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Health Effects from the Inhalation of Oxidant Air Pollutants as Related to the Immune System

Final Report
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ABSTRACT

These investigations dealt with further clarification of links between ozone inhalation and lung diseases. Increases in allergic lung disease occurred among our test populations of mice following inhalation of ozone, thus establishing a cause and effect relationship between the toxic inhalant and enhancement of a specific health problem. Allergic enhancement was detected in an experiment with an ozone concentration of 0.13 ppm. When the ozone level was reduced to 0.10 ppm, the effect disappeared. The ozone threshold is now considered to be a concentration less than 0.13 ppm and greater than 0.10 ppm.

An adjuvant substance was used to increase the effectiveness of the antigen (allergen) for inducing immunologic responses in the body. The adjuvant was inactivated Bordetella pertussis cells. This is the same material that is used to immunize against whooping cough in children. In mice receiving adjuvant, there was a significant enhancing effect for allergic sensitization from ozone at the level of 0.10 ppm.

Work was begun to study the allergic enhancement by ozone in an animal model where the allergic reaction would be analogous to an asthma attack in human subjects. Three preliminary trials led to the development of a protocol for the aerosol sensitization of guinea pigs and the elucidation of disease responses in the lungs.

In past experiments an ozone effect was detected as an alteration in the severity of viral pneumonia wherein the ozone exposed animals experienced a milder influenza process. It was considered likely that inhaled ozone could inactivate virus as it was transferred from infected to noninfected cells in the airways. An experiment was run at the ozone level of 0.16 ppm to determine if the observed effect would persist. This ozone concentration was chosen because it is frequently encountered in the environment and because in vitro studies showed that it had only a minimal, and delayed, effect for influenza virus inactivation. The experiment revealed that the ozone effect had persisted, and significantly less mortality occurred in the ozone exposed animals.

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SUMMARY

These investigations dealt with further clarification of links between ozone inhalation and lung diseases. Ozone has been shown by others to act as a powerful oxidizing agent producing histologically recognizable damage to the lining epithelial cells of the respiratory tract. Immunology offers real promise in determining events set in motion by air pollutants. Increases of immunological activity in the lung are clear indicators of injury to the tissues. Increases in allergic lung disease occurred among our test populations of mice following inhalation of ozone, thus establishing a cause and effect relationship between the toxic inhalant and enhancement of a specific health problem. Changes in the pattern of immunological defenses of the lung against infecting virus (influenza virus) followed exposure to ozone and offered evidence that the susceptibility of lung cells to the virus had been altered. In this summary comments will be made first about allergic lung sensitization, and this will be followed by remarks concerning viral pneumonitis (influenza infection).

I. Studies on Allergic Enhancement from Ozone Inhalation in Mice

Mice were chosen as the experimental model animal for 3 reasons. 1) The immune system of mice has been more thoroughly investigated than that of any other species of animal. 2) Other investigators consider the mouse the best model animal system for studying immunoglobulin E (IgE) antibody responses as it relates to man. IgE is the class of antibodies responsible for allergic reactivity. 3) The unit cost of mice is low enough to permit the use of statistically valid numbers of animals in comparison groups.

The procedure for allergic sensitization was aerosolization of an allergen (ovalbumin) over approximately 30 minutes in a chamber containing the test mice. When this was repeated 4 times at approximately 10 day intervals, a small percentage of the mice would become sensitized to the inhaled protein. The allergic reactivity of these animals was compared to mice that were exposed to ozone in addition to the allergen contact. Mice were held in specially designed environmental chambers and exposed continuously to ozone for 4 days. They were then given the aerosolized allergen contact and held in ambient air for approximately 6 days. The process was repeated 4 times.

Since the lung is not a dominant shock organ in allergically sensitized mice, it was necessary to reveal allergic sensitization by another means. The test procedure chosen was induction of anaphylactic shock following the intravenous injection of ovalbumin. Shocked animals became listless and prostrate, and death occurred in many instances within 20 minutes of the injection. A human subject experiencing an asthma attack from inhalation of an allergen can also go into anaphylactic shock if the allergen is injected. Thus, shock is associated with general anaphylaxis, and asthma is a syndrome of local anaphylaxis.

The mice were rested in ambient air for 1 week following the last contact with the allergen, and both groups were injected with ovalbumin to determine the numbers in each set that had become sensitized. Previous experiments had demonstrated that ozone exposure at levels of 0.64, 0.40, 0.24, and 0.16 ppm would enhance the development of an allergic state to the

inhaled allergen. Work in the current project was to determine the threshold concentration of ozone producing this effect. Allergic enhancement was detected in an experiment with an ozone concentration of 0.13 ppm. When the ozone level was reduced to 0.10 ppm, the effect disappeared, since there was no statistically significant difference between animals held in ambient air versus those exposed to 0.10 ppm of ozone. The ozone threshold is now considered to be a concentration less than 0.13 ppm and greater than 0.10 ppm.

