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Modulation of *N*-nitrosodiethylamine-induced hamster lung tumors by ozone

Hanspeter Witschi[a], Dennis W. Wilson[b] and Charles G. Plopper[c]

[a]*Institute of Toxicology and Environmental Health,* [b]*Department of Veterinary Pathology, School of Veterinary Medicine and* [c]*Department of Anatomy and Cell Biology, School of Veterinary Medicine, University of California, Davis (USA)*

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Summary

Male Syrian Golden hamsters were treated with subcutaneous injections of *N*-nitrosodiethylamine (DEN), 20 mg/kg, twice a week for 24 weeks. Half the animals were kept in filtered air and the other half was exposed continuously to an atmosphere of 0.8 ppm of ozone. After 6 months, no more DEN injections were given and all animals were kept in air until termination of the experiment at 7 months. It was found that the animals kept in ozone developed half as many peripheral lung tumors as did the animals kept in air; however, the difference was not statistically significant. Tumors of the trachea, bronchi, nasal cavity and liver developed with the same incidence whether the animals were exposed to ozone or not. It was concluded that ozone, an agent known to produce cell proliferation in the respiratory tract, does not enhance the development of tumors in the peripheral lung or in the nasal cavity of hamsters.

Key words: *N*-nitrosodiethylamine (DEN); Syrian golden hamster; Lung tumors; Ozone; Cell proliferation; Tumor modulation

Introduction

Ozone is a common air pollutant. It is also a highly reactive chemical that readily interacts with biological targets [1]. In isolated cell systems ozone increases the occurrence of chromosome breakage and of chromatid-type deletions and enhances cell transformation [2–4]. Inhaled over prolonged time periods (6–15 months), ozone produces irreversible proliferative changes in the respiratory tract of mice [5–7]. On occasion an increase in the incidence of pulmonary adenomas has been observed [8,9]. Although epidemiological studies have to date failed to provide unequivocal evidence that ozone is a risk factor in human lung cancer, it has been listed as a potential contributor to lung carcinogenesis in man [10].

Proliferation of the epithelial cells lining the respiratory tract is a common event following exposure to oxidant air pollutants [11]. Intermittent or sustained cell proliferation often modulates tumor development in the lung or in the nasal cavity [12,13]. Ozone is known to produce diffuse cell proliferation throughout the

Correspondence to: Hanspeter Witschi, ITEH, University of California, Davis, CA 95616, USA.

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respiratory tract and thus conceivably could modify lung tumor development. So far, the only experiments designed to test this hypothesis have given equivocal results; they show that ozone either enhances [8] or reduces [9] the development of pulmonary adenomas in mice. We therefore decided to examine the effects of continuous exposure to ozone on lung tumor development in hamsters treated with a carcinogenic dose of *N*-nitrosodiethylamine. Our results show that, under the conditions of the present experiment, ozone did not enhance the development of chemically induced lung tumors but rather it seemed to adversely affect tumor formation.

Materials and methods

Experimental animals

Male LVG Syrian Golden Hamsters were purchased from Charles River Canada, St. Constant, Quebec, Canada and entered the experiment when 7–11 weeks old. Upon arrival, the animals were attributed at random to four groups such that the body weights in the different groups were as homogeneously distributed as possible. The animals were housed in plastic cages on conventional bedding and the cages were placed into 4.2 m^3 Hinners-type exposure chambers at the inhalation facility, California Regional Primate Research Center, University of California, Davis. The animals were kept on a 12-h light/12-h dark cycle with free access to conventional lab chow and water. They were given a few days acclimatization period in the chambers before initiation of experimental protocols.

Treatment and exposure

Four treatment groups were formed. Group 1 (24 animals) was kept in air and treated with DEN. Group 2 (24 animals) was exposed to ozone and treated with DEN. Group 3 (16 animals) was kept in air and injected with 0.9% NaCl and group 4 (16 animals) was exposed to ozone and injected with NaCl. Specifically, treatments were as follows: DEN, purchased from Eastman-Kodak, Rochester NY, was diluted with 0.9% NaCl to a final concentration of 1% DEN. Animals were injected subcutaneously (s.c.), in the region of the flank organ, twice a week, with 0.2 ml/100 g body weight of the DEN solution, a dose of 20 mg/kg of DEN per injection. Control animals received an equal volume of 0.9% NaCl s.c.

Ozone was generated by silent electric arc discharge through a stream of medical grade 100% oxygen. The ozone formed was mixed into a stream of filtered air and humidity was kept at 50%. Ozone concentrations were measured with a Dasibi UV photometer and chamber air flow was maintained at 30 chamber vol./h. The nominal ozone concentration in the chamber was 0.8 ppm and measured concentrations were 0.79 ± 0.05 ppm (mean and SD from 20 000 measurements).

