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Enhancement of Allergic Lung Sensitization in Mice by Ozone Inhalation (42733)

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Abstract. Inhaled ozone was found to exert an enhancing effect for allergic lung sensitization when mice contacted an aerosolized allergen. The animals were exposed to ozone concentrations of 0.24, 0.16, 0.13, and 0.10 ppm. After 4 days of continuous ozone exposure, the mice had allergen contact from an aerosolized solution of ovalbumin. The animals were then maintained in ambient air for several days before the cycle of ozone and aerosolized allergen was repeated over four allergen contact cycles. Mice were rested in ambient air for a week after the last allergen contact, and they were then tested for allergic sensitization by the intravenous injection of 2 mg of ovalbumin to induce anaphylactic shock in allergic individuals. The control groups of mice were maintained in ambient air throughout the experiment, but they experienced identical allergen contact with the ozone-exposed mice. The phenomenon of allergic enhancement from ozone inhalation was detected at 0.24, 0.16, and 0.13 ppm of ozone. The enhancing effect disappeared at 0.10 ppm of ozone. The study indicated a potential for increasing the number of allergically sensitized individuals when various allergens are inhaled during periods of high ozone exposure with the consequent adverse changes on respiratory membranes. The significance to human health of the allergic enhancement phenomenon by ozone needs investigation.

Evidence has been presented to show that ozone inhalation induces airway hyperreactivity, and this may aggravate lung disease in persons affected with asthma (1, 2). An additional health problem from ozone inhalation appears as an enhancing effect for the development of allergic lung sensitization to inhaled allergens. This phenomenon was reported by Osebold *et al.* (3) in experiments using mice as the model mammalian respiratory system with animals exposed to 0.64 and 0.40 ppm of ozone. The numbers of immunoglobulin E (IgE) containing cells in the lungs showed a positive correlation with increasing allergic sensitivity among ozone-exposed animals, which suggested that immunologic responses in lung tissue were necessary for establishment of the allergic state (4). Allergen-specific homocytotropic antibodies were detected in the serum of the aerosol-sensitized mice (4). Recently, Biagini *et al.* (5) reported that exposure to ozone at 1.0 ppm enhanced the development of allergy to inhaled platinum in monkeys. The present study probed the occurrence of allergic enhancement by ozone at concentrations more frequently encountered in the environment, and determined an ozone level where the enhancing effect disappeared.

Materials and Methods. *Animals.* Animals were a specific pathogen-free (SPF) line of Swiss–Webster female mice, 5–6 weeks of age, which were shipped in covered cages (Hilltop Lab Animals, Inc., Scottsdale, PA). Mice were first acclimatized for a few days while housed in an isolation room with 15 air exchanges/hr at a temperature of approximately 22°C.

The animals were monitored by the supplier for a variety of infectious agents including the genera *Mycoplasma, Pasteurella, Klebsiella,* and the pneumonia virus of mice. At varied times mice were necropsied and subjected to microbiological examination by the laboratory at the University's vivarium. There was no evidence to suggest that intercurrent infections played a role in the test results. At the terminus of each experiment necropsies were made on aliquots of animals from all tested groups. The only gross and histologic lesions encountered were those compatible with Type 1 hypersensitivity.

Ozone exposure. Mice were exposed to ozone concentrations ranging from 0.24 down to 0.10 ppm in stainless-steel chambers

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at the Environmental Chamber Facilities of the California Primate Research Center. Ozone concentrations conformed to the Official Environmental Protection Agency standard and were continuously monitored by ultraviolet photometric ozone analyzers (Dasibi Environmental Corp., Glendale, CA). Control animals were housed in identical chambers in an environment of filtered ambient air. A silent electrical discharge ozone generator was used to produce ozone from bottled oxygen. The ozone was admitted into the chamber after mixing with room air. Air supplied to each chamber was passed through a C-B-R filter (Mine Safety Appliance Corp., Pittsburgh, PA), with air flow through the chamber set at 15 vol changes/hr. When elevated ozone levels were used the exposure was continuous except for the interruption to clean cages, which required opening chambers to ambient air for approximately 15 min each day.

