results of Health Checks in Hamsters[a]

O_3 Experiment[b]	
Gross pathology	No gross lesions
Histopathology	No significant lesions (lung, liver, duodenum, pancreas, ileum, cecum, kidney, spleen)
Parasitology	No parasites observed
Microbiology	No pathogenic bacteria recovered from nasopharynx, ileum, or cecum
Serology	Not performed
Clinical pathology	Not performed
Case diagnosis	No evidence of naturally occurring spontaneous or infectious disease
NO_2 Experiment[c]	
Gross pathology	No gross lesions
Histopathology	No significant lesions
Parasitology	No enteric helminths, protozoa, or ectoparasites observed
Microbiology	*Pasteurella pneumotropica*, nasopharynx
Serology	No titers detected, basic profile
Case summary	Cultural evidence of *P. pneumotropica* in all animals sampled

[a] Examination results from health checks on three hamsters per shipment, conducted at the beginning of the study, by the Comparative Pathology Laboratory, University of California at Davis School of Veterinary Medicine.

[b] Two shipments of animals received in January 1989.

[c] One shipment of animals received in February 1990.

tained in wire-mesh modules fabricated from stainless steel and were housed in 4.2-m^3 Hinners-type exposure chambers. The animals were kept on a 12-hour light and 12-hour dark cycle with free access to conventional lab chow and water. Animals exposed to O_2 or room air were housed in animal rooms at the Institute for Toxicology and Environmental Health (ITEH), University of California at Davis. They were housed in conventional plastic shoe-box cages on hardwood bedding, on a 12-hour light and 12-hour dark cycle with free access to conventional lab chow and water. The feed used in all experiments was Purina Rodent Laboratory Chow No. 5001, purchased from Purina Mills, St. Louis, MO.

TREATMENT OF ANIMALS WITH DIETHYLNITROSAMINE

To produce tumors in the respiratory tract, hamsters were injected with DEN, twice a week, at a dose of 20 mg/kg body weight. Before each injection, the animals were weighed individually and 0.2 mL/100 g body weight of the 1% DEN solution was injected subcutaneously. Injections were made into the right or left rear flank area of the animals, in the region of the flank organ. After the injection, slight pressure was applied momentarily to avoid leakage of fluid from the injection site. Control animals were injected with a corresponding volume of saline (0.2 mL/100 g body weight). After treatment, the animals were immediately returned in their cages to the corresponding exposure atmospheres.

GENERATION OF EXPOSURE ATMOSPHERES

Oxygen

An atmosphere of 65% O_2 was created in 450-L plastic tents by metering 100% medical O_2 into a stream of filtered compressed air to a final O_2 concentration of 65%. Oxygen concentrations in the chamber were measured daily with an oxygen meter. Actual chamber concentrations were 64.5% (SD 3.3%, n measurements = 120).

Ozone

Ozone was generated from vaporized liquid medical-grade O_2 with a silent electric arc discharge ozonizer (Erwin Sander Corp., Giessen, Germany). The O_3 was mixed into a stream of filtered air, and both O_3 and O_2 were conveyed through Teflon lines to the mixing inlet of the exposure chamber. Humidity was kept at 50%. Chamber air flow was maintained so that the chamber volume of air changed 30 times per hour. Ozone concentrations in the chamber were measured with a calibrated Dasibi ultraviolet photometer (model 1003-AH, Dasibi Environmental Corp., Glendale, CA). Data were recorded every two minutes directly onto an IBM-AT computer (IBM Corp., Armonk, NY) for later analysis. The Dasibi O_3 analyzer was calibrated against a Dasibi ultraviolet photometer (model 1008PC, serial number 2534), which was calibrated against a National Institute of Standards and Technology standard reference photometer (serial number 4) located at the California

Air Resources Board Assurance Standards Laboratory. The nominal O_3 concentration in the chamber was 0.8 ppm. Measured concentrations were 0.77 ppm (SD 0.03, *n* measurements = 12,000).

Nitrogen Dioxide

Exposure to NO_2 was achieved by bubbling nitrogen through the liquid dimer dinitrogen tetroxide, held at 0°C (Freeman and Hayden 1964; Freeman 1968). The resultant NO_2 was conveyed to the mixing inlet of the exposure chamber through stainless-steel lines. Chamber concentrations were measured with a Dasibi chemoluminescent nitrogen NO_2 analyzer (model 2108). The monitor was calibrated using the Environmental Protection Agency gas-phase titration method (U.S. Environmental Protection Agency 1988) using a nitric oxide standard with O_3 in a Dasibi gas calibrator (model 1005-CE-2, serial number 041). The nominal chamber concentration was 15 ppm NO_2. Actual chamber concentrations were 14.75 ppm (SD 2.63, *n* measurements = 2,258).

PATHOLOGY

Tissue samples for histopathological analysis were collected from animals killed at the scheduled end of the experiment and from animals found dead or killed because of poor clinical condition (body weight falling below 80 g or animals showing visible signs of distress). All animals were killed by an overdose of pentobarbital, and complete autopsies were performed. Lungs were fixed by perfusion through the vascular bed with 10% neutral buffered formalin, excised en bloc (larynx, trachea, mediastinum, heart, and lungs) and preserved in the same fixative. Other organs collected at autopsy included kidneys, adrenals, liver, testes, brain, nasal cavities, pancreas, and femur. All tissues were stored in formalin until the end of a particular experiment, when they were shipped from the University of California at Davis to the University of Tennessee at Knoxville. Upon receipt, tissues were trimmed, dehydrated, and embedded in paraffin. Conventional sections were cut and stained with hematoxylin and eosin.

Special immunocytochemical stains were performed on selected samples of lung tissue. Immunocytochemical stains for mammalian bombesin, immunoreactive calcitonin, and immunoreactive keratin were performed using the Vectastain ABC-Kit (Vector, Burlingame, CA). Deparaffinized lung sections were incubated with primary antibody (1:1,000) for 48 hours at 4°C. The primary antibodies specific for the carboxyl terminal sequences of bombesin and calcitonin were supplied by Dr. K.L. Becker of the Department of Endocrinology, Veterans' Administration Hospital, George Washington University, Washington, DC. The primary antibody (1:100) for keratin was purchased from Lipshaw (Detroit, MI) and was specific for 56 and 64 kDa cytokeratins. Controls included sections without primary antiserum, sections treated with excess antigen for calcitonin and keratin, sections of the thyroid gland and sections of the skin.

A review of lung slides from animals exposed to combinations of DEN with air and DEN with O_3 was conducted by a pathology working group on July 12, 1990. Members of the group were: Drs. Eugene McConnell, independent consultant, Raleigh, NC (Chairman); Marvin Kuschner, Department of Pathology, State University of New York at Stonybrook; David G. Kaufman, Department of Pathology, University of North Carolina at Chapel Hill; Jeff Everitt, Chemical Industry Institute of Toxicology, Research Triangle Park, NC; Adi F. Gazdar, National Cancer Institute Medical Oncology Branch, Naval Hospital, Bethesda, MD; and Gary Boorman, National Institute for Environmental Health Sciences, Research Triangle Park, NC. A written report was provided to the principal investigators upon completion of the review.

EXPERIMENTAL DESIGN

Details on the conduct of each experiment are given in the Results section.

STATISTICS

Comparison of incidence of histopathological lesions between animals exposed to air pollutants and animals kept in filtered air was done by chi-squared analysis (not corrected for continuity).

RESULTS

OZONE EXPERIMENT

The goal of this experiment was to test the hypothesis that O_3 would modulate the development of DEN-induced lung tumors in hamsters.

Experimental Design

The animals used in this experiment arrived in January 1989 in two different shipments that were two weeks apart. Animals in shipment Number 1 were divided at random into two groups and were assigned to be injected with DEN and exposed either to filtered air (Group 1) or to 0.8 ppm O_3 (Group 2). Animals in shipment Number 2 were divided at random into two groups, and the animals were assigned to be injected with saline and exposed to filtered air (Group 3)

Table 2. Experimental Design[a]

Group Number and Treatment	*n*	Necropsy Schedule
O_3 Experiment		
1. DEN + filtered air[b]	40	20 animals after 16 weeks 20 animals after 24 weeks
2. DEN + 0.8 ppm O_3[b,c]	40	20 animals after 16 weeks 20 animals after 24 weeks
3. NaCl + filtered air[d]	20	All animals after 24 weeks
4. NaCl + 0.8 ppm of O_3[c]	40	20 animals after 16 weeks 20 animals after 24 weeks
5. DEN + 65% O_2[e]	10	10 animals after 12 weeks
NO_2 Experiment		
1. DEN + filtered air[f]	40	21 animals after 16 weeks 19 animals when moribund (between 24 and 32 weeks)
2. DEN + 15 ppm NO_2[f,g]	40	20 animals after 16 weeks 20 animals when moribund (between 24 and 35 weeks)
3. NaCl + filtered air[d]	20	All animals after 35 weeks
4. NaCl + 15 ppm NO_2[g]	40	20 animals after 16 weeks 20 animals after 35 weeks
5. DEN + room air[h]	20	When moribund (between 24 and 32 weeks)
6. DEN + 65% O_2[h]	20	When moribund (between 16 and 32 weeks)

[a] In the O_3 experiment conducted in 1989, the animals in groups 1 through 4 were housed within the inhalation chambers at the CRPRC in wire-mesh–bottom cages. The animals in group 5 were kept in an oxygen tent in plastic shoe-box cages with hardwood bedding at the ITEH. In the NO_2 experiment conducted in 1990, the animals in groups 1 through 4 were housed in the inhalation chambers located at the CRPRC in wire-mesh–bottom cages. The animals in groups 5 and 6 were housed in plastic shoe-box cages with hardwood bedding at the ITEH.

[b] DEN injected twice a week for 16 weeks.

[c] O_3 exposure terminated after 16 weeks.

[d] For complete statistical analysis, 20 animals exposed to NaCl and filtered air also should have been examined after 16 weeks. However, because no spontaneous lung tumors have ever been found in hamsters of this age, it was decided to omit this group in the interest of reducing the total number of animals used.

[e] DEN injected twice a week for 8 weeks.

[f] DEN injected twice a week for 24 weeks.

[g] NO_2 exposure terminated after 24 weeks.

[h] DEN injected twice a week for 20 weeks.

or to 0.8 ppm O_3 (Group 4). The different treatment groups are listed in Table 2.

All animals in Groups 1 through 4 received subcutaneous injections twice a week of DEN (20 mg/kg) or saline (0.2 mL/100 g body weight) while being exposed to O_3 or filtered air, 23 hours per day, 7 days per week. Injections and pollutant exposure lasted for 16 weeks. After 16 weeks, 20 animals from each group treated with DEN and air, DEN and O_3, and saline and O_3 were killed. For the animals remaining at this time, all DEN injections stopped, and the O_3 atmosphere was replaced by filtered air. These animals were allowed to survive without any further treatment until 24 weeks after the beginning of the experiment, when they were killed by pentobarbital overdose. Throughout the entire experimental period, the animals in Groups 1 through 4 were housed in wire-mesh cages within the inhalation chambers at the CRPRC.

A separate group of 10 hamsters (six from shipment number 1 and four from shipment number 2) served as positive controls for the induction of lung tumors of neuroendocrine origin (Group 5). The animals were injected with 20 mg/kg of DEN two times a week for eight weeks. They were housed at ITEH in conventional plastic shoe-box cages on hardwood bedding within a plastic tent. An atmosphere of 65% O_3 was maintained within the plastic tent for 24 hours a day. After eight weeks, the DEN injections were stopped, but O_3 exposure was continued. The animals were killed 12 weeks after the beginning of the experiment.

Weight Gain

Data on weight gain in the four treatment groups are shown in Figure 1. Control animals in Group 3 (saline and air) gained weight initially at a rapid rate, and, after 12

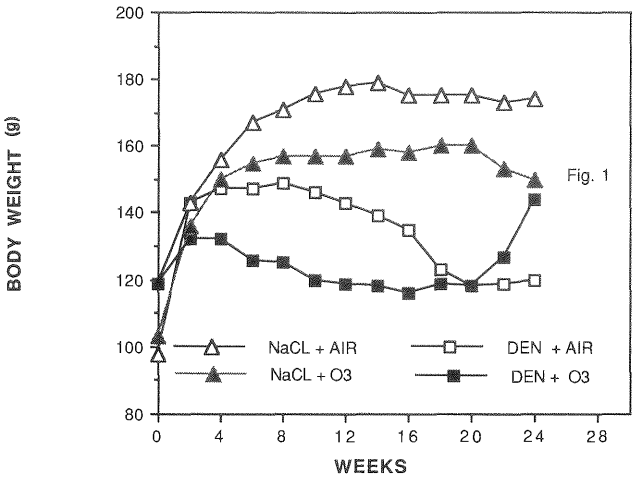

Figure 1. Weight gain of hamsters during the O_3 experiment. Male Syrian golden hamsters were injected subcutaneously with DEN (20 mg/kg) twice a week for 16 weeks, or with saline, and were either exposed to 0.8 ppm O_3 for 23 hours a day, 7 days a week, or kept in filtered air. After the 16th week, the animals received no further treatment and all animals were kept in air until being killed at 24 weeks.

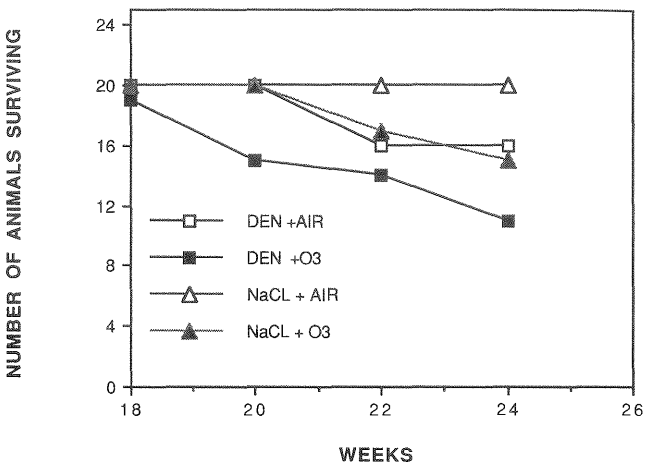

Figure 2. Survival of animals in the O_3 experiment from week 18 to the end of the experiment (same animals as in Figure 1).

weeks, remained stable until the end of the experiment. Animals in Group 4 (saline and O_3) gained weight during the first six weeks and then remained stable at a lower plateau than animals in Group 3. Some weight loss was observed during the last four weeks. All animals treated with DEN (Groups 1 and 2) gained substantially less weight than the hamsters in Groups 3 and 4, with the animals in Group 2 (DEN and O_3) demonstrating the lowest body weights during the first 16 weeks of the experiment. It is noteworthy, however, that animals in this group rapidly gained some weight after the treatment with DEN and O_3 had stopped.