The animals were exposed to 0.8 ppm of ozone for 23 h a day, 7 days per week. Air controls were kept in an identical chamber, ventilated with filtered air only. All the animals were treated with DEN or NaCl and exposed to ozone or filtered air for a total period of 6 months. After 6 months, no further DEN injections were given and from this time on all animals were kept in an atmosphere of filtered air.

Pathology

Animals were killed when found in clinically poor condition (body weight falling below 80 g) or at the end of the experiment. For sacrifice, animals received a pentobarbital overdose. At autopsy, all organs were inspected and lungs, trachea, liver, kidneys, adrenals, pancreas, femur and brain as well as any additional macroscopically gross lesions were fixed in neutral buffered formalin. Conventional paraffin sections were prepared for all tissues and examined for the presence of tumors.

Data analysis

The histopathological findings were tabulated and the incidence of tumors found was calculated as a percentage of tumor bearing animals in each group. Comparisons between groups were made with the chi-square test and a P-value of 0.05 or less was considered to be significant.

Results

Survival rates

Data on weight gain in the four different treatment groups are shown in Fig. 1. During the first 2 months, animals kept in air and given saline injections rapidly gained weight and then stayed stable until the end of the experiment. Animals kept in ozone and injected with NaCl initially gained less weight than the controls and remained stable at a lower plateau. Animals treated with DEN and air gained weight only during the first 12 weeks and then gradually lost weight again. The animals treated with ozone and DEN showed a similar pattern but their highest body weights never reached the weight of animals treated with DEN alone.

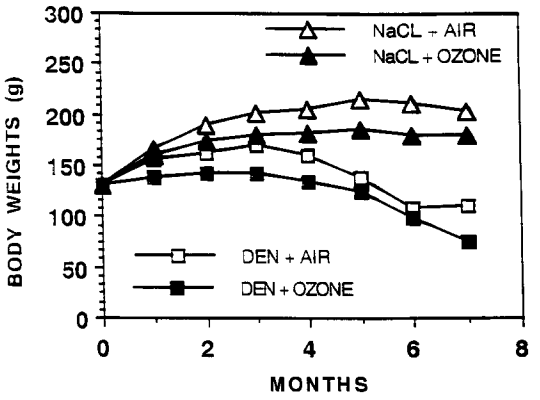

Fig. 1. Weight gain of hamsters during the experiment. Male Syrian Golden hamsters were treated with DEN, 20 mg/kg s.c., twice a week, for 24 weeks (square symbols) or with injections of saline (triangles) and exposed to 0.8 ppm of O_3, 23 h a day, 7 days a week (closed symbols) or kept in filtered air (open symbols). After the 24th week, the animals received no further treatment and all animals were kept in air until sacrifice between 24 and 34 weeks.

All animals treated with DEN survived during the first 15 weeks of the experiment. After this time, many animals were found dead or had to be killed because of poor clinical condition. Survival rates are shown in Fig. 2. As in previous studies, the cause of death in these animals was most likely suffocation due to developing bronchial and tracheal tumors [14]. Animals kept in ozone seemed to die somewhat later than animals kept in air, but the difference was not statistically significant. Twenty-nine weeks after the beginning of the experiment, all animals treated with DEN had died or had to be sacrificed. At this time, the experiment was terminated and the animals in the control groups were killed. Fourteen animals in each of the two control groups (NaCl and air or NaCl and ozone) were still alive at this time; two animals in each group having been found dead during the course of the experiment.

Tumor incidence

Data on tumor incidence are shown in Table I. In animals treated with DEN and exposed to ozone, the overall incidence of lung tumors was only half the incidence of lung tumors found in animals treated with DEN and kept in filtered air. Despite a 50% reduction in tumor incidence, however, the difference between controls and ozone-exposed animals was statistically not significant. Ozone exposure had practically no effect on the incidence of bronchial, tracheal or nasal tumors. On the other hand, in animals exposed to ozone, the incidence of liver tumors was somewhat higher than in the animals exposed to air. The increase was statistically not significant. No tumors were found in animals injected with 0.9% NaCl and exposed to ozone or kept in air.

Histopathology of the neoplastic alterations

There was no difference between animals treated with DEN and kept in air and animals given DEN and exposed to ozone in the morphology of tumors in the nasal cavity, trachea and bronchi, peripheral lung or liver. Nasal tumors were adenocar-

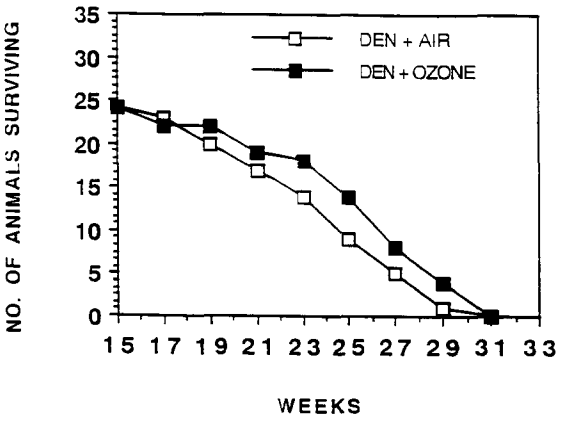

Fig. 2. Survival of animals treated with DEN and kept in air (open squares) or exposed to 0.8 ppm of O_3 (closed squares) between week 15 and 31 of the experiment.