Aerosolization of allergen. Mice were allergically sensitized to a crystalline form of ovalbumin (OA) obtained from the chicken egg. At intervals, the mice were exposed to nebulized OA in a Tri-R Airborne Infection Apparatus (Tri-R Instruments, Inc., NY). The nebulizer was designed to yield aerosol droplets of less than 3.0 μm (range of 0.5 to 3.0 μm). Approximately 50 animals could inhale the aerosol in a given run. Mice representing various groups were placed in the chamber for each operating cycle, and were exposed to nebulized OA (20 mg/ml solution in sterile distilled water). The 12-ml volume of OA solution was nebulized in approximately 30 min. Relative humidity in the chamber was about 50% at 22°C.

Experimental design. The basic experimental design is shown in Fig. 1. After 4 days of continuous exposure, the mice had allergen contact from the aerosolized OA solution. The animals were then maintained in ambient air for several days before the cycle of ozone and aerosolized allergen was repeated over four allergen contact cycles. Periods when the animals were held in ambient air and then returned to the ozone chambers were to simulate the intermittent phasing of low and then high air pollution episodes that may occur in the environment. Animals were rested in ambient air for a week after the last allergen contact and were then tested for allergic sensitization.

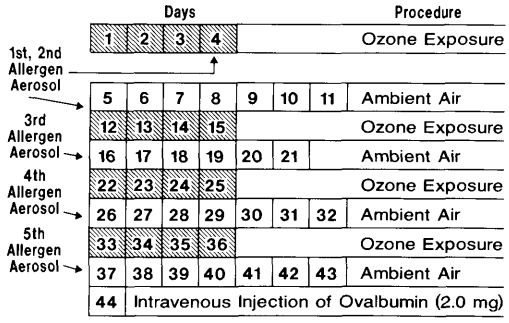

FIG. 1. Experimental design for testing the allergic enhancement effect of ozone on mice containing an inhaled allergen. The ozone exposures were continuous over 4-day periods, which were repeated four times. Ozone concentrations varied in different experiments from 0.10 to 0.24 ppm. The allergen used in an aerosol for the sensitization of mice, and for iv injection to induce anaphylactic shock in sensitized animals, was a 20 mg/ml solution of ovalbumin.

Test for allergic sensitization. Since the lung is not a dominant shock organ in allergically sensitized mice, it was necessary to reveal allergic sensitization by other means. The test procedure chosen was induction of anaphylactic shock following the iv injection of 2 mg of OA (6). In sensitized animals the signs of increased respiration were apparent 1 to 2 min after the injection of antigen. Cyanosis was obvious as darkening of the eye and ear color. Within 10 min many sensitized animals were prostrate from the generalized vascular changes. Deaths occurred 20 to 40 min after the injections in some mice. The minimal symptoms used to score anaphylaxis were increased respiration, cyanosis, ruffled fur, and listlessness. Mice were watched carefully over a 2-hr period, and those destined to survive the anaphylactic shock gradually increased their activity as the syndrome subsided.

Sensitization of positive control mice. Mice were sensitized to OA by two ip injections, at an interval of about 27 days. The injections were 1 ml in volume containing 1 μg of OA in 1 mg of alum precipitate.

Data analysis. Experiments were performed at different times to test the responses of separate lots of mice obtained

from the same source. The individual trials offered accumulative information regarding allergic enhancement from ozone inhalation.

Mice were randomized at the time of OA injection and the scoring for anaphylaxis. The statistical significance of the difference was determined between the OA aerosol-sensitized and ozone-exposed groups of mice versus the control groups which were subjected to only the OA aerosol. Groups were compared first by the χ^2 and, if that procedure indicated significance at the 5% level, the data were analyzed for probability by the Fisher exact test.

Results. Four experiments were performed to analyze the enhancement of allergic lung sensitization by inhaled ozone. These were probing experiments to determine the threshold for this effect and, consequently, there was a stepwise decrease in the ozone concentrations employed. An overview of the results, showing ozone concentrations and the numbers of responding mice, is presented in Fig. 2. Probability values are reported in Table I. The results were interpreted to mean that significant allergic enhancement from ozone exposure occurred in all ozone concentrations down through 0.13 ppm. At the level of 0.10 ppm, the enhancement effect from ozone disappeared.