Survival

All animals in the four groups survived for the first 16 weeks of the experiment (Figure 2). Between 16 and 24 weeks, nine animals in the DEN and O_3 group were either found dead or had to be killed because of extreme weight loss (below 80 g) or because of their poor clinical condition. In the DEN and air group, four animals had to be killed before the scheduled date for the same reasons. In the 23rd week of the experiment, five animals exposed to saline and O_3 were found dead. The likely cause of death for these animals was severe gastrointestinal infection, manifested by acute diarrhea. One week later, when the remaining animals in the saline and O_3 group were killed, cecal swabs and gastrointestinal tract tissue were sent to the University of California at Davis Comparative Pathology Laboratory. The laboratory report listed moderate chronic diffuse typhylocolitis [*sic*] with goblet cell hyperplasia and multiple focal crypt regeneration in three animals, and acute ulceration of the squamous gastric mucosa of unknown etiology in one animal. Bacteriology and serology were inconclusive, and precise etiologic diagnosis of the acute gastrointestinal disease was not established. All other tissues were examined with the material from the rest of the study; however, histopathology of the respiratory tract and other major organs did not provide a clue for the premature deaths. All animals kept in air and given saline injections survived until 24 weeks.

Tumor Incidence

The histopathological lesions are listed in Table 3. For the animals killed 16 weeks after the beginning of the experiment, significantly more peripheral lung lesions were found in Group 2 (DEN and O_3) and Group 3 (saline and O_3) than in Group 1 (DEN and air). Originally, these peripheral lung lesions were diagnosed as small adenomas of potentially neuroendocrine differentiation (Figure 3). The diagnosis of "tumor," as opposed to "hyperplasia," was based on the compact appearance of the lesion and the organoid growth pattern. The suspicion that the lesion might be neuroendocrine was based on our experience that tumors with neuroendocrine differentiation, induced by DEN and hyperoxia or NNK and hyperoxia (Schuller et al. 1988, 1990a), have a compact, highly cellular appearance and lack glandular features. On the other hand, Clara cell–derived DEN-induced adenomas usually grow along preexisting structures (e.g., bronchi/bronchioli or alveoles) during the early stages of development and demonstrate a distinct papillary or glandular growth pattern.

Immunocytochemical stains for mammalian bombesin and calcitonin were negative in all these sections. Immunostains on representative slides from these experiments and with identical antisera also were conducted by Dr. Adi Gazdar of the National Cancer Institute. They also failed to yield positive staining; however, the normal neuroendocrine cells

Table 3. Incidence of Histopathological Lesions in Hamsters Exposed to Ozone and Examined 16 Weeks After the Beginning of Treatment[a]

Location (Lesion)	Treatment Groups		
	DEN + Air (n = 19)[b]	DEN + O_3 (n = 20)	NaCl + O_3 (n = 19)[b]
Bronchiolar hyperplasia[c]	2 (11%)	11 (55%)[d]	15 (79%)[d]
Lung (adenomas)[c]	2 (11%)	1 (5%)	0 (0%)
Bronchi (papillary polyps)	2 (11%)	0 (0%)	0 (0%)
Trachea (papillary polyps)	19 (100%)	9 (45%)[d]	0 (0%)
Nasal cavities (adenomas and carcinomas)	NA[e]	NA	NA
Liver (hepatocellular carcinomas)	0 (0%)	0 (0%)	0 (0%)
Liver (hepatocellular adenomas)	0 (0%)	0 (0%)	0 (0%)
Liver (necrosis and bile duct proliferation)	12 (63%)	0 (0%)[d]	0 (0%)
Bronchopneumonia/abscesses	2 (11%)	2 (10%)	1 (5%)

[a] Animals were injected with DEN (20 mg/kg) twice a week (controls injected with saline) and kept in either 0.8 ppm O_3 or filtered air at CRPRC for 23 hours a day, 7 days a week, for 16 weeks.

[b] Originally 20 animals per group; tissues of one animal lost during processing.

[c] In this experiment, lung tissues were reviewed by a pathology working group. Diagnosis listed represents a group consensus.

[d] Significantly different ($p < 0.05$) compared with DEN + air group.

[e] NA = not available. Tissues for examination of nasal passages not available in this experiment.

present in these slides did not yield positive staining either. This may be a false negative result caused by overfixation of tissues that were stored too long in neutral buffered formalin. After a review of the slide material by the pathology working group, the original diagnosis of these lesions as small peripheral adenomas was replaced by the diagnosis of "hyperplasia." Accordingly, lesions like the one presented in Figure 3, together with epithelial hyperplasias proliferating along preexisting airways and into the bronchiolar lumen (Figures 4 and 5), are listed in Tables 3 and 4 as "bronchiolar epithelial hyperplasias."

Lung tumors were found in one animal (5%) in Group 2 (DEN and O_3); two animals (11%) in Group 1 (DEN and air) had lung tumors. Immunocytochemical stains yielded negative reactions for calcitonin and keratin in the tumors. The immunocytochemical stain for mammalian bombesin showed a weak positive reaction in the single lung tumor

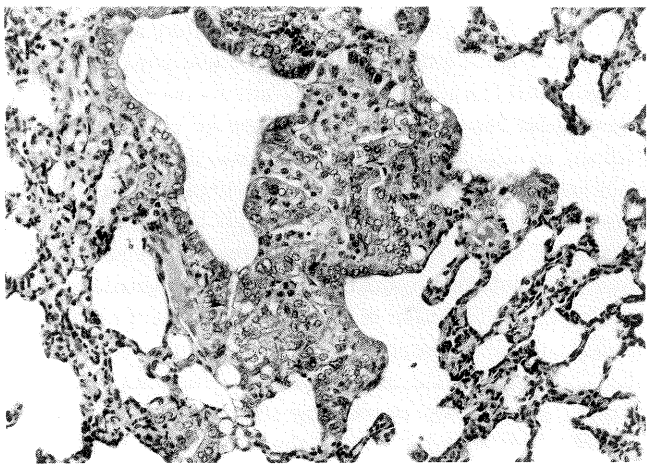

Figure 3. Peripheral lung lesion in a hamster treated with DEN and O_3, originally diagnosed as a small peripheral adenoma with potentially neuroendocrine differentiation. The original diagnosis was based on the compact appearance of the lesion, along with an organoid growth pattern and lack of glandular features. The tumor is different from Clara cell–derived-DEN induced adenomas, which usually grow along preexisting structures (bronchi, bronchioles, or alveoli) during their early stages of development and demonstrate a distinct glandular or papillary growth pattern. The lesion was called "hyperplasia" by the pathology working group. Magnification is × 120.

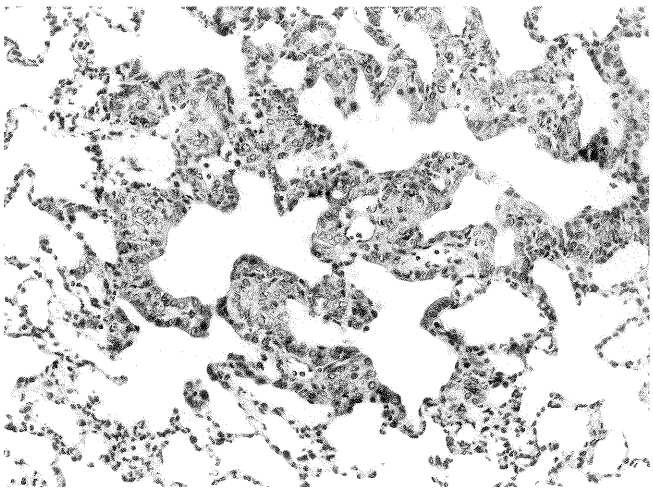

Figure 4. Epithelial hyperplasia in an animal treated with O_3 and DEN. Epithelial cells have proliferated along preexisting peripheral airways. In contrast with the lesion illustrated in Figure 3, proliferated cells are diffuse, and no organoid pattern is discernible. Magnification is × 100.

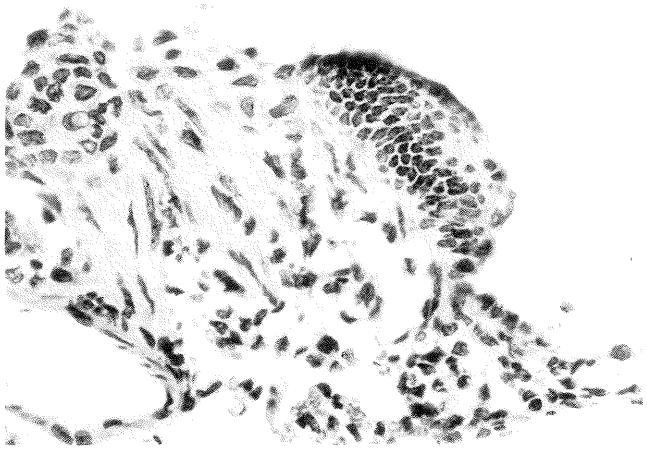

Figure 5. Epithelial hyperplasia in an animal treated with O_3 with DEN. A multilayered focus of proliferated cells arises from the bronchiolar epithelium and projects into the lumen of the airway. Magnification is × 200.

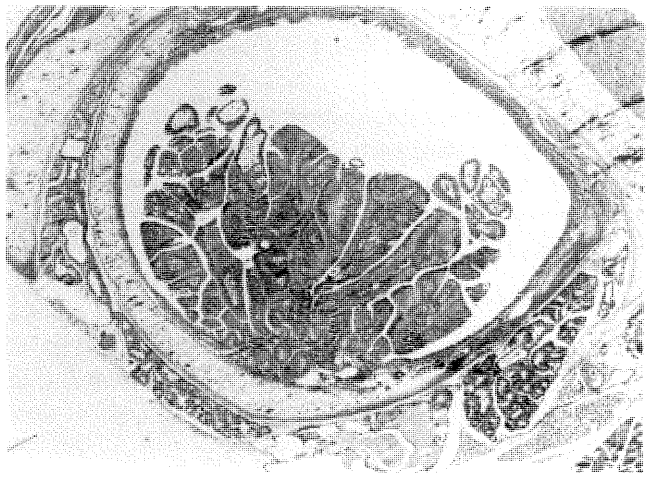

Figure 6. Papillary polyp of the trachea in an animal treated with DEN. This neoplasm typically projects as an intraluminal mass attached to the tracheal mucosa and submucosa by a slender fibrous stalk. The size of these tumors depends on the plane of the section. However, as in the present example, the tumors may often almost obstruct the tracheal lumen. The tumors consist of multiple papilliferous growths lined by single or double layers of low cuboidal to flat epithelium. The mitotic rate is low. Magnification is × 50.

found in the group treated with DEN and O_3. All tumors in Group 1 (DEN and air) were negative. Efforts to enhance the positive immunoreaction by increasing the concentration of antiserum to 1:500 and exposing the slides at room temperature rather than at 4°C failed.

As expected, DEN produced a substantial number of neoplastic lesions in the trachea and bronchi. Tracheal papillary polyps (Figure 6) were found in 100% of all animals treated with DEN and air. Exposure to O_3 seemed to prevent the development of these tumors, and the incidence was significantly lower ($p<0.01$) in Group 2 (DEN and O_3) than in Group 1 (DEN and air). The tumors consisted of multiple papilliferous growths lined by a single-to-double layer of low

Table 4. Incidence of Histopathological Lesions in Hamsters Exposed to Ozone and Examined 24 Weeks After the Beginning of Treatment[a]

Location (Lesion)	Treatment Groups: DEN + Air[b] (n = 20)	DEN + O_3[c] (n = 20)	NaCl + O_3[d] (n = 19)
Bronchiolar hyperplasia[e]	2 (10%)	10 (50%)[f]	13 (68%)[f]
Lung[e] (adenomas)	6 (30%)	3 (15%)	0 (0%)
Bronchi (papillary polyps)	0 (0%)	2 (10%)	0 (0%)
Trachea (papillary polyps)	19 (95%)	19 (95%)	0 (0%)
Nasal cavities (adenomas and carcinomas)	NA[g]	NA	NA
Liver (hepatocellular carcinomas)	1 (5%)	0 (0%)	0 (0%)
Liver (hepatocellular adenomas)	1 (5%)	0 (0%)	0 (0%)
Liver (necrosis and bile duct proliferation)	20 (100%)	12 (60%)[f]	0 (0%)
Bronchopneumonia/abscesses	4 (20%)	3 (15%)	0 (0%)

[a] Animals were injected with DEN (20 mg/kg) twice a week (controls injected with saline) and kept in either 0.8 ppm O_3 or filtered air at CRPRC for 23 hours a day, 7 days a week, for 16 weeks. After this time, all treatments were terminated and the animals were kept in filtered air until killed. In the animals exposed to saline and air, no abnormal histopathological findings were observed; these animals are not listed in the table.

[b] Four animals had to be killed for the reasons outlined in footnote c.

[c] Between 16 and 24 weeks, 9 of the 20 animals were either found dead or had to be killed because of extreme weight loss or poor clinical condition.

[d] Originally 20 animals; tissues of one animal lost to cannibalism. In the 23rd week, 5 of the 19 animals were found dead, most likely from gastrointestinal infection.

[e] In this experiment, lung tissues were reviewed by a pathology working group. Diagnosis listed represents a group consensus.

[f] Significantly different ($p < 0.05$) compared with DEN + air group.

[g] NA = not available. Tissues for examination of nasal passages were not available in this experiment.

cuboidal flat epithelium; mitotic figures were sparse. Bronchopneumonic foci were found in 5% to 10% of the animals exposed to air or O_3.

A somewhat surprising observation was made in the livers of the animals treated with DEN. Although more than 50% of the animals treated with DEN and air showed signs of DEN-induced hepatotoxicity, no such lesions were found in the animals exposed to O_3. The DEN-induced toxic liver lesions consisted of foci of hepatocytes with vacuolized cytoplasm or focal proliferation of bile duct epithelia or both.

Histopathological analysis of the animals that were killed at week 24 of the experiment (including animals that died or were killed before the scheduled killing because of their poor clinical appearance) are given in Table 4. All but one of the animals exposed to DEN and air had tracheal papillary polyps. In these animals, there was a higher incidence of lung adenomas than had been found at 16 weeks. A representative tumor is depicted in Figure 7. The Clara cell–derived tumors consisted of tall columnar cells growing in a distinct glandular and/or papillary pattern. Mitotic rates were low.