TABLE I

INCIDENCE OF TUMORS IN HAMSTERS TREATED WITH DEN AND EXPOSED TO OZONE

Animals were treated with DEN s.c., 20 mg/kg, twice a week or with NaCl and exposed either to O_3 or to filtered air for 6 months. After 6 months, no more DEN injections were given and all animals were kept in filtered air. No tumors were found in a group of 16 animals treated with injections of NaCl and kept in filtered air throughout.

Tumor location	DEN + air (n = 24)	DEN + O_3 (n = 24)	NaCl + O_3 (n = 16)
	No. of animals with tumors (%)		
Lung	10 (42)	5 (21)	0 (0)
Bronchi	7 (29)	5 (21)	0 (0)
Trachea	23 (96)	21 (88)	0 (0)
Nasal cavity	8 (33)	6 (25)	0 (0)
Liver	9 (38)	14 (58)	0 (0)

cinomas, composed of sheets and cords of moderately stratified epithelial cells overlying a fibrovascular stroma. These masses disrupted turbinate structure and small nests of similar cells occasionally were infiltrating the bones of the nasal cavity.

Tracheo-bronchial neoplasms were adenomatous polypoid growths lined by either columnar secretory cells or, more often, by stratified squamous keratinizing epithelium. Respiratory epithelium adjacent to the squamous variants often had undergone squamous metaplasia.

Parenchymal lung tumors were largely bronchiolo-alveolar adenomas with characteristic papillary growths of a single layer of cuboidal epithelium with a basally polar nucleus overlying a thin fibrovascular stroma. These tumors were generally well circumscribed by a thin rim of connective tissue and compressed the adjacent parenchyma. Occasional animals had somewhat larger but still compressive growths composed of stratified squamous keratinizing epithelium forming solid clusters divided by connective tissue septa. The centers of these clusters frequently contained aggregates of keratin.

A mixture of proliferative lesions was present in the livers of DEN-treated hamsters. The most prominent change was a spectrum of hepatocellular proliferations ranging from altered foci to hepatocellular carcinomas. Intermediate in this change were small proliferative nodules of well differentiated hepatocytes confined to one acinus. Hepatocellular carcinomas were larger aggregates of less well differentiated cells forming trabecular cords that invaded adjacent acini. The other proliferative lesion in these livers was multiple foci of biliary cystadenoma formation.

Non-neoplastic ozone-induced lesions in the lung

The major changes in the lungs of ozone-exposed animals, when compared to unexposed controls, were found in the central acinar region. In the lungs of animals exposed to air, the distal bronchioles were generally lined by simple cuboidal

epithelium containing a mixture of ciliated cells and non-ciliated cells. The terminal bronchioles generally ended abruptly with openings into alveolar ducts. Only in a small number of centriacinar areas was there cuboidal bronchiolar epithelium present in alveolar duct regions. In a small number of bronchioles in some of the animals, there were areas which contained more than one layer of epithelial cells and appeared to be somewhat like nodules. There were few inflammatory cells observed in this region.

In the lungs of animals that had been exposed for 6 months to ozone, the terminal bronchioles were lined by simple cuboidal epithelium with both ciliated and non-ciliated cells. The cuboidal epithelium did not end abruptly with the transition to alveolar ducts. Most of the centriacinar region observed in all of the exposed animals contained multiple sites of bronchiolar epithelial hyperplasia and as many as one or two generations of alveolar duct distal to the terminal bronchiole. In numerous cases, these extensive bronchiolarized alveolar ducts also contained small areas of what appeared to be multiple layers of nucleated cells. In the alveoli immediately adjacent to the distal bronchiole, there was extensive thickening of the septum. The interalveolar septum was thickened and often infiltrated with inflammatory cells, primarily lymphocytes.

Discussion

It has been speculated for a long time that air pollutants might represent an additional risk factor in lung cancer development. Ozone, NO_2 and SO_2 as well as certain aldehydes are gases usually listed as being most likely to have such an effect. However, repeated reviews of the available evidence found little experimental support for the possibility that gaseous air pollutants do cause lung cancer in experimental animals [16–18].