Each experiment required a series of control animal sets to reveal the appropriate responses (or lack of responses) by the mice following the injection of allergen to provoke shock in sensitized animals. Table II presents the characteristic series of animal groups used, and the test results obtained with ozone at 0.13 ppm. The critical group for comparison purposes was that contacting aerosolized allergen, but maintained in filtered ambient air, which served as the control group for comparison with the group experiencing equal contact with the allergen but exposure to ozone in addition. Small sets of control animals were tested in each experiment to delineate the reliability of the anaphylactic shock system and the reagents employed.

TABLE I. PROBABILITY VALUES FOR ALLERGIC SENSITIZATION OF MICE MAINTAINED IN FILTERED AMBIENT AIR VERSUS MICE EXPOSED TO OZONE[a]

Ozone level (ppm)	Probability[b]	Significance
0.24	$P < 0.0001$	+
0.16	$P < 0.0195$	+
0.13	$P < 0.0132$	+
0.10	$P > 0.50$	−

[a] In a given experiment the contact with aerosolized allergen was equal for the two compared groups. Ozone exposures were continuous for 4-day periods, and were repeated intermittently in four cycles over approximately 6 weeks.

[b] Probability calculated by the Fisher exact test. The numbers of animals used are recorded in Fig. 2.

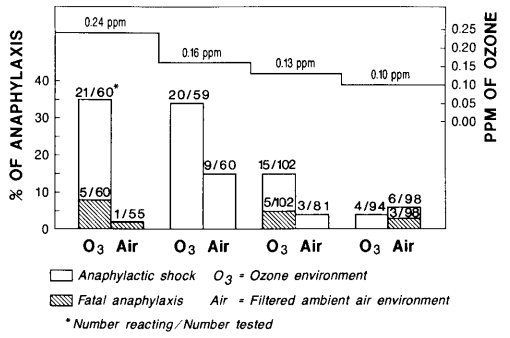

FIG. 2. Anaphylactic reactivity of mice following allergen contact. Groups of mice exposed intermittently to raised ozone levels were compared with mice breathing ambient air. Ozone enhanced allergic sensitization of the animals at 0.24, 0.16, and 0.13 ppm. The enhancing effect was not seen at 0.10 ppm.

TABLE II. ALLERGIC RESPONSES OF MICE TO 2 mg OF OVALBUMIN INJECTION INTRAVENOUSLY[a]

	Total mice	Anaphylactic shock	
		No.	Percentage
Test groups			
Ozone and allergen	102	15	14.7%
Ambient air and allergen	81	3	3.7%
Conrol sets			
Unmanipulated	10	0	
Ozone only	10	0	
Ovalbumin immunized	19	19	100%
Ozone and ovalbumin immunized	10	10	100%

[a] Groups exposed to 0.13 ppm of ozone were managed according to the regimen outlined in Fig. 1.

The complete lack of response in the normal unmanipulated controls (mice maintained in ambient air without exposure to ozone or contact with allergen) indicated that there were no toxic effects following the intravenous injection of the 2 mg dose of ovalbumin. The "ozone only" test set indicated that the inhalation of ozone under these circumstances caused no alteration of responses in animals lacking contact with the allergen. Two sets of animals were used to show that mice were, indeed, anaphylactically sensitized if the OA antigen had been injected prior to administration of the shocking dose. The exposure to ozone did not alter the effect on animals that had received the OA-sensitizing injections. In summary, the animal control sets showed that the anaphylactic shock reaction was universally absent in animals that had not contacted OA prior to the shocking injection, whereas anaphylaxis did develop in animals that had allergen injections prior to iv administration of the shocking antigen dose.

Discussion. An adverse health effect from ozone exposure has been demonstrated as enhancement of lung sensitization to an inhaled allergen in a mammalian animal. This warrants the attention of the medical community and groups concerned with health hazards in the environment. A growing body of literature presents positive correlations of reduced pulmonary capacity and increased severity of lung diseases with the presence of high levels of air pollutants (7–13).

In earlier studies we showed that continuous exposure of mice to 0.40 or 0.64 ppm of ozone was accompanied by immunological changes in the lungs (14). Plasma cells and lymphocytes synthesizing IgA accumulated along the respiratory mucous membranes. The results obtained were consonant with the hypothesis that a loss of membrane integrity in the lung made it likely that the barrier to antigenic substances would be compromised and immunological responses could follow. Hogg *et al.* (15) presented evidence to suggest that increased membrane permeability following ozone inhalation resulted from damage to the tight junctions between epithelial cells.