The group treated with DEN and O_3 demonstrated a slight increase in lung tumor incidence (15%) and a marked increase in tracheal tumors compared with what had been found at 16 weeks, whereas the incidence of bronchiolar hyperplasia remained about the same. An example of a lung tumor found in the DEN and O_3 group is given in Figure 8. The tumor clearly expresses a differentiation that is at variance with the tumor shown in Figure 7. Instead of the tall columnar tumor cells that typically grow in a gland-like pattern in adenomas caused by DEN alone, the cells in the lesions caused by DEN and O_3 are round to slightly oval and grow in dense nests and clusters in an organoid pattern. However, immunostains were again negative, and no definite diagnosis of the suspected neuroendocrine differentiation could be made. After reviewing the tumors induced by DEN and O_3, including the example shown in Figure 8, the pathology working group stated that the tumors induced by DEN and O_3 could not be classified separately from the ones found in the DEN and air group.

The incidence of bronchiolar hyperplasias in the group treated with saline and O_3 was slightly lower than it had been after 16 weeks, although still significantly higher ($p<0.05$) than in animals in the DEN and air group. Representative slides of bronchiolar hyperplasia from all groups were subjected to immunocytochemical stains for calcitonin and bombesin, but yielded negative reactions.

As had been seen in the animals killed at 16 weeks, O_3 continued to have some mitigating effect on the development of liver damage. The incidence of toxic liver lesions (necrosis and bile duct proliferation) was significantly lower ($p<0.05$) in the animals exposed to DEN and O_3 than in those exposed to DEN and air. About 20% of the animals treated with DEN had bronchopneumonic foci, regardless of whether they had been kept in filtered air or in an atmosphere of O_3.

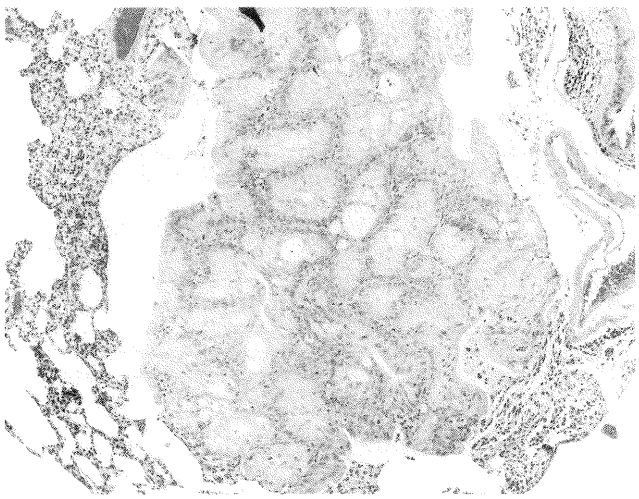

Figure 7. Peripheral lung adenoma induced by DEN alone. This Clara cell–derived tumor typically consists of tall columnar cells that grow in distinct glandular or papillary patterns, or both. The mitotic rate is low. Magnification is × 100.

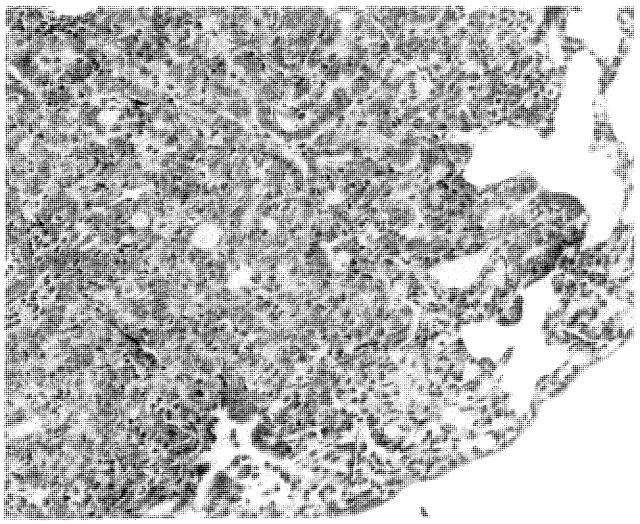

Figure 8. Peripheral adenoma of potentially neuroendocrine differentiation in a hamster exposed to DEN and O_3. This tumor clearly expresses a differentiation that is different from the adenoma shown in Figure 7 and induced by DEN alone. Instead of the tall columnar tumor cells that typically grow in a gland-like pattern in the adenomas caused by DEN alone, the cells comprising this lesion are round to slightly oval and grow in dense nests and clusters in an organoid pattern. The mitotic rate is low. The pathology working group stated that all tumors induced by DEN and O_3, including this example, were not different from those induced by DEN alone. Magnification is × 100.

Positive Controls

A separate group of 10 hamsters was exposed to DEN while being kept in conventional plastic shoe-box cages on hardwood bedding in a hyperoxic environment. The experimental protocol for this group was similar to that for our previous experiments, which had yielded neuroendocrine lung tumors in hamsters simultaneously exposed to hyperoxia and DEN (Schuller et al. 1988, 1990b). However, a few modifications in carcinogen and O_2 exposure were made. In previous studies the animals were exposed to 70% O_2; in this study, they were exposed to 65% O_2. The animals were treated twice a week with subcutaneous injections of DEN (20 mg/kg). Although the dose of DEN injected was identical to that used in the previous studies, the injection solution was half the concentration of that used previously. After eight weeks, DEN treatments were stopped, whereas O_2 exposure continued. In a previous study, the animals were killed at 8 weeks (Schuller et al. 1988), whereas in the present study they were given a four-week recovery period from DEN while being kept in O_2.

From the very beginning of the experiment, the animals started to loose weight, and three animals died before the 10th week of the experiment. The remaining animals were killed 12 weeks after the beginning of DEN and O_2 exposure. Histopathological analysis of the lungs showed tumors in the lungs of two animals. However, neither normal pulmonary neuroendocrine cells nor the tumors in these sections stained positively with the various immunostains that were applied.

NITROGEN DIOXIDE EXPERIMENT

The goal of these experiments was to test the hypothesis that NO_2 would modulate the development of DEN-induced lung tumors in hamsters.

Rationale for Modifying Experimental Design

Analysis of the tumor data from the O_3 experiment raised some concern with regard to the number of lung tumors found in animals that were designed to serve as controls. In hamsters injected with DEN and kept in filtered air, overall lung tumor incidence (30% at 24 weeks) was substantially lower than expected from observations made in previous studies. Using an identical total dose of DEN, but at twice the concentration as that adopted for the present study, a lung tumor incidence of approximately 60% usually was found in animals treated with DEN and kept in air. Between 60% and 100% of the animals exposed to DEN and hyperoxia developed lung tumors (Schuller and McMahon 1985; Schuller et al. 1988, 1990b). Moreover, the long fixation time of tissue specimens in the current study (as evidenced by the negative reaction of normal neuroendocrine lung cells) precluded an identification of neuroendocrine lung tumors by immunocytochemistry. In the absence of published data on an unequivocal effect of the nitrosamine concentration used in injection solutions on tumor response, we considered that the animals may have been killed too early in the present O_3 experiment. This, in turn, might not have allowed for the full expression of DEN carcinogenicity and a possible promoting effect of the air pollutant under study. The protocol for the NO_2 study was modified accordingly.

Experimental Design

At the beginning of the experiment, a total of 180 hamsters, received in a single shipment, were separated randomly into six different treatment groups. The body weights in the different groups were distributed as homogeneously as possible. The six groups were assigned the following treatments: Group 1 (n = 40) was injected with DEN and kept in filtered air; Group 2 (n = 40) was injected with DEN and exposed to 15 ppm NO_2; Group 3 (n = 20) was injected with 0.9% NaCl and kept in filtered air; Group 4 (n = 40) was injected with 0.9% NaCl and exposed to NO_2. Positive controls were as follows: Group 5 (n = 20) was injected with DEN and kept in room air; Group 6 (n = 20) was injected with DEN and exposed to hyperoxia (65% O_2) (Table 2).

The animals for the NO_2 study were housed in inhalation chambers in stainless-steel wire-mesh–bottom cages at the CRPRC Inhalation Facility. They were given subcutaneous injections of DEN (20 mg/kg) twice a week, for a total of 24 weeks. Exposure to 15 ppm NO_2 was for 23 hours a day, seven days a week. After 16 weeks, half of the animals in Groups 1, 2, and 4 were killed, and the tissues prepared for histopathological analysis. The DEN injections and exposure to NO_2 continued for the remaining animals until 24 weeks after the beginning of the experiment. From this point on, no further DEN injections were given, and the NO_2 atmosphere was replaced by filtered air. Twenty-eight weeks after the beginning of the experiment, the last animals in Group 1 were found dead or had to be killed; after 35 weeks, all animals in Group 2 were dead. At this time, all control animals (Group 3) were killed, and the experiment was terminated.

Weight Gain

Average weights of the animals in all four groups are shown in Figure 9. Within the first 12 weeks, all animals except the ones treated with DEN and NO_2 gained weight at a normal rate. After 16 weeks, all animals injected with DEN began to loose weight rapidly, whereas animals injected

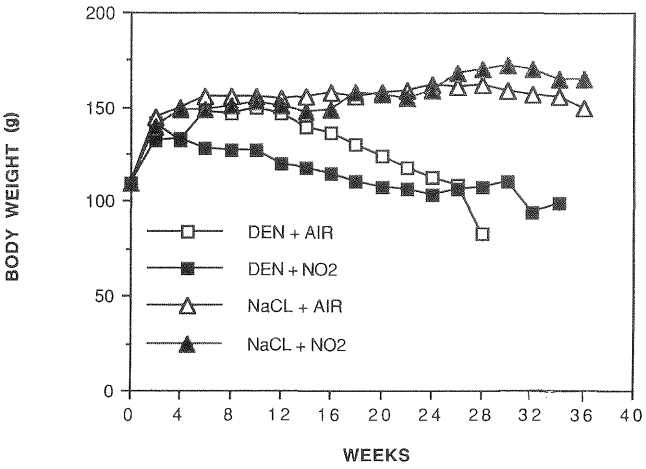

Figure 9. Weight gain of hamsters during the NO_2 experiment. Male Syrian golden hamsters were injected subcutaneously with DEN (20 mg/kg) twice a week for 24 weeks, or with saline, and were either exposed to 15 ppm NO_2 for 23 hours a day, 7 days a week, or kept in filtered air. After the 24th week, the animals received no further treatment and all animals were kept in air until being killed between 24 and 34 weeks.

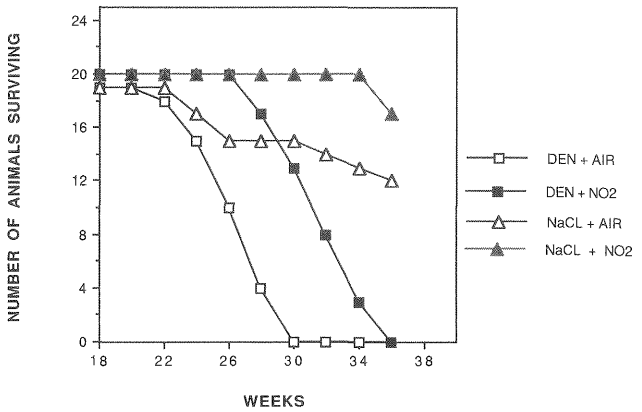

Figure 10. Survival of animals in the NO_2 experiment from week 18 to the end of the experiment (same animals as in Figure 9).

with 0.9% NaCl maintained weight at a more or less steady rate.

Survival Rates

Up to 16 weeks after the beginning of the experiment, all animals had survived, with the exception of two animals from Group 1 (DEN and air) that died accidentally. After 20 weeks, many animals in the groups treated with DEN died spontaneously or had to be killed because of extreme weight loss and poor clinical condition. Most likely, these early deaths were caused by tumors in the trachea, bronchi, or nasal passages, or by extensive liver tumors. Survival rates were different for animals kept in air and those exposed to NO_2. In the latter group, survival was distinctly better toward the end of the experiment (Figure 10). After 28 weeks, all animals treated with DEN and air had died or had to be killed. The animals remaining in Group 2 (DEN and NO_2) died or had to be killed between weeks 31 and 35, at which time the experiment was ended. Between weeks 22 and 35, several animals in the air and NaCl group died. A prominent clinical sign was diarrhea, although autopsies revealed no apparent reason for the premature deaths. Almost all of the animals injected with NaCl and kept in NO_2 survived for 35 weeks.

Tumor Incidence

Sixteen weeks after the beginning of the experiment, animals treated with DEN and exposed to NO_2 had no lung

Table 5. Incidence of Histopathological Lesions in Hamsters Exposed to Nitrogen Dioxide and Examined 16 Weeks After the Beginning of Treatment[a]

Location (Lesion)	Treatment Groups		
	DEN + Air (n = 21)	DEN + NO_2 (n = 20)	NaCl + NO_2 (n = 20)
Bronchiolar hyperplasia	0 (0%)	0 (0%)	0 (0%)
Lung (adenomas)	2 (9%)	0 (0%)	0 (0%)
Bronchi (papillary polyps)	2 (9%)	1 (5%)	0 (0%)
Trachea (papillary polyps)	17 (81%)	5 (25%)[b]	0 (0%)
Nasal cavities (adenomas and carcinomas)	10 (48%)	3 (15%)[b]	0 (0%)
Liver (hepatocellular carcinomas)	0 (0%)	0 (0%)	0 (0%)
Liver (hepatocellular adenomas)	0 (0%)	0 (0%)	0 (0%)
Liver (necrosis and bile duct proliferation)	21 (100%)	19 (95%)	0 (0%)
Bronchopneumonia/abscesses	0 (0%)	5 (25%)	2 (10%)

[a] Animals were injected with DEN (20 mg/kg) twice a week (controls injected with saline) and kept in either 15 ppm NO_2 or filtered air for 23 hours a day, 7 days a week, for 24 weeks. After this time, all the animals were killed.

[b] Significantly different ($p < 0.05$) compared with DEN + air group.

Table 6. Incidence of Histopathological Lesions in Hamsters Exposed to Nitrogen Dioxide and Examined 24 to 32 Weeks After the Beginning of Treatment[a]

Location (Lesion)	Treatment Groups		
	DEN + Air (n = 19)	DEN + NO_2 (n = 20)	NaCl + NO_2 (n = 20)
Bronchiolar hyperplasia	0 (0%)	0 (0%)	0 (0%)
Lung (adenomas)	5 (26%)	0 (0%)[b]	0 (0%)
Bronchi (papillary polyps)	3 (16%)	0 (0%)	0 (0%)
Trachea (papillary polyps)	19 (100%)	15 (75%)[b]	0 (0%)
Nasal cavities (adenomas and carcinomas)	16 (84%)	16 (80%)	0 (0%)
Liver (hepatocellular carcinomas)	3 (16%)	0 (0%)	0 (0%)
Liver (hepatocellular adenomas)	1 (5%)	8 (40%)[b]	0 (0%)
Liver (necrosis and bile duct proliferation)	18 (95%)	20 (100%)	0 (0%)
Bronchopneumonia/abscesses	2 (11%)	0 (0%)	0 (0%)

[a] Animals were injected with DEN (20 mg/kg) twice a week (controls injected with saline) and kept in either 15 ppm NO_2 or filtered air for 23 hours a day, 7 days a week, for 24 weeks. After this time, all treatments were terminated, and the animals were kept in filtered air until killed (between 24 and 32 weeks after the beginning of the experiment). In the animals exposed to saline and air, no abnormal histopathological findings were observed; these animals are not listed in the table.