An added factor was introduced in a previous experiment where the ozone concentration was reduced to 0.16 ppm. The new factor was an adjuvant substance used to increase the effectiveness of the antigen (allergen) for inducing immunologic responses in the body. The adjuvant used was inactivated bacterial cells of the organism named Bordetella pertussis. This bacterium is the cause of whooping cough in children and the same inactivated organisms are used in the routine immunization of infants and young children. Experimental work by others has shown that this organism contains a protein with the capacity for modifying immune regulation in the body in a way that favors synthesis of the IgE class of antibodies. In our past studies, this adjuvant markedly increased the numbers of allergically sensitized animals, and in each experiment the ozone exposure continued to reveal allergic enhancement above that encountered in animals maintained in ambient air.

In the present project period, the study made at 0.10 ppm of ozone included groups of animals that were given a single injection of B. pertussis adjuvant. The outcome for animals receiving the adjuvant was quite different from the result in animals lacking the adjuvant. As previously stated, this latter group did not reveal allergic enhancement from the 0.10 ppm of ozone. However, when the pertussis adjuvant factor was present, the low ozone concentration of 0.10 ppm still produced a highly significant enhancing effect for allergic sensitization. This finding raises a health question. Does the segment of the population undergoing whooping cough immunization experience added risk of developing respiratory allergies in the presence of elevated levels of environmental ozone?

II. Preliminary Studies on Allergic Enhancement from Ozone in Guinea Pigs

Work was begun during this project to study the allergic enhancement by ozone in an animal model where the allergic reaction would be analogous to an asthma attack in human subjects. The allergically sensitized animal, and the asthmatic human, are both in an anaphylactically sensitized state. That is, an injection of the allergen intravenously can put them into a state of anaphylactic shock. Shock is the method used to test for sensitization in mice because their lungs do not respond strongly as shock organs. However, the lungs of guinea pigs respond to allergic reaction from inhaled allergen with symptoms resembling those occurring in the human lung during an asthma attack. The central feature of this is contraction of bronchiolar smooth muscle and the accompanying effects of airway restriction. Three preliminary trials were performed to develop a suitable protocol with respect to the sensitization schedule, and appropriate allergen concentrations for both sensitization and the elicitation of disease response in the lungs. Experiments will be run in the ensuing project

period with a protocol including three sensitizing exposures to aerosolized ovalbumin in a concentration of 1 mg/ml. Comparisons in allergic reactivity will be made between guinea pigs maintained in ambient filtered air and those exposed to 4 days of continuous ozone exposure at 0.20 ppm. Lung reactivity will be determined when a 20 mg/ml concentration of ovalbumin is aerosolized as a fourth contact with the allergen.

III. Study on the Effect of Ozone on the Influenza Process

Our past work on viral pneumonia in mice infected with aerosolized human influenza virus was performed at ozone concentrations of 0.40 and 0.64 ppm. An ozone effect was detected as an alteration in the severity of viral pneumonia wherein the ozone exposed animals experienced a milder process. Influenza is often a fatal disease in mice, and it was found that significantly fewer mice died when the animals were maintained in a continuously elevated ozone environment before and after infection by virus inhalation. Two factors were considered as probable mechanisms for this ozone effect. When we did in vitro investigations, it was found that ozone at the levels used in the mouse studies could readily inactivate influenza virus. It was considered likely that inhaled ozone could inactivate virus as it was transferred from infected to non-infected cells in the airways. In this current project an experiment was run at an ozone level of 0.16 ppm to determine if the observed effects would persist. This ozone concentration was chosen because it is frequently encountered in the environment and because in vitro studies showed that it had only a minimal, and delayed, effect for influenza virus inactivation. The experiment showed that the ozone effect did persist, and significantly less mortality occurred in the ozone exposed animals. A second factor may be contributing to this phenomenon. Ozone may modify the respiratory membranes in ways that lessen the degree of virus replication in lining cells of the airways. Metaplastic changes in the cells of the membranes may be considered a change of one kind of tissue cell for another. The abnormal airway lining surface in ozone exposed lungs is, no doubt, a tissue response to a persisting, injurious irritant. This could mean that replacement cells were changed with respect to their ability for serving as host cells to the influenza virus. It would require in depth virological studies to pursue this aspect of the mechanism. Such a study is not now proposed as a project for Air Resources Board support.

IV. Publications during this Support Period

During this support period, 6 studies appeared in print wherein the Air Resources Board was cited for support. These publications are identified in the body of this report along with brief resumes on the content of the papers.

CONCLUSIONS

I. The threshold for allergic enhancement to an inhaled allergen by ozone exposure was found to lie between 0.10 and 0.13 ppm in the animal model. This ozone concentration is frequently present in the environment of man, and its potential consequences for increasing the numbers of individuals

with respiratory allergies poses a serious question for the prevention of human illness.