A variety of proteins from plant, animal, or microbial sources can be allergens for humans. In the present study ovalbumin was used to mimic the inhalation of environmental allergens. Ovalbumin is a useful experimental allergen since its size of 44,000 Da places it in that range of proteins which are small enough to be absorbed through mucous membranes and large enough to be sufficiently complex to function as immunogens (16). The contact between environmental allergens and susceptible individuals can be prolonged and nearly continuous (i.e., the pollen of plants or the presence of animal dander from pets). However, allergen contact was necessarily limited in the experiments, and the times of contact were estimated to be periods when the antigenic stimulation might be effective.

We reported that damage to the respiratory membranes from ozone inhalation could be monitored by quantitating the serum albumin levels in respiratory secretions (14). On day 4 of ozone exposure a peak of serum albumin in secretions indicated that this was a period of impaired membrane integrity, and a time when extrinsic antigen (allergen) might easily gain access to immunocompetent cells in the tissues underlying the epithelium. The rationale for presenting airborne antigen after 3–4 days of continuous ozone exposure was based on the anticipation that sensitization might occur at that time.

A brief account of events in this type of allergy includes the following considerations. Immunoglobulin E is well established as the antibody responsible for sensitizing individuals for asthmatic attacks and anaphylactic shock (17). Accumulating investigations by others have shown the relevance of study on IgE responses in laboratory rodents to the analogous events in humans (17, 18). The B-lymphocytes synthesizing IgE are located in lymphoid tissue of the lung under the epithelial lining of the airways (19). Antigenic stimulation of these cells follows the inhalation of allergens which gain access to the subepithelial spaces. This leads to the synthesis of IgE with specificity for the allergen. The IgE molecules fix to mast cell receptors for the Fc region of the antibody and thus sensitize the mast cell. If the allergen is reintroduced, as by inhalation, the resulting antigen–antibody reaction causes the mast cells

to degranulate and release pharmacologically active substances, producing the tissue responses seen in an asthma attack. An individual experiencing asthma from allergen inhalation can also experience anaphylactic shock if the allergen is injected in sufficient quantity. Thus, shock is associated with generalized anaphylaxis, and asthma is a syndrome of local anaphylaxis.

Mice dying from anaphylactic shock revealed the expected evidence of circulatory collapse with venous engorgement (6). Organs such as the lungs, liver, and spleen appeared uniformly engorged with blood. Histologic examination of lungs sometimes revealed folding of airway membranes suggestive of smooth muscle contraction. However, the airways remained sufficiently patent to prevent distention of the lungs with entrapped air. The mice appeared to die of anoxia due to the stagnation of blood into thin-walled vessels.

Controlled experimentation on animals offers a means for determining alterations in the immune responses of the respiratory tract as mediated by the presence of air pollutants. Ozone has been shown to act as a powerful oxidizing agent that produces histologically recognizable damage to the lining epithelial cells of the respiratory tract (20, 21). The problems accruing from high ozone levels will persist in the foreseeable future as by-products from the use of internal combustion engines. It is likely that rising ozone levels can increase the total number of asthmatic patients in two ways. First, once an asthmatic state becomes established, it may be predicted that polluted air will irritate the airways of the lung and augment the onset of bronchospasm, thus increasing the number of clinical asthma attacks during high-pollution episodes (1, 7). Second, the number of allergically sensitized individuals might rise through the mechanism described in this report when allergens are inhaled during periods of ozone attack on respiratory membranes. Our earlier reports (3, 4) demonstrated the phenomenon of enhancement by ozone for allergic lung sensitization. The present study revealed significant increases in sensitivity to an inhaled allergen in each of three experiments when the concentrations of ozone were 0.13, 0.16, and 0.24 ppm.

In the animal model the threshold for allergic enhancement to an inhaled allergen by ozone exposure was found to lie between 0.10 and 0.13 ppm. This ozone concentration is frequently present in the environment of human populations, and the magnitude of this ozone effect for human health is, as yet, undetermined. The potential for increasing the number of individuals with respiratory allergies could be large, which poses a question for the prevention of human illness.

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