[b] Significantly different ($p < 0.05$) compared with DEN + air group.

tumors (as opposed to two lung tumors in the DEN and air group) and had significantly fewer ($p<0.05$) tumors in the trachea and the nasal passages than the animals treated with DEN and kept in air (Table 5). No liver tumors were found, although signs of hepatotoxicity were present in all animals except one. No tumors were detected in the animals injected with NaCl and exposed to NO_2. Bronchopneumonic foci were found in 17.5% of all the animals exposed to NO_2.

Tumor data for the animals found dead or killed between 24 and 32 weeks after the beginning of the experiment are listed in Table 6. No lung tumors were found in the animals treated with DEN and exposed to NO_2. The incidence of tracheal tumors rose to 75%, but remained significantly lower than in the animals treated with DEN and kept in filtered air. The incidence of nasal tumors in animals injected with DEN was the same whether they had been exposed to NO_2 or to air. The incidence of hepatocellular carcinomas (Figure 11) seemed slightly higher in animals kept in air, whereas a higher incidence of hepatocellular adenomas (Figure 12) was found in the animals exposed to NO_2. No tumors were found in animals injected with NaCl

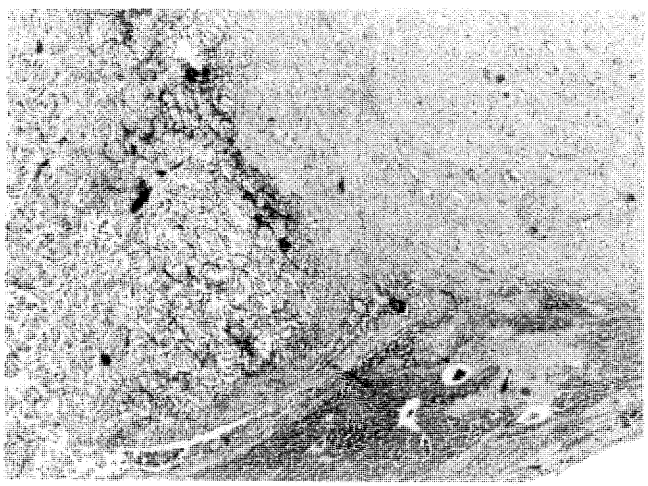

Figure 11. Hepatocellular carcinoma in an animal treated with DEN. These tumors are large, space-occupying masses that invade the normal liver parenchyma and result in destruction of hepatic architecture. The tumor cells are pleomorphic with eosinophilic cytoplasm, prominent nucleoli, occasional multinucleation, and frequent mitosis. Magnification is × 40.

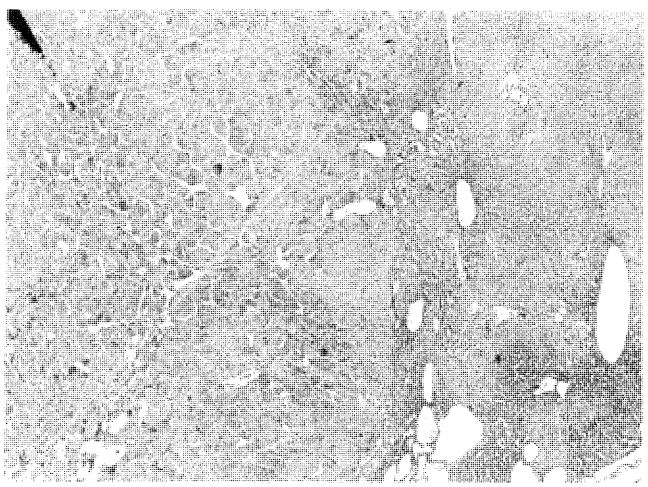

Figure 12. Hepatocellular adenoma in an animal treated with DEN. This lesion is well circumscribed, lacks lobular architecture, and does not invade surrounding liver parenchyma. The eosinophilic hepatocytes comprising this lesion are generally homogeneous with very little or absent pleomorphism. Magnification is × 40.

and exposed to NO_2 or filtered air. Bronchopneumonic foci were found in 11% of the animals treated with DEN and exposed to filtered air.

Positive Controls

An additional group of 40 animals received in the same shipment was kept in the animal facility at the ITEH in conventional plastic shoe-box cages on hardwood bedding. All animals in this group received subcutaneous injections of DEN (20 mg/kg) twice a week. Half of the animals were kept in a hyperoxic environment (65% O_2 in the inspired air); the other half were kept in room air. Weight gain and survival data are shown in Figures 13 and 14. Animals treated with DEN and kept in air gained weight during the first six weeks, but then lost it rapidly. Animals exposed to DEN and O_2 gained less weight for a shorter time period; only toward the end of the experiment, when the DEN injections were stopped after 20 weeks, did the few surviving animals regain some weight. The first animals exposed to DEN and O_2 began to die after six to eight weeks; after 20 weeks, only 35% of the animals were still alive. At this time, it was decided to stop all further DEN treatments. After 28 weeks, all animals exposed to DEN and O_2 had died or had to be killed. In the group treated with DEN and air, all animals survived for the first 16 weeks. However, between 24 and 32 weeks, the animals treated with DEN and air also began to die; their survival rates were practically identical to those seen in animals exposed to filtered air and treated with DEN at the CRPRC (Figure 10).

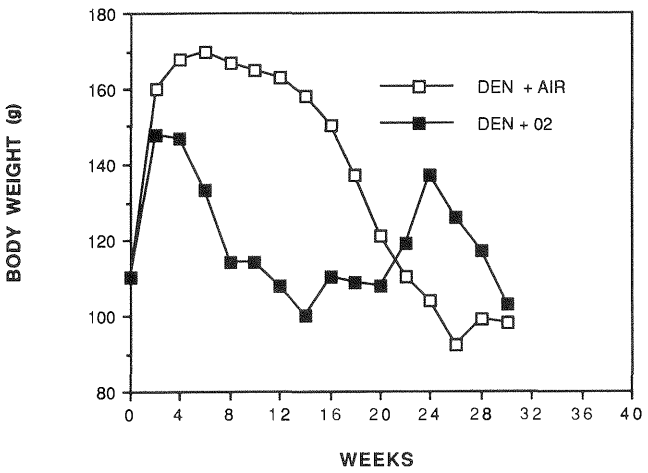

Figure 13. Weight gain of hamsters during the O_2 experiment. Male Syrian golden hamsters were injected subcutaneously with DEN (20 mg/kg) twice a week for 20 weeks, and either exposed to 65% O_2 for 24 hours a day, 7 days a week, or kept in room air. After the 20th week, the animals received no further treatment with DEN and remained in O_2 or in air until they were killed.

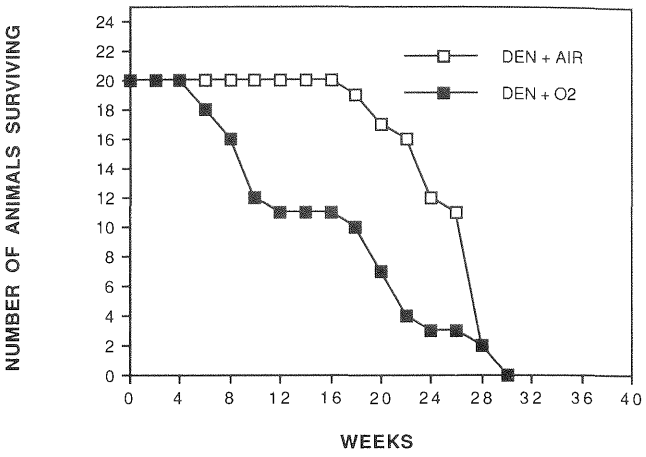

Figure 14. Survival of animals in the O_2 experiment from the beginning to the end of the experiment (same animals as in Figure 13).

Tumor Incidence

Tumor incidence in the two groups is listed in Table 7. For animals treated with DEN and kept in air, we found a 58% incidence of lung tumors, a 100% incidence of tracheal and nasal tumors, and a 37% incidence of liver tumors. In the DEN and O_2 group, quite a few animals died early during the experiment. Thus, for these animals, there may have been insufficient time for full tumor expression caused by the DEN treatment. Therefore, Table 7 lists the tumor incidence for only those animals that survived for 16 weeks or longer. The animals exposed to hyperoxia had no lung tumors and had significantly fewer tumors in the nasal cavity, and the liver (both hepatocellular carcinomas and adenomas) than the animals kept in air. However, the incidence of tracheal tumors was comparable in the two groups, as were signs of toxic liver damage caused by the DEN and the incidence of bronchopneumonic foci.

DISCUSSION

The experiments described in this report were designed to test a specific hypothesis. Previous work has shown that tumors expressing neuroendocrine markers develop within a short time and with high incidence in hamsters exposed to a carcinogenic dose of DEN and a hyperoxic environment (Schuller et al. 1988). Transformation of preexisting alveolar type II cells into cells with neuroendocrine function appears to be a key element in the development of such tumors (Schuller 1988; Schuller et al. 1988, 1990b; Nylen et al. 1990). The available data on type II alveolar cell proliferation caused in the lungs of laboratory animals by the two air pollutants justified testing the hypothesis that a com-

Table 7. Incidence of Histopathological Lesions in Hamsters Exposed to Hyperoxia[a]

Location (Lesion)	Treatment Groups: DEN + Room Air (n = 19)[b]	Treatment Groups: DEN + O_2 (n = 11)[c]
Bronchiolar hyperplasia	0 (0%)	4 (36%)
Lung (adenomas)	11 (58%)	0 (0%)[d]
Trachea (papillary polyps)	19 (100%)	11 (100%)
Nose (adenomas and carcinomas)	19 (100%)	7 (64%)[d]
Liver (hepatocellular carcinomas)	7 (37%)	0 (0%)[d]
Liver (hepatocellular adenomas)	10 (53%)	1 (9%)[d]
Liver (necrosis and bile duct proliferation)	19 (100%)	11 (100%)
Bronchopneumonia/abscesses	3 (16%)	3 (27%)

[a] Animals were injected with DEN (20 mg/kg) twice a week for 20 weeks and kept in either air or an atmosphere of 65% oxygen. Animals were killed 24 to 32 weeks after the beginning of the experiment.

[b] These animals are referred to in the text as "positive controls" in the NO_2 experiment. They were housed at ITEH. Originally 20 animals; tissues of one animal were lost to cannibalism.

[c] Only those animals that survived longer than 16 weeks are included in this calculation.

[d] Significantly different ($p < 0.05$) compared with DEN + air group.

bined exposure to either oxidant pollutant and DEN would favor the development of neuroendocrine lung tumors in hamsters. The alternative hypothesis was that the two air pollutants might provide an overall, nonspecific cocarcinogenic or promoting stimulus for the development of tumors in the respiratory tract of hamsters.

We did not find evidence to support either hypothesis. Also, under the experimental conditions used in this study, which differed in several aspects from the experimental design of our previous hyperoxia and DEN hamster studies (Schuller et al. 1988, 1990a), we induced significantly fewer lung tumors, and the neuroendocrine nature of such lesions could not be established, due to overfixation of tissues. The outcome of the two experiments therefore needs some analysis.

CONSISTENCY OF INTRAEXPERIMENTAL FINDINGS

It is important to compare the findings made in the O_3 and NO_2 experiments for internal consistencies. The two experiments were conducted within a two-year time period. They involved two different batches of DEN from the same supplier and three different shipments of outbred Syrian golden hamsters, received from the same source. The preparation of DEN solutions and procedures followed for treatment and autopsy of the animals were performed by the same personnel. In the O_3 experiment, animals received DEN injections for 16 weeks, whereas in the NO_2 study, animals received DEN for 24 weeks. Despite this somewhat different treatment regimen, the two control groups, i.e., the animals treated with DEN, kept in filtered air, and killed at the end of the experiment, had a remarkably similar incidence of tumors in the lung, bronchi, trachea, and liver, and in signs of hepatic DEN toxicity (Table 8). Tumor production thus was consistent in animals that were treated with DEN, kept in wire-mesh cages, and exposed to filtered air in the same location (inhalation facility of the CRPRC). On the other hand, quite different tumor incidences were found in a group of hamsters treated with DEN and kept on bedding in conventional shoe-box cages (Table 7, DEN and air).

CONSISTENCY OF INTEREXPERIMENTAL FINDINGS

The overall incidence of lung tumors in hamsters treated with DEN and kept in filtered air was lower than expected. In several previous studies, lung tumor incidence in hamsters treated with DEN had been 50% to 60% (Schuller and McMahon 1985; Schuller et al. 1988; Schuller et al. 1990a,b). Our apparent inability to achieve a similarly high incidence raised some concern. A possible explanation for the discrepancy was discovered in the analysis of the NO_2 study. Hamsters of the same shipment, originally distributed at random to six different treatment groups and handled throughout the experiment by the same personnel, were eventually housed under different conditions. Animals assigned to the NO_2 experiment were kept in wire-mesh cages at the inhalation facility of the CRPRC. Animals assigned to the hyperoxia experiment were kept in conventional plastic shoe-box cages on hardwood bedding at the animal facility of the ITEH. When data for tumor incidence are compared between animals exposed to DEN and air at

Table 8. Tumor Incidence in Hamsters Treated with Diethylnitrosamine, Exposed to Filtered Air, and Kept in Wire-Mesh Cages[a]

Location (Lesion)	Treatment Groups	
	DEN + Air (O_3 Experiment) (n = 20)	DEN + Air (NO_2 Experiment) (n = 19)
Lung (adenomas)	6 (30%)	5 (26%)
Bronchi (papillary polyps)	0 (0%)	3 (16%)
Trachea (papillary polyps)	19 (95%)	19 (100%)
Liver (hepatocellular carcinomas)	1 (5%)	3 (16%)
Liver (hepatocellular adenomas)	1 (5%)	1 (5%)
Liver (necrosis and bile duct proliferation)	20 (100%)	18 (95%)

[a] Animals listed in this table are the filtered air controls from the O_3 and NO_2 experiments, and were killed 24 to 32 weeks after the beginning of treatment (data from Tables 4 and 6). These animals were kept in wire-mesh cages in Hinners-type chambers at the inhalation facility of the CRPRC while being given subcutaneous injections of DEN (20 mg/kg) twice a week. The two experiments were conducted one year apart.

the CRPRC and animals exposed to DEN and air at the ITEH, differences become obvious (Table 9). Significantly fewer lung and liver tumors were found in the animals housed in the wire-mesh cages.