It has also been examined repeatedly whether gaseous air pollutants enhance or 'promote' the development of respiratory tract tumors induced by known carcinogens such as polycyclic aromatic hydrocarbons or nitrosamines. The available evidence remains conflicting. Some studies imply air pollutants as promoting agents [19–23], whereas others show no effect or even a mitigating effect [24–28]. Data on ozone are particularly conflicting: in vitro ozone has been found to enhance cell transformation or to selectively kill cancer cells. The effects of ozone on cells in vitro most probably depend upon experimental design [3,29,30]. So do the results of in vivo experiments, where ozone has also been found to enhance or to prevent tumor development [18,31].

In the present study, we did not find that concomitant exposure to ozone and to a carcinogen would enhance peripheral lung or nasal tumor development in hamsters. Rather, ozone appeared to inhibit the development of tumors in the lung periphery, although in the present study the difference between ozone and air-exposed animals was statistically not significant. Nevertheless, our present results agree with some observations made in a previous study [32]. In hamsters treated with DEN and exposed to 0.8 ppm of ozone for 4 months, the incidence of lung tumors, although small, was half of that found in animals kept in air (Table II). When animals, treated with DEN and exposed to ozone for 4 months were allowed to

recover in air for 2 additional months without any further treatment, lung tumor incidence in hamsters exposed to ozone and DEN again was half as high as in animals treated with air alone (Table II). When the three experiments were analyzed together with the Mantel-Haenszel procedure [33] the difference between controls and ozone-exposed animals was statistically significant. We have therefore three experiments in which we obtained evidence that ozone reduced lung tumor incidence by 50% compared to animals kept in air. This makes it tempting to conclude that ozone inhibits the development of chemically induced lung tumors in hamsters. Such a conclusion is reinforced by statistically significant evidence that ozone inhibits lung tumor development in mice [8].

At present, we have no explanation why ozone might decrease the development of tumors in the respiratory tract. There are several possibilities. Ozone or products from ozone reactions with other molecules could react with the DEN to detoxify it; ozone is well known to react with amines and with reactive species such as nitrosamines. Furthermore, ozone initiates lipid peroxidation and nitrosamines might act as inhibitors in this process and thereby be destroyed. Thus, exposure to ozone might simply reduce the effective dose of DEN to which the animals are exposed (Pryor WA, pers. commun.). Ozone has also been shown to affect the cytochrome P-450 system in pulmonary Clara cells [34], an event that conceivably could mitigate the effects of a chemical carcinogen through enhancement of detoxification pathways.

Another protective mechanism might act more at the cellular than at the molecular level. Current thinking on the pathogenesis of many cancers attributes a substantial role to cell proliferation [35]. It is tempting to speculate that increased cell proliferation in the respiratory tract will enhance tumorigenesis. Some experimental evidence supports this view [36,37] whereas other observations show that tumor development in the respiratory tract is inhibited in the presence of increased cell turnover [38,39]. Increased cell turnover caused by exposure to air pollutants is mostly a result of cell death [11]. If this is a non-selective process for DEN-exposed cells, then the relative initiated population at risk for transformation would become reduced through repeated episodes of cell death. Cells might be shed into the lumen of the tracheobronchial tree and be replaced eventually with non-initiated cells. The

TABLE II

INCIDENCE OF PERIPHERAL LUNG TUMORS IN HAMSTERS TREATED WITH DEN AND EXPOSED TO OZONE: CUMULATIVE DATA

Experiment + Treatment	DEN + AIR[a]	DEN + O_3[a]
A: DEN and O_3 for 4 months[b]	2/19	1/20
B: DEN and O_3 for 4 months and 2 months recovery[b]	6/20	3/20
C: DEN and O_3 for 6 months and 2 months recovery[c]	10/24	5/24

[a]No. of tumor bearing animals/total no. of animals per group.
[b]Data from Witschi et al. [32].
[c]This paper.
Analysis of the entire data set according to the Mantel-Haenszel procedure [33] gave a value of $P < 0.05$.

question appears to be important in view of the observations that two common air pollutants, ozone [8,32 and this paper] and NO_2 [28,39] have been found to interfere with the development of tumors in the respiratory tract. However, it should be noted that these effects were only seen in animals exposed to comparatively high levels of the oxidant pollutants, 0.8 ppm for ozone and 15 ppm for NO_2. In rats treated with a chemical carcinogen and exposed to much lower levels of ozone or NO_2, the oxidant gases have been claimed to enhance tumorigenesis, although the results reported are marginal and not statistically significant [21,40].

In summary, we have observed that ozone does not enhance the development of chemically induced tumors in the respiratory tract of hamsters. There is even a possibility that ozone exposure inhibits or at least delays tumor development. While ozone is known to produce cellular hyperplasia in the respiratory tract, this fact alone does not automatically imply that tumor development must or will be enhanced. The role of ozone in the eventual pathogenesis of lung cancer and the role of cell proliferation in respiratory tract carcinogenesis needs further experimental evaluation and critical analysis.

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