This finding was in all likelihood more than spurious. Analysis of the literature shows that on occasion animals housed in wire-mesh cages do tend to develop fewer lung tumors. In a study involving several thousand mice, an effect of caging on the development of spontaneous lung tumors was reported (Nettesheim et al. 1970). Overall lung tumor incidence in C57BL/6 mice kept in wire-mesh cages was 3.0%, as opposed to 5.5% in animals housed in conventional shoe-box cages. At that time, this was explained by the fact that mice kept in shoe-box cages survived for an average of 26 weeks longer than animals kept in wire-mesh cages. In a later, unpublished experiment, we found that strain A mice, given a single injection of 20 mg/kg of 3-methylcholanthrene developed an average of 27 tumors per lung within 16 weeks when kept in shoe-box cages. In animals housed in wire-mesh cages, only 16 tumors per lung developed (Table 10). This experiment showed that tumor formation may be suppressed by wire-mesh caging even if the life span of the animals is not a factor.

Tumor development thus may be affected by housing conditions, among other factors. Animals kept in inhalation chambers in wire-bottom cages may develop fewer tumors than animals undergoing the same treatment with a systemic carcinogen while being kept in conventional housing on bedding. The observations further point out the role of general animal husbandry in carcinogenesis studies. The possibility needs to be considered that it may be inappropriate to select carcinogen doses for an inhalation study, designed to test a cocarcinogenic effect, based on results obtained in previous studies in which animals were housed on bedding. However, it also should be noted that in the present study, housing appeared only to affect incidence of

Table 9. Comparison of Tumor Incidence in Animals Kept in Wire-Mesh Cages and Animals Kept in Shoe-Box Cages[a]

Location (Lesion)	Shoe-Box Cages (n = 19)	Wire-Mesh Cages (n = 19)
Lung (adenomas)	11 (58%)	5 (26%)[b]
Bronchi (papillary polyps)	1 (6%)	3 (15%)
Trachea (papillary polyps)	19 (100%)	19 (100%)
Nose (adenomas and carcinomas)	19 (100%)	16 (80%)
Liver (hepatocellular carcinomas)	7 (37%)	3 (16%)
Liver (hepatocellular adenomas)	10 (53%)	1 (5%)[b]
Liver (necrosis and bile duct proliferation)	20 (100%)	18 (96%)

[a] All hamsters were received on the same date and injected subcutaneously with DEN (20 mg/kg) twice a week for 20 to 24 weeks; they were killed 24 to 32 weeks after the beginning of the experiment. Animals kept in shoe-box cages are the same animals as in Table 7 (DEN + air) and were housed at ITEH. Animals kept in wire-mesh cages are the same ones as in Table 6 (DEN + air) and were housed at CRPRC.

[b] Significantly different ($p < 0.05$) compared with animals kept in shoe-box cages.

Table 10. Influence of Housing on Lung Tumor Development in Strain A Mice[a]

	n	Housing Conditions	Number of Tumors per Lung (mean ± SEM)
Group A	26	Shoe-box cages	26.7 ± 2.6
Group B	23	Wire-mesh cages	15.9 ± 2.4[b]

[a] Unpublished data. All animals were received in the same shipment. When animals were five to six weeks old, they were treated with one intraperitoneal injection of 3-methylcholanthrene (20 mg/kg). Half of the animals were kept in conventional plastic shoe-box cages with hardwood bedding (group A). The other half of the animals were kept in wire-mesh cages for 12 weeks and exposed for six hours a day, five days a week, to filtered air in an inhalation chamber (group B). After 12 weeks, the animals from group B were returned to shoe-box cages. All animals were killed 16 weeks after injection of the carcinogen, and the lung tumors were counted using the method of Witschi and Morse (1983).

[b] Significantly lower ($p < 0.05$) compared with animals kept in shoe-box cages.

lung and liver tumors. In the trachea or in the nasal cavity, similar tumor incidences were found, regardless of housing conditions.

Having offered a tentative explanation for the lower-than-expected tumor incidence in the inhalation experiments, we now can compare tumor incidence in the one group kept in plastic cages on conventional bedding and treated repeatedly with DEN with the results from similar studies conducted elsewhere. Table 11 shows that our tumor data compare favorably with observations made in outbred Syrian golden hamsters injected with DEN and conducted in 3 different locations during the past 8 years (Schuller and McMahon 1985; Schuller et al. 1988, 1990a).

AIR POLLUTANTS AND ENHANCEMENT OF LUNG CANCER

It has been suspected for a long time that air pollutants may enhance the development of lung cancer. Ozone, NO_2, and sulfur dioxide (SO_2) are usually the agents listed as being most likely to have such an effect. Other inhalants include various aldehydes and gaseous or particulate pollutants that are commonly encountered in polluted urban atmospheres.

The available experimental evidence was reviewed in detail by Nettesheim and Schreiber (1975) and by Nettesheim and colleagues (1981). In the 1981 review, the authors came to the conclusion that there was little experimental evidence that gaseous air pollutants would increase the risk of developing lung cancer. Noteworthy in this review is the mention of a large unpublished study with over 1,000 hamsters. No evidence of carcinogenic or cocarcinogenic effects was found for three reactive gases: formaldehyde, acrolein, and NO_2, alone or in combination. The only air pollutant suspected as a possible exception was SO_2. This particular conclusion was based on findings of Laskin and coworkers (1976), in which a synergistic interaction between intratracheally instilled benzo[*a*]pyrene and inhaled SO_2 was described. Although they did not include detailed data on tumor incidence, the findings of Pauluhn and associates (1985) seemed to confirm the original observation of Laskin and colleagues (1976). However, a thorough reevaluation of the experiments conducted by Laskin and coworkers failed to provide evidence that SO_2 would enhance tumor development in the respiratory tract (Gunnison et al. 1988). The experiments may have been somewhat inconclusive because of the high incidence of lung tumors (greater than 90%) in the animals treated with carcinogen alone. This situation obviates the detection of eventual promoting effects.

A study designed to examine possible cocarcinogenic effects of a mixture of SO_2 and NO_2 in DEN-induced respiratory tract tumors in hamsters suggested a positive effect of

Table 11. Tumor Incidence in Hamsters Treated with Diethylnitrosamine and Exposed to Air (Historical Controls)[a]

	Tumor Location and Incidence			
Reference	Lung	Trachea	Nose	Liver
Schuller and McMahon 1985	6/10 (60%)	10/10 (100%)	ND[b]	ND
Schuller et al. 1988	7/12 (60%)	ND	ND	ND
Schuller et al. 1990a	6/12 (50%)	10/12 (84%)	4/12 (32%)	6/12 (50%)
This study	11/19 (58%)	19/19 (100%)	19/19 (100%)	7/19 (37%)

[a] This table lists the number of tumor-bearing animals per total number of animals per group in different experiments conducted over a six-year period. All animals were treated for 20 to 24 weeks with subcutaneous injections of DEN (17 to 20 mg/kg) twice a week. They also were kept in shoe-box cages.

[b] ND = not done.

exposure (Heinrich et al. 1982). However, these findings could not be duplicated later (Heinrich et al. 1989). Equally inconclusive are studies with other air pollutants, particularly acetaldehyde and tobacco smoke. Several of these studies yielded conflicting results. In hamsters treated with DEN, acetaldehyde did not enhance tumor development, and, in one experimental group, even seemed to inhibit tumor development (Feron and Kruysse 1977; Feron et al. 1982). Formaldehyde appeared to enhance tumor multiplicity in the upper respiratory tract of hamsters treated with DEN (Dalbey 1982). Tobacco smoke had been reported to be a respiratory cocarcinogen in early studies with hamsters (Dontenwill 1964; Wynder and Hoffmann 1967; Karbe and Kostner 1974), but no evidence for a cocarcinogenic effect of tobacco smoke inhalation was found in a more recent study (Hecht et al. 1983). On the other hand, tobacco smoke and some other inhalants seem to enhance tumor development in the lungs of mice (Keast et al. 1985).

As far as the two oxidant air pollutants O_3 and NO_2 are concerned, results also remain conflicting. The evidence has been recently reviewed (Witschi 1988). Depending upon experimental design, O_3 either enhances (Hassett et al. 1985) or mitigates (Last et al. 1987) the development of lung tumors in mice. A recent study examined a possible cocarcinogenic effect of low levels of NO_2 in the lungs of rats treated with the systemic carcinogen *N*-bis-(2-hydroxypropyl)-nitrosamine (Ichinose et al. 1991). An apparent increase in tumor incidence was found in this study. The difference from the control group was, however, statistically not significant (2.5% lung tumor incidence in carcinogen-treated controls vs. 12.5% lung tumor incidence in rats given *N*-bis-(2-hydroxypropyl)-nitrosamine and exposed for 17 months to 4 ppm NO_2). Also conflicting are results on the effects of air pollutants on the formation of cancer cell colonies in the lungs ("metastases") following intravenous injection of cancer cells into the tail veins of mice. Both O_3 and NO_2 have been found to enhance or inhibit pulmonary metastasis. The outcome of the experiments apparently depends very much on experimental design, e.g., the temporal relationship between intravenous injection of tumor cells and the beginning or end of the exposure to the pollutant (Richters and Kuraitis 1983; Kobayashi et al. 1987; Weinbaum et al. 1987; Richters 1988). Exposure to the air pollutant prior to tumor cell injection usually increases the number of metastatic colonies in the lungs. Oxidant exposure following intravenous cell injection often has the opposite effect.

In summary, there is at present no clear picture regarding whether air pollutants enhance or possibly suppress pulmonary carcinogenesis. Evidence for either of the two possibilities is available. Unfortunately, as seen below, the pilot experiments described in the present report also fail to clarify further this important question.

INTERPRETATION OF THE RESULTS OF THE PRESENT STUDY

We did not obtain any evidence for a direct carcinogenic action of O_3 or NO_2. In the animals exposed to the pollutants alone, no lung tumors developed, although O_3 produced marked bronchiolar hyperplasia. However, no definite conclusions may be drawn from this observation. The experiments were terminated much earlier than is usual for a conventional bioassay for carcinogenesis.

In animals treated with a carcinogen and exposed to an air pollutant, we did not find evidence for cocarcinogenesis. If anything, the two oxidant pollutants appeared to inhibit the development of certain respiratory tract tumors or at least to delay their development. Papillomas in the bronchi or the trachea have been found in virtually every experiment in which hamsters were treated with a carcinogenic dose of DEN (Herrold 1964; Feron et al. 1982; Schuller and McMahon 1985; Heinrich et al. 1989). Incidence of tracheal tumors is often close to 100%. The most significant finding in our experiment was that in groups of animals exposed to O_3 or NO_2, the incidence of tracheal tumors was significantly lower in animals killed immediately after the termination of oxidant exposure. The average incidence of tracheal tumors in the two groups combined was 30%, as opposed to a nearly 100% incidence in the animals kept in air. Once the animals had been removed from the oxidant atmosphere and were no longer treated with DEN, the incidence of tracheal papillomas increased. Two months later, the tumor incidence was similar to that found in the control groups.

Similar observations were made for tumors in the nasal cavity, which are also readily induced by DEN treatment. Nitrogen dioxide inhibited the development of nasal tumors as long as the animals were exposed to the pollutant. Once the exposure ended, nasal tumors were found in similar incidence in animals exposed to NO_2 and animals exposed to air. Unfortunately, due to a technical problem, data on nasal tumors are not available for the O_3 experiment.

A somewhat different pattern was found with regard to lung tumors. In animals exposed to O_3, a few lung tumors developed. At both 16 weeks and 24 weeks, the number of tumors in the animals exposed to O_3 was lower than in the animals kept in air; however, due to the low overall number of animals bearing tumors, the difference was statistically not significant. No positive evidence was obtained that any of the tumors in the animals exposed to O_3 displayed neu-

roendocrine differentiation. The effect of NO_2 appeared to be less equivocal; no lung tumors were found in the animals exposed to NO_2 at any time. The data seem to agree with the observation by Henschler and Ross (1966) that exposure of mice to NO_2 seemed to prevent rather than to enhance lung tumor development. These observations are at variance with the recent findings of Ichinose and colleagues (1991), who reported a suggestive, but not a significant, enhancing effect of exposure to NO_2.

A noteworthy observation in the animals exposed to O_3 was a consistent proliferation of bronchiolar epithelial cells, both in animals treated with DEN and animals treated with NaCl. Pronounced proliferation of pulmonary neuroendocrine cells is a hallmark of DEN treatment (Reznik-Schuller 1976a,b) and also has been described in the lungs of monkeys exposed to O_3 (Castleman et al. 1980). However, special immunostains did not reveal the presence of bombesin or calcitonin in the foci of hyperplastic bronchiolar cells. This prohibits a final diagnosis. Reasons for this failure, including the absence of a positive reaction for bombesin or calcitonin in all slides examined remain unclear. The possibility must be considered that the tissues in this experiment had been kept too long in neutral buffered formalin (for logistic reasons) and were not, as in other experiments, embedded in paraffin after a comparatively short fixation period with a different fixative (Linnoila et al. 1984; Pack et al. 1986). Others have commented on the difficulties in using bombesin-like immunoreactivity as a marker for tumors of neuroendocrine cell origin (Hirsch et al. 1990). The absence of hyperplasia in cells resembling neuroendocrine cells in the animals exposed to NO_2 would agree with the observations made in hamster lungs by Palisano and Kleinerman (1980).

In the experiments on the tumor-modulating effects of O_2, animals treated with DEN and hyperoxia had a lung tumor incidence that was significantly lower than in comparable studies (Schuller et al. 1988): 20% at 12 weeks in the positive controls for the O_3 study and 0% at 24 to 32 weeks in the positive controls for the NO_2 study (Table 7). The reasons for this are not clear but may include different animal husbandry, or different DEN treatment (identical total dose of DEN administered, but at half the concentration of that used in previous studies (Schuller and McMahon 1985; Schuller et al. 1988). Another factor may be that the recovery times from the discontinuation of treatment until the animals were killed were different from those in the above cited studies. An inhibition of tumorigenesis in the liver by hyperoxia correlates well with previously reported observations (Schuller et al. 1990a), whereas the decrease in nasal cavity tumors seen in hamsters exposed to hyperoxia was not found in previously published studies.

In both experiments, the occurrence of bronchopneumonic foci in the lungs of animals in the different treatment groups ranged from 0% to 27%. On the average, 10% of all animals had bronchopneumonic foci. The role of respiratory tract infection in the modulation of lung tumor development in laboratory animals has been discussed (Nettesheim et al. 1981). In rats treated with a nitrosamine that is carcinogenic to lungs, the presence of chronic murine pneumonia enhanced respiratory tract carcinogenesis (Schreiber et al. 1972). Thus, one might anticipate similar findings with hamsters treated with DEN. In the experiments listed in Tables 3 through 7, a total of 24 of all the animals treated with DEN had histopathological signs of bronchopneumonia. Of these 24 animals, four (17%) developed lung tumors. Accordingly, 165 animals treated with DEN were pneumonia-free, and among these animals, 26 (16%) had lung tumors. We cannot conclude from our experiments that respiratory tract infection would modulate nitrosamine-induced carcinogenesis in hamster lungs.

Finally, in comparing published tumor incidences in hamsters injected subcutaneously with DEN while being maintained in ambient air (Table 11), it is apparent that a significant incidence of liver tumors was described only in this report and in one other published paper (Schuller et al. 1990a); no liver tumors were observed in the older studies (Schuller and McMahon 1985; Schuller et al. 1988). Reasons for this shift in tumor distribution are unknown at the present time, although a genetically linked change in organ susceptibility seems possible in hamsters descended from two original colonies at Charles River in 1949 and 1951 and operated as a closed colony since that time.

CONCLUSIONS

It seems quite certain that the development of lung cancer in humans may be modulated by multiple factors. Asbestos workers or uranium miners who smoke have a higher risk of developing lung cancer than nonsmokers. Thus, it seems reasonable to consider interactions between different carcinogenic stimuli as an important element in the pathogenesis of lung cancer development. Many experimental studies in animals also show that exposure to a lung-specific carcinogen and an apparently otherwise noncarcinogenic stimulus may enhance lung tumor development. However, other animal studies show no effect or, on occasion, even a reduction in lung tumors from exposure to a noncarcinogenic inhalant.

At present, there is no known common mechanism to explain why, on occasion, tumor development in the respiratory tract may be enhanced. Current thinking on the patho-

genesis of many cancers attributes a substantial role to cell proliferation (Butterworth et al. 1991). In organs such as the liver or kidney, cell proliferation induced by a variety of stimuli is thought to be a contributing factor to tumor development. For this reason, the validity of conventional carcinogenesis bioassays has been questioned (Ames and Gold 1990), although this view has not gone unchallenged (Weinstein 1991). With regard to enhanced tumor development in the respiratory tract, it is tempting to speculate that enhanced turnover and proliferation of the cells lining the airways enhances respiratory tract carcinogenesis (Little et al. 1978; Keenan et al. 1989a,b). Unfortunately, it is not possible to make such a general statement at present. Data reviewed previously show that agents known to produce damage and repair to the epithelium lining the respiratory tract on occasion do enhance tumor development, whereas under other experimental circumstances they have no effect or may even inhibit tumor development (Nettesheim et al. 1981; Witschi 1988, 1991). The experiments described in this report do not confirm the hypothesis that increased cell turnover brought about by O_3 or NO_2 would enhance tumor development in hamster lungs. Rather, they suggest that, on occasion, the two gases may inhibit tumor formation.

The clearest evidence for such a possibility was obtained in the experiment in which the combined effects of NO_2 and DEN were examined. Although NO_2 usually evokes a hyperplastic response in the respiratory tract, its effects on tumor development were inhibitory rather than stimulatory. As far as O_3 is concerned, the data are less clear-cut. Nevertheless they show that O_3 does not increase the tumor yield in the lung. Ozone, according to its chemical reactivity, certainly has the potential to act as a carcinogen (Pryor 1991). In vitro studies suggest that O_3 may act as a cocarcinogen (Borek et al. 1986, 1988), as does at least one in vivo study with mice (Hassett et al. 1985). In the present study, no evidence for a potential cocarcinogenic action of O_3 was obtained. The data are in agreement with the observations of Thomassen and colleagues (1991), who did not find evidence for a cocarcinogenic effect of O_3 in animals exposed to the pollutant in vivo. The most conflicting results were obtained with O_2. In the lungs of several species, O_2 produces cell hyperplasia after acute and chronic exposure (Adamson and Bowden 1974; Lindenschmidt et al. 1986b; Tryka et al. 1986). Oxygen has been found to inhibit tumor development in the lungs of mice and rats (Lindenschmidt et al. 1986a,b), whereas it enhances tumor development in hamsters (Schuller et al. 1988, 1990a,b).

In summary, the results presented in this report, for the time being, do not allow the drawing of definite conclusions. The role of two common air pollutants as possible additional risks in the etiology of lung cancer in animals or humans remains uncertain.

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ABBREVIATIONS

APUD	amine precursor uptake decarboxylase (cells)
CRPRC	California Regional Primate Research Center
DEN	diethylnitrosamine
ITEH	Institute of Toxicology and Environmental Health
N_2	nitrogen
NaCl	sodium chloride
NO_2	nitrogen dioxide
O_2	oxygen
O_3	ozone
SO_2	sulfur dioxide

INTRODUCTION

Ozone and nitrogen dioxide are highly reactive oxidant gases that are major components of urban air pollution. When inhaled, these pollutants can induce a range of effects in biological systems. One major unanswered question is whether exposure to oxidant air pollutants causes or contributes to the development of lung cancer. In 1987, the Health Effects Institute (HEI) began a small research program to address this issue. The HEI funded three investigators to examine the carcinogenic potential of ozone. Drs. Carmia Borek and David Thomassen tested the ability of ozone to transform cells in vitro; Dr. Kenneth Donaldson investigated the ability of inflammatory leukocytes from ozone-exposed rats to injure pulmonary epithelial cells. (The results of two of these studies have been published in HEI Research Reports [Donaldson et al. 1991; Thomassen et al. 1992]). At the same time, the National Toxicology Program initiated a major animal bioassay in which two species of animals (Fischer-344 rats and B6C3F1 mice) were exposed to ozone alone and ozone in combination with a known lung carcinogen. In 1988, HEI funded a fourth study in its ozone carcinogenicity program. In that study, which forms the basis of this report, the investigators proposed to use a newly developed animal model for neuroendocrine lung cancer to determine whether exposure to oxidant pollutants modulates the development of these highly malignant tumors. The proposal for this project originated from HEI's Request for Preliminary Applications (RFPA) process.

The HEI occasionally issues RFPAs that solicit brief proposals on topics outside of those defined by the more focused Requests for Applications. In response to RFPA 87–4, which was issued in the summer of 1987, Dr. Hanspeter Witschi, from the University of California at Davis, submitted a preliminary application for a project entitled "Air Pollutants and Neuroendocrine Lung Cancer." The Health Research Committee encouraged Dr. Witschi to submit a full proposal, which he did in April 1988. The 18-month project began in January 1989, and total expenditures were $237,954. The Investigators' Report was submitted in March 1992 and was accepted for publication by the Health Review Committee in July 1992. During the review of the Investigators' Report, the Review Committee and the investigators had an opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. This Commentary is intended to place the Investigators' Report in a broad scientific context, to discuss the strengths and weaknesses of the study, and to address the public health implications of the findings.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a), (b)(1), (g), and (h) and Sections 207(c)(4), (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 NAAQS have been established for six air pollutants: nitrogen dioxide, ozone, carbon monoxide, particulate matter, lead, and sulfur dioxide. These pollutants, which are primary and secondary emission products from transportation and industrial sources, are expected to cause health effects in people who receive sufficiently high exposures. A recent survey indicates that approximately 70 million people live in areas of the United States where at least one air quality standard—most frequently the ozone standard—is exceeded (U.S. Environmental Protection Agency 1992). The NAAQS are set as threshold concentrations, that is, concentrations of a specific pollutant below which no adverse human health effects are expected. They are set by the EPA Administrator and are based on a detailed evaluation of the available scientific literature, with a scheduled review at five-year intervals to incorporate relevant new information. Because determining appropriate standards for emissions of oxidants and their precursors depends, in part, on an assessment of the health risks that they present, research into their health effects is essential to the informed regulatory decision-making required by the Clean Air Act.

SCIENTIFIC BACKGROUND

OXIDANT AIR POLLUTANTS

The air we breathe contains a number of pollutants, some of which are derived from the combustion of fossil fuels and the atmospheric transformation of these combustion prod-

ucts. Ozone and nitrogen dioxide are two oxidant air pollutants that are formed from combustion products. Nitrogen dioxide is derived primarily from nitric oxide, a combustion product from industrial operations, the generation of electric power, and motor vehicles (Finlayson-Pitts and Pitts 1986). In the atmosphere, nitric oxide is oxidized to form nitrogen dioxide (Urone 1976). A minor amount (between 5% and 10%) of the total oxides of nitrogen emitted by combustion processes is already in the form of nitrogen dioxide (U.S. Environmental Protection Agency 1982).

The current NAAQS for nitrogen dioxide is 0.053 parts per million (ppm)*, as an annual arithmetic mean concentration; this means that the average of measurements taken over a 12-month period at any given site should not exceed 0.053 ppm. In 1991, the annual mean concentrations of nitrogen dioxide ranged from slightly below 0.01 ppm at the less polluted measuring sites to approximately 0.05 ppm at the sites in highly polluted areas; the median value was slightly greater than 0.02 ppm (U.S. Environmental Protection Agency 1992). Nitrogen dioxide also is an important indoor air pollutant. Heating and cooking appliances that utilize natural gas, kerosene, coal, and wood are potential sources of indoor nitrogen dioxide. Short-term peaks of 0.2 ppm or higher can be produced when unvented gas stoves or space heaters fueled by gas or kerosene are used in an enclosed space (Leaderer et al. 1986; Samet et al.1987).

Ozone is a highly reactive gas and a major constituent of photochemical smog. Ozone is not emitted directly into the atmosphere, but is formed by complex photochemical reactions between nitrogen oxides and volatile organic compounds in the presence of sunlight. Both volatile organic compounds and nitrogen oxides are emitted from mobile and stationary sources (U.S. Environmental Protection Agency 1992). The current NAAQS for ozone is 0.12 ppm, a level not to be exceeded for more than one hour once per year. Summertime peak hourly levels of ozone range from 0.05 ppm in rural sections of the United States to as high as 0.3 ppm in some urban areas. The EPA estimated that in 1991, over 69 million people lived in counties in which the levels of ozone exceeded the NAAQS (U.S. Environmental Protection Agency 1992).

Data from epidemiological, clinical, and animal studies indicate that exposure to sufficiently high concentrations of either ozone (reviewed by the U.S. Environmental Protection Agency 1986, 1988a,b; Lippmann 1989) or nitrogen dioxide (Morrow 1984; Samet et al. 1987; reviewed by the U.S. Environmental Protection Agency 1993) can damage tissues or cells and cause adverse effects on the respiratory system. The effects of exposure to either pollutant depend on the concentration of the gas and the duration of the exposure. In some exercising human subjects, short-term exposures to the same levels of ozone that occur in ambient settings cause a transient decrement in respiratory function measurements (Folinsbee et al. 1988) and an influx of inflammatory cells into the alveoli (Koren et al. 1989; Devlin et al. 1991). In animals, ozone exposure has been shown to damage type I epithelial cells that line the alveoli (Barry et al. 1985; Chang et al. 1991) and to cause proliferation of type II cells (Chang et al. 1991).

Short-term exposures to extremely high concentrations of nitrogen dioxide (in excess of 50 to 100 ppm) cause severe damage to lung tissue and can lead to fatal pulmonary edema (Muelenbelt and Sangster 1990). The consequences of inhaling nitrogen dioxide at concentrations that occur in typical indoor and outdoor settings (0.01 to 0.2 ppm) or even in the high ambient exposure range (0.2 to 1 ppm) have been more difficult to characterize. In animals, long-term exposures to concentrations of nitrogen dioxide in excess of 1 ppm resulted in permanent damage to the epithelium of the centriacinar region of the lung. At lower concentrations (approximately 0.5 ppm), the morphologic effects have been subtle and often reversible (Kubota et al. 1987).

Because ozone and nitrogen dioxide are highly reactive gases and cause damage to the cells and tissues of the lungs when inhaled at sufficiently high concentrations, there is speculation that these pollutants could contribute to the development of lung cancer. Although cigarette smoking is the major cause of lung cancer, inhaled air pollutants also may be factors in the pathogenesis of the disease. The following section discusses the animal models that have been used to test this hypothesis, with a focus on the hamster model of neuroendocrine lung cancer.

NEUROENDOCRINE CELL LUNG CANCER

Carcinomas of epithelial cells that line the conducting airways of the lungs are classified according to histologic characteristics. Carcinomas are classified as either squamous cell carcinoma, adenocarcinoma, large cell carcinoma, small cell carcinoma, or combinations thereof (Robbins and Kumar 1987). Small cell lung carcinoma, which represents approximately 25% of human lung cancers, is a highly malignant form of lung cancer and is strongly associated with cigarette smoking. Small cell lung carcinomas are composed primarily of cells with neuroendocrine characteristics.

Neuroendocrine cells are found as single cells or in clusters, termed neuroepithelial bodies, along the respiratory tract (reviewed by Becker and Gazdar 1983, and by Gould et al. 1983). The characteristic feature of neuroendocrine cells is the large number of secretion granules located

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

in the bottom portion of the cells. These granules contain several polypeptides, such as mammalian bombesin (also called gastrin releasing peptide), calcitonin, serotonin, neuro-specific enolase, and leu-enkephalin. These substances can regulate the diameter of airways and blood vessels and can induce cell division. Neuroendocrine cells constitute a small percentage of the lung epithelial cells in human adults, but are much more numerous in the airways of fetuses and newborns. Although the function of neuroendocrine cells is not well understood, their numbers and secretory activity change in response to various environmental stimuli. Both high (hyperoxia) and low (hypoxia) levels of oxygen in the inspired air cause an increase in the number of these cells. The number of cells, their distribution, and their content of mitogenic peptides during fetal gestation suggest that these cells also participate in lung development (Sunday et al. 1990). Because of similarities between neuroendocrine cells and the cells found in small cell lung carcinomas, it is presumed, although not proven, that the neuroendocrine cell is the cell of origin in these tumors. Alternatively, the expression of neuroendocrine characteristics by these tumor cells could occur during tumor development from a pluripotent stem cell.

Studying small cell lung carcinomas has been difficult because of an absence of good experimental models. In 1988, Drs. Schuller, Becker, and Witschi reported that they had developed a model for inducing neuroendocrine neoplasms in the lungs of Syrian golden hamsters (Schuller et al. 1988). In this system, animals were exposed continuously to hyperoxia (70% oxygen) for eight weeks, while receiving subcutaneous injections of the carcinogen, diethylnitrosamine (DEN) twice each week. The lesions that developed had numerous cytoplasmic granules when examined by electron microscopy, were positive for markers of neuroendocrine cells, such as calcitonin and bombesin, and were diagnosed as neuroendocrine lung cancers. In a subsequent study, Schuller and colleagues (1990) reported that, although the cells in these tumors had neuroendocrine cell characteristics, they might have been derived from alveolar type II epithelial cells. In their model of neuroendocrine cell neoplasia, the investigators speculated that exposure to hyperoxia caused proliferation of the neuroendocrine cells and that these dividing cells might be more susceptible to carcinogenic stimuli than nondividing cells. Cell proliferation was one of the critical features of their model.

MULTISTAGE CARCINOGENESIS

Carcinogenesis is a multistage process that includes genetic and nongenetic changes in target cells (Harris 1991; International Agency for Research on Cancer 1991). Tumors develop as a result of changes in normal cellular processes that control growth and differentiation. Numerous chemicals, as well as viruses and physical agents, have been implicated as carcinogens. Initiation, the first step in carcinogenesis, may occur when a carcinogen reacts with DNA, causing genetic changes. Subsequent replication of initiated cells can "fix" and propagate DNA lesions in succeeding cell generations. Genetically damaged cells, which persist in tissues after an initiating agent has been removed, can give rise to a tumor during the ensuing stages of promotion and progression.

The importance of cell proliferation in the promotional stage of carcinogenesis has been highlighted recently (Ames and Gold 1990; Cohen and Ellwein 1990; Preston-Martin et al. 1990). Although several lines of evidence support the hypothesis that cell proliferation contributes to some forms of cancer, the role and necessity of cellular proliferation in carcinogenesis is not clear (Weinstein 1991). It is reasonable to investigate whether specific agents that stimulate cell proliferation, such as oxidant gases, can enhance tumor development.

OXIDANTS AND LUNG CANCER

Ozone is a highly reactive molecule that theoretically could influence the development of lung tumors by either genotoxic or nongenotoxic mechanisms. Ozone, by itself, has not been shown to react with DNA in vivo. However, it is thought that the products from the decomposition of ozone (such as oxygen free radicals) or the products from ozone's reaction with cell membranes (such as lipid peroxides) can damage DNA (reviewed by Mustafa 1990, and by Pryor 1991). Ozone inhalation also elicits an influx of inflammatory cells into the lungs (Devlin 1991; Donaldson et al. 1991). These inflammatory cells are an additional source of oxygen free radicals. Ozone exposure also induces cellular proliferation. Hyperplasia of alveolar type II cells (Evans et al. 1976; Barry et al. 1985), cuboidal bronchiolar cells (Fujinaka et al. 1985), and neuroendocrine cells (Castleman et al. 1980) has been reported in animals exposed to ozone. Thus, a cascade of reactions initiated by ozone may damage DNA, induce cell proliferation, or both.

Evidence that ozone might play a role in tumor development is suggested by the findings that, under some experimental conditions, inhalation of ozone induces benign lung tumors in A/J mice, a strain that exhibits a high spontaneous incidence of lung tumors. The incidence of mice with lung tumors has been observed to be significantly higher in A/J mice exposed to 0.5 ppm ozone for six months than in control animals exposed to air alone (Hassett et al. 1985). Furthermore, when Last and coworkers (1987) exposed both A/J and Swiss Webster mice (a strain with a low spon-

taneous incidence of lung tumors) to 0.4 and 0.8 ppm ozone intermittently for 18 weeks, ozone exposure did not change the incidence or frequency of tumors in the Swiss Webster mice. However, the 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.

Both groups of investigators also examined the effect of chronic ozone exposure on the development of chemically induced lung tumors. A/J mice exposed to 0.5 ppm ozone intermittently for six months and injected with the carcinogenic chemical urethane after each exposure developed more lung adenomas than mice treated only with urethane, suggesting that ozone acted as a cocarcinogen (Hassett et al. 1985). However, when urethane treatment preceded the ozone exposure, tumor frequency either was unchanged (Hassett et al. 1985) or decreased (Last et al. 1987), depending on the concentration of ozone. Thus, the results are inconsistent, and it is not possible to conclude from the animal data whether ozone is a carcinogen, a cocarcinogen, or both.

The evidence supporting a role of nitrogen dioxide as a carcinogen also is inconclusive. Six-month exposures of A/J mice to 1 or 5 ppm nitrogen dioxide had no effect on lung tumor development; animals exposed to 10 ppm nitrogen dioxide exhibited a slight increase in the incidence and frequency of lung adenomas, but only when compared with a pooled control group (Adkins et al. 1986). In rats exposed to 4 ppm nitrogen dioxide for 17 months and treated with the carcinogen *N*-bis(2-hydroxypropyl) nitrosamine, tumor incidence was higher in oxidant-exposed animals, but the increase was not statistically significant (Ichinose et al. 1991). However, exposure to high concentrations of this oxidant (in excess of 0.5 ppm) elicited the entry of inflammatory cells into the lungs and caused a hyperplastic response of type II cells (Evans et al. 1972, 1975). Different effects have been reported on neuroendocrine cells. In rats, nitrogen dioxide exposure increased the number of neuroendocrine cells (Kleinerman et al. 1981), but decreased the number of this cell type in exposed hamsters (Palisano and Kleinerman 1980).

Interpreting the significance of the tumor data for both oxidants is difficult because of the high incidence of spontaneous adenomas in the A/J strain of mice (see Witschi 1988). Strains with a high spontaneous incidence of lung tumors are generally more susceptible to the effects of lung carcinogens than strains with low spontaneous tumor rates. In his review, Witschi (1988) noted that, in different oxidant studies, there was a wide variability in tumor incidence and multiplicity in the untreated control groups. For example, in the ozone studies, the positive results may not have been due entirely to a substantial increase in the number of lung adenomas in mice exposed to ozone, but rather to abnormally low tumor incidence in the control mice.

The currently available experimental evidence does not conclusively implicate either ozone or nitrogen dioxide as pulmonary carcinogens. Exposure to either oxidant causes epithelial cell proliferation and therefore has the potential to enhance chemical-induced carcinogenesis. There is some evidence that such exposures may modulate tumorigenesis in animals, but the results and their interpretation are unclear (see Witschi 1988). Further exploration of the carcinogenic or cocarcinogenic potential of these two oxidants is needed. In particular, because of reported effects of nitrogen dioxide on neuroendocrine cell proliferation, it would be helpful to determine whether either of these oxidants mimics the effect of hyperoxia on neuroendocrine cell tumor development.

JUSTIFICATION FOR THE STUDY

Based on their previous work showing the effects of hyperoxia on neuroendocrine cell tumor development, Drs. Witschi and Schuller proposed to determine whether neuroendocrine lung tumors would develop in hamsters treated with DEN and exposed to air pollutants. Their premise was that oxidants stimulate neuroendocrine cell proliferation and might therefore mimic the effects of hyperoxia.

The HEI Research Committee was enthusiastic about the neuroendocrine cell tumor model developed by the investigators and considered Drs. Witschi and Schuller well qualified to conduct the proposed study. After discussions between the Research Committee and the investigators, it was agreed that the investigators would use ozone and nitrogen dioxide as the pollutants and that the study would be limited to determining whether a single dose of either pollutant had any effects on lung tumor development. It was understood that because the concentrations of pollutants were high (15 ppm nitrogen dioxide and 0.8 ppm ozone), the results could not be applied to human risk assessment. The primary objective of the investigation was to establish whether oxidant exposure enhanced DEN-induced lung tumors in the hamster model. Thus, levels of pollutants that are much higher than those found in ambient air were used in this study.

SPECIFIC AIMS AND STUDY DESIGN

The primary objective of this study was to determine whether exposure to either ozone or nitrogen dioxide

Table 1. Experimental Design for the Ozone Experiment

Group	Shipment	*n*	Carcinogen Treatment	Oxidant Exposure	Duration of Treatment and Exposure (weeks)	Necropsy Schedule (weeks)	Housing (caging/local environment/location)
1	1	40	DEN	Air	16/16	16 and 24	Wire-mesh/chamber/CRPRC
2	1	40	DEN	0.8 ppm Ozone	16/16	16 and 24	Wire-mesh/chamber/CRPRC
3	2	20	Saline	Air	16/16	16	Wire-mesh/chamber/CRPRC
4	2	40	Saline	0.8 ppm Ozone	16/16	16 and 24	Wire-mesh/chamber/CRPRC
5	1, 2	10	DEN	65% Oxygen	8/12	12	Shoe-box–type/room/ITEH

modulates the development of chemically induced tumors in the respiratory tract of Syrian golden hamsters. The investigators explored whether either pollutant specifically increases the number of pulmonary neuroendocrine tumors induced by DEN. They conducted two preliminary studies to explore these questions. In the first experiment, animals were injected subcutaneously with 0.2 mL/100 g of body weight of either saline or a 1% solution of DEN (to obtain a dose of 20 mg/kg of body weight) twice weekly and were exposed continuously to 0.8 ppm ozone for 16 weeks. The animals for this experiment were received in two shipments and allocated to different treatment groups. In the second experiment, hamsters were injected with DEN or saline twice weekly and were exposed continuously to 15 ppm nitrogen dioxide for 16 or 24 weeks. For a putative positive control group, the investigators injected animals with DEN and exposed them to 65% oxygen. Dr. Witschi supervised the exposures of the animals at the University of California at Davis and Drs. Schuller and Breider conducted the histopathologic examination of tissues at the University of Tennessee. (Dr. Breider only examined the slides from the nitrogen dioxide experiment.) Ozone and nitrogen dioxide concentrations were measured directly in the inhalation chamber. Tables 1 and 2 in this Commentary, which are expanded versions of Table 2 from the Investigators' Report, summarize some of the key features of the two experimental protocols.

Animals were killed according to a predetermined schedule or when moribund. Animals that died before the scheduled date also were evaluated. Thirteen different areas of tissue, including the lungs, trachea, larynx, and nasal cavities, were collected, and the tissues were fixed by standard methods for pathologic examination. Specifically, the lungs were perfused through the vasculature with 10% formalin, removed from the animals, and preserved in the same fixative until the end of the experiment. The fixed tissues were shipped from the University of California at Davis to the University of Tennessee, where they were prepared for histological analysis. Conventional tissue sections with hematoxylin and eosin were analyzed by Drs. Schuller and Breider. The types and location of lesions, such as hyperplasia, tumors, papillary polyps, evidence of infections,

Table 2. Experimental Design for the Nitrogen Dioxide Experiment

Group	Shipment	*n*	Carcinogen Treatment	Oxidant Exposure	Duration of Treatment and Exposure (weeks)	Necropsy Schedule (weeks)	Housing (caging/local environment/location)
1	3	40	DEN	Air	16 or 24	16 to 32	Wire-mesh/chamber/CRPRC
2	3	40	DEN	15 ppm Nitrogen Dioxide	16 or 24	16 to 32	Wire-mesh/chamber/CRPRC
3	3	20	Saline	Air	24	32	Wire-mesh/chamber/CRPRC
4	3	40	Saline	15 ppm Nitrogen Dioxide	16 or 24	16 and 32	Wire-mesh/chamber/CRPRC
5	3	20	DEN	Air	20	24 to 32	Shoe-box–type/room/ITEH
6	3	20	DEN	65% Oxygen	20	16 to 32	Shoe-box–type/room/ITEH

and necrosis, were recorded for each animal in each group. Specific immunostaining was carried out for calcitonin and bombesin, two peptides known to distinguish neuroendocrine cells from other pulmonary epithelial cells.

After the investigators had completed their experiment with ozone, the HEI Research Committee asked a panel of pathologists to perform an independent evaluation of a subset of the microscopic slides. The six pathologists, who had extensive experience with cancers of the respiratory tract in animals and humans, discussed the classification of the lesions observed in the ozone experiment. As a result of their discussions, the investigators changed some of their original diagnoses from neoplasms to focal hyperplasia. Details on the composition of the panel and their findings are provided in the Investigators' Report.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The investigators completed their proposed air, ozone, and nitrogen dioxide exposures of DEN- and saline-treated animals. They also obtained morphologic data on tissues from all the animal groups; however, they were unable to determine whether the hyperplastic cells and tumor cells had neuroendocrine cell characteristics.

ASSESSMENT OF METHODS AND STUDY DESIGN

The overall design of the study was appropriate for the question under investigation. Hamsters were allocated into 11 treatment groups: five groups for the ozone experiment, which was conducted in 1989, and six groups for the nitrogen dioxide study, which was conducted a year later (see Tables 1 and 2 of this Commentary). Each of the two treatment variables (DEN and the pollutant) had an appropriate control (saline and air), which allowed for a two-factor design. Thus, each experimental condition could be compared with data from a well-matched control group. The exposures were carried out under controlled conditions. The chamber concentrations of ozone and nitrogen dioxide were measured directly; thus, the actual exposure concentrations were carefully documented. In principle, this design should have allowed for a definitive interpretation of findings related to the combined DEN and pollutant exposures.

There are, however, aspects of the study design and experimental procedures that compromise the interpretation of the study results. For example, although the animals from individual shipments, were assigned randomly to the different treatment groups, the mean body weights at the beginning of the experiments varied among groups. In the ozone experiment, the animals were received from the supplier in two shipments and there was a 20% difference in the initial starting weights of hamsters between the DEN-injected groups (120 g) and the saline-injected groups (100 g). Also, during the course of the experiments, serious weight losses occurred in some of the groups (see Figures 1, 9, and 13 of the Investigators' Report), and a number of animals died spontaneously or had to be killed prematurely (see Figures 2, 10, and 14). These deaths were attributed to tumors or to gastrointestinal and pulmonary infections of unknown etiology. In approximately 10% of the hamsters (excluding the saline plus air treatment group, for which the data were not given), foci of pneumonia were noted during routine pathologic analysis. It is unclear whether the difference in body weight, both at the beginning and during the experiments, or the presence of infections had any influence on the outcome of the experiments.

Problems also occurred in the histopathologic evaluation of the lung tissue. Although routine histologic pathology was performed successfully on all tissues, the investigators could not identify neuroendocrine cells or their derivatives in lung tissues from any of the treatment groups, including the putative positive control group. Thus, the investigators were unable to determine whether the hyperplastic or tumor cells expressed characteristics of neuroendocrine cells.

STATISTICAL ANALYSES

The investigators used Chi-squared analysis to determine whether there were any differences between pollutant-exposed and air-exposed animals. This is a valid method of analysis; however, because the investigators had set up a two-factorial design for their experiments (DEN, pollutant, both, or neither), a two-factor analysis of variance also could have been used. A two-factor analysis of variance systematically evaluates the potential statistical significance of the effects of DEN, the effects of the pollutant, and whether there is any synergy between the two treatments. However, because the outcome of these experiments was mostly negative, the use of paired comparisons instead of a two-way analysis of variance is not critical for interpreting the results.

Although there appear to be differences in weight gain among the experimental groups, this finding was not supported by any statistical analysis. Indications of variability or precision, such as standard deviations or standard errors, were not provided in Figures 1, 9, or 13. The variable weight gain among the treatment groups, which was one of the few positive findings of these experiments, could have been substantiated by a two-factor analysis of variance.

The negative control group (saline plus air) showed no tumors or lesions, according to the footnotes in Tables 4 and 6 of the Investigators' Report. This finding justifies, to some degree, the investigators' decision to use only 20 animals and to kill them after 24 weeks, rather than having an additional 20 animals to be killed after 16 weeks. This design assumes that the number of tumors and lesions would be zero at 16 weeks. However, to conduct a comprehensive statistical analysis, data for a negative control group would be needed at 16 weeks. Without these data, the direct carcinogenic effect of the pollutant or the spontaneous lesion rate at this time during the experiment cannot be assessed.

RESULTS AND INTERPRETATION

The results of this study were largely negative with respect to the original hypothesis posed by the investigators. Contrary to expectations, combined exposures either to DEN plus ozone or DEN plus nitrogen dioxide did not result in an increase in the number of animals with respiratory tract tumors. Furthermore, of those tumors observed, it was not possible to determine whether the cells had characteristics of neuroendocrine cells.

The investigators were unable to reproduce their earlier model for the induction of pulmonary neuroendocrine lung cancer in hamsters exposed to DEN and hyperoxia. There are several possible explanations for the discrepancy between these findings and those reported earlier (Schuller et al. 1988). First, as the investigators suggest, the absence of specific histochemical staining for bombesin and calcitonin may have been due to a loss of antigenicity of the polypeptides. The investigators speculate that their methods of tissue fixation, the long periods of tissue storage before paraffin embedding, or both, may have compromised their histochemical analysis. The loss of antigenicity due to inadequate fixation or long storage is well known to histologists and protein chemists and might have been anticipated. As an alternative to immunostaining, the investigators could have characterized the hyperplastic cells and tumors by electron microscopy, as they did in their earlier work (Schuller et al. 1988). However, this approach was not proposed in this study.

Second, it is conceivable that the cells did not have these characteristics and were derivatives of another cell type, such as alveolar type II cells. It is unfortunate that the tumors and hyperplastic cells were not tested for the presence of specific markers for type II cells (for example, surfactant proteins) because the investigators postulated that these cells were the cell of origin observed in their second DEN-hyperoxia study (Schuller et al. 1990).

A third possibility is that some changes in the neuroendocrine cells may have been missed because of the design of the schedule for killing the animals. In their original study, Schuller and coworkers examined the lesions after eight weeks of treatment (Schuller et al. 1988). In the present study, the animals were killed 16 or 24 weeks after starting the DEN treatment and ozone exposure regimen. In the nitrogen dioxide experiment, the animals were killed even later (after 16 to 32 weeks). Furthermore, some animals were maintained in air for an additional eight weeks after the ozone or nitrogen dioxide exposures were terminated, which may have been conducive to regression of the hyperplasia. Recently, regression of neuroendocrine hyperplasia has been reported in the hamster model. Sunday and Willett (1992) examined the kinetics of pulmonary neuroendocrine hyperplasia in hamsters treated continuously with DEN and hyperoxia (60% oxygen). They found an induction of neuroendocrine cell differentiation in the lungs of hamsters treated with DEN and hyperoxia. The peak period of neuroendocrine cell hyperplasia, as demonstrated by a variety of markers, occurred 9 to 14 weeks after the initiation of treatment; after 14 weeks, the lesions rapidly regressed. The spontaneous regression of pulmonary neuroendocrine cell hyperplasia induced by combined treatment with DEN and hyperoxia was not known when this study was designed.

Finally, the hamsters in this study may have been less susceptible to carcinogenesis than those used in the earlier study. It is also possible that the findings in the earlier study may have been misinterpreted. In the absence of specific stains or confirmatory transplantation experiments, it may be difficult to distinguish between hyperplastic lesions and early neoplasia. There was unanimous agreement among the six members of the independent Pathology Working Group that the tumors in representative reference slides from the previous study had characteristics consistent with a diagnosis of neuroendocrine neoplasia: namely, positive bombesin stain along with electron micrograph descriptions, as provided in the publication by Schuller and coworkers (1988); however, the Working Group noted that in the reference slides it reviewed from the earlier study, the positive staining material was for the most part on the lumenal side of the cell rather than near the basilar side, as would be expected for a neuroendocrine cell. Regarding the lung lesions in the present study, the Pathology Working Group reviewed 16 slides from all exposure groups in the ozone experiment. In almost all cases, the reviewers were unanimous in their diagnosis. With respect to the large airway papillomas, the Working Group's diagnosis was consistent with that of the original pathologist. For 10 lesions that were originally diagnosed as peripheral neoplasms, how-

ever, the Working Group's diagnosis was bronchiolar epithelial hyperplasia. The Working Group also considered three lesions originally classified as possible neuroendocrine-type tumors to be peripheral adenomas. The Working Group, therefore, did not confirm the diagnosis of neuroendocrine tumors in any of the 11 cases examined in this study.

The investigators observed a variable incidence of lung adenomas, tracheal and bronchial papillary polyps, and bronchiolar hyperplastic lesions in animals exposed to DEN. They noted that exposure to either ozone or nitrogen dioxide in combination with DEN appeared to suppress the formation of lung adenomas. However, these results should be interpreted cautiously for several reasons. First, the total number of tumor-bearing animals was small, and only in the nitrogen dioxide experiment at the later necropsy (24 to 32 weeks) was there a significant difference between the animals exposed to DEN plus pollutant and the control animals exposed to DEN plus air. Second, it has been known for more than 50 years that dietary restriction in animals causes a reduction of spontaneous and chemically induced tumors (Tannenbaum 1942; Simopoulos 1987). Eliminating nutrient intake as a variable would require the inclusion of pair-fed controls. The average weights of the animals treated with DEN plus either pollutant were much lower than those of the other groups, a factor that may have contributed to the observed delay in the induction of some respiratory tract tumors. For these reasons, the lower number of tumors in the DEN plus ozone groups or the DEN plus nitrogen dioxide groups should not be attributed to a protective effect of either pollutant. Witschi and colleagues (1993) recently reported that hamsters exposed to DEN and 0.8 ppm ozone had half as many peripheral lung tumors as did the animals kept in air; however, the difference was not statistically significant.

There also were large differences in the occurrence of lung adenomas among the various subgroups exposed to DEN plus air, with values ranging from 9% to 58%. Some of this variability appears to be due to the fact that the tumor rates were greater in those hamsters killed at the later time points than in those animals killed after 16 weeks. In addition, the investigators noted that the type of container in which the animals were housed influenced the number of tumor-bearing animals. In this study, there was a lower incidence of respiratory tract tumors in DEN-treated animals housed in wire-mesh cages than in those housed in shoe-box–type plastic cages. In the nitrogen dioxide experiment, 58% of the animals housed in plastic boxes had tumors, whereas only 26% of the animals housed in wire-mesh containers had tumors (see Table 9 of the Investigators' Report). However, other explanations, such as the fact that the animals were housed in different facilities (see Table 2 of this Commentary), are also possible. Because of the considerable variability in this control group and the small differences in the number of tumor-bearing animals between the DEN plus air groups and the DEN plus pollutant groups, we cannot conclude from the current study that either ozone or nitrogen dioxide protects against the formation of lung tumors.

The investigators did not address the possibility that exposure to ozone or nitrogen dioxide may alter the metabolism of DEN, rendering it biologically inactive or changing its accessibility to target cells. The delay in appearance of tracheal papillary polyps and the much lower prevalence of liver necrosis and biliary obstruction in the DEN plus ozone groups than in the DEN plus air groups are consistent with the hypothesis that there are oxidant-induced differences in DEN metabolism. If further studies confirm these preliminary observations, it will be important to characterize the metabolism and turnover times of DEN in target tissues of control and pollutant-exposed animals.

The investigators were unable to confirm the findings from their earlier study showing that hyperoxia increases the number of lung tumors in animals treated with DEN (Schuller et al. 1988). In the positive control group from the nitrogen dioxide experiment, the investigators found no tumors in the animals exposed to DEN plus 65% oxygen, but they did find that 58% of the animals exposed to DEN plus air had tumors. The reason for this discrepancy from their earlier work is not obvious. The failure to produce tumors in the positive control group raises several interesting issues, some of which were discussed by the investigators in their report. They suggest that differences in experimental procedures, such as animal housing, the schedule of killing the animals, and the dilution of the DEN, may have been responsible. The hamsters used for the DEN plus oxygen treatment groups also may have been different in some unknown way (such as genetic, health, or dietary) from those animals studied previously. The investigators did not, however, discuss the possibility that the high occurrence of tumors they observed previously with the DEN plus oxygen may have resulted from some unknown variable rather than the hyperoxia per se, or that their earlier results may have been misinterpreted. Additional experiments are needed to address and resolve this issue.

The failure of ozone or nitrogen dioxide to enhance the number of lung tumors challenges the general perception that chemically induced tumors develop more readily in tissues undergoing a more rapid rate of cell proliferation than in quiescent tissues. Such a relationship appears to be true for cultured cells, but may not be true for all tissues in the more complex environment of an intact animal. For example, in humans, certain tissues with rapid turnover rates (intestinal epithelium, skin, hematopoietic tissues) do not

have higher tumor rates than tissues that divide more slowly. Furthermore, in this study, the extent of proliferation caused by exposure to the ozone, nitrogen dioxide, or oxygen is not known. In the ozone experiment, the investigators noted an increase in bronchiolar hyperplasia, which suggests an increase in cell division. However, because the investigators did not measure cell turnover, it is only presumed that cell division rates increased. Thus, although cell proliferation was an important feature of the hypothesis, its role in tumor induction was not clarified in this study.

IMPLICATIONS FOR FUTURE RESEARCH

These studies do not answer the question of whether inhaling ozone or nitrogen dioxide modulates the occurrence of chemically induced tumors. The original hypothesis presented by the investigators must be tested under experimental conditions that are more rigorously controlled than those in the present study. In particular, animal housing conditions and dietary intake should be comparable in different treatment groups, and animal tissues should be examined at a wider range of time periods so that histopathological changes can be assessed in more detail. Specific methods and markers could be used to evaluate the cell composition of any observed lung tumors. Consideration should be given to determining whether tumor cells contain amplified *myc* oncogenes, which have been demonstrated in cell lines (Little et al. 1983) and tumor cells (Wong et al. 1986) from human small cell lung cancers. Finally, obtaining information on the metabolism of DEN in the various exposure groups would be of considerable interest.

Because these studies did not verify the investigators' earlier findings that exposing hamsters to hyperoxia and DEN produced neuroendocrine cell tumors in the lung, it will be important to determine the reasons for this discrepancy and the role of hyperoxia in inducing neuroendocrine cell neoplasia. The availability of an animal model for neuroendocrine cell neoplasia remains important for studying the pathogenesis of highly malignant small cell lung carcinoma and the role of environmental factors in the etiology of this disease.

CONCLUSIONS

Dr. Witschi and colleagues used a model that they had developed for inducing neuroendocrine lung tumors in Syrian golden hamsters to evaluate whether inhaling either ozone or nitrogen dioxide enhances tumor development. Because hyperoxia causes proliferation of neuroendocrine cells, and both ozone and nitrogen dioxide cause hyperplasia of airway and alveolar epithelial cells, the investigators postulated that these highly reactive oxidant pollutants would increase the number of respiratory tract tumors, particularly neuroendocrine cell tumors, in DEN-treated hamsters.

In this study, inhaling 0.8 ppm ozone or 15 ppm nitrogen dioxide did not increase the occurrence of respiratory tract tumors in DEN-treated hamsters. In contrast to their earlier findings (Schuller et al. 1988), the investigators found that combined treatment with hyperoxia and DEN failed to produce neuroendocrine cell tumors in the hamster lungs. Although there were some differences in the experimental protocol of this study and that of the investigators' previous work, the reason for the discrepancy in results is not known. The negative findings may have resulted from overfixation of the lung tissues and a resulting loss of antigenicity of the neuroendocrine cell markers. Alternatively, the lesions may not have contained cells with neuroendocrine characteristics, or the lesions may have regressed by the time the animals were killed. Another explanation is that the earlier findings may have been misinterpreted. Because no lung tumors with neuroendocrine characteristics could be demonstrated in the putative positive control groups (that is, the DEN plus hyperoxia groups), no definitive conclusions can be reached regarding the tumor enhancing potential of ozone or nitrogen dioxide.

Some effects of oxidant exposure were noted in the occurrence of nonneuroendocrine lesions. In nitrogen dioxide–exposed, DEN-treated hamsters, the occurrence of lung adenomas was less frequent than in air-exposed, DEN-treated animals. In ozone-exposed, DEN-treated hamsters, there were fewer tracheal papillary polyps and liver lesions, which are characteristic signs of DEN toxicity, than in animals exposed to DEN and air. These observations may have been due to an effect of oxidant exposure on DEN metabolism or some other protective mechanism, but should be interpreted cautiously because of differences in dietary intake, animal housing conditions, and the small numbers of tumor-bearing animals. Exposure to ozone alone caused bronchiolar cell hyperplasia, but nitrogen dioxide had no effect on cellular hyperplasia at the times studied.

Whether exposure to oxidant air pollutants contributes to lung cancer remains an unanswered question. As expected, ozone or nitrogen dioxide exposure alone did not result in lung tumors. The exposure periods were relatively short (four to six months), and these experiments were not designed to assess the direct carcinogenicity of these pollutants. The cocarcinogenicity results (DEN plus ozone or DEN plus nitrogen dioxide) are also inconclusive because no neuroendocrine tumors were observed in the lungs of the putative positive control group (DEN plus hyperoxia).

Although there was some suggestion that these oxidant pollutants may delay the development of nonneuroendocrine tumors of the respiratory tract, such a conclusion is premature, given the small number of tumor-bearing animals and the differences in the health status and body weights of the animals in the various treatment groups.

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