II. When the adjuvant, killed Bordetella pertussis bacteria, was injected into mice to heighten the immune responses to an allergen, the low level of 0.10 ppm of ozone was still capable of enhancing allergic sensitization. Therefore, the threshold ozone concentration for this health effect is less than 0.10 ppm, when acting in consort with B. pertussis adjuvant.

III. Allergic enhancement by ozone should be demonstrated in an animal model where allergic reactivity occurs in the lung, in a process analogous to human asthma. A suitable protocol for this study has been developed, and the experimentation will continue in the ensuing project period.

IV. Enough work has been done to establish that ozone inhalation, even at the low level of 0.16 ppm, will reduce the severity of pneumonia in the mouse from influenza virus infection. The mechanism for this effect has not been established.

V. During this project period, 6 publications have appeared detailing work concerning these investigations.

RECOMMENDATIONS

I. An adverse health effect from ozone exposure has been demonstrated as enhancement of lung sensitization to an inhaled allergen in the mammalian lung. This should be brought to the attention of the medical community, and groups concerned with environmental protection. The use of this information for setting air quality standards needs consideration.

II. Further study is required to clarify the adjuvant effect from B. pertussis vaccine when acting in consort with ozone exposure to augment allergic responses against inhaled allergens. We have found that a single injection of pertussis vaccine into mice, along with a low ozone concentration of 0.10 ppm, enhances the allergic responses. The same level of ozone did not enhance in the absence of pertussis organisms. This finding raises the question of a link between the large number of respiratory allergies in children, and the presence of increased environmental ozone, along with the routine whooping cough immunization of children with a series of B. pertussis injections. Experimentation on the amount of B. pertussis vaccine required to produce this effect in ozone exposed mice is an important matter that needs answering.

III. Studies should be completed toward the demonstration of allergic enhancement from ozone exposure, where allergic sensitivity is detected as lung reaction to inhaled allergen. The process in the guinea pig lung is analogous to an asthma attack in man. The study will make this health effect from ozone more readily understood, with respect to both mechanism and significance.

IV. One more experiment is to be run during the ensuing project period to complete the studies of ozone effects on viral pneumonitis. Extensive edema fluid collects in the lungs during a severe influenza infection. In the

mouse model, edema reaches its peak on day 8 following infection, and fatalities accumulate over the following days. We have also found that ozone exposure increases the amount of fluid in the airways, and this reaches its maximum after 4 days of continous ozone exposure. The severity of the disease process could be increased if the two edemagenic effects occurred simultaneously. Such circumstances could easily occur in the natural situation. The planned experiment will examine the ozone effect on influenza when exposures to virus and ozone are timed to maximize the edemagenic effects.

The mechanism by which inhaled ozone reduces the severity of influenza in mice is of considerable interest to the field of virology. Investigation of the matter would lie within the purview of medical science research agencies. It is suggested that funds from the Air Resources Board need not be used for this purpose.

PROJECT REPORT

Health Effects from the Inhalation of Oxidant Air Pollutants as Related to the Immune System

(A1-054-32)

I. Purpose and General Background to the Project

The appropriate stance to take regarding acceptable levels of air pollutants in California is affected by the increasing need to determine what actually happens to mammalian tissue following pollutant exposure. A growing body of epidemiological literature presents positive correlations of lung diseases and reduced pulmonary capacity with the presence of high levels of air pollutants [1,2,3]. However, skeptics can question the interpretations of such investigations by pointing to uncontrolled variables, and the fact that positive evidence of tissue changes are not shown by such studies. If air pollutants are shown to adversely affect the respiratory tract by enhancing the likelihood of developing asthma, or by altering the recovery from respiratory infections, this information would have impact on public attitudes toward the control of air pollution. Controlled experimentation on animals offers the means for determining alterations in the immune responses of the respiratory tract as mediated by the presence of air pollutants. Ozone has been shown by others to act as a powerful oxidizing agent that produces histologically recognizable damage to the lining epithelial cells of the respiratory tract [4,5].

Immunology offers real promise in determining events set in motion by air pollutants. Increases of immunological activity in the lung are clear indicators of injury to the tissues. Increases in allergic lung disease (asthma) among a test population of animals following inhalation of an air pollutant establishes a cause and effect relationship between the toxic inhalant and enhancement of a specific health problem. Changes in the pattern of immunological defenses of the lung against infecting virus (influenza virus) following exposure to ozone offers evidence that the susceptibility of lung cells to the virus has been altered.

The immune system is primarily concerned with the body's handling of foreign substances entering the tissues. If the foreign substance provokes an immune response, it is referred to as an antigen. For example, in an influenza attack the influenza virus acts as an antigen, and the immune responses to the viral antigen are specific antibodies and cells reacting with virus, which lead to its destruction and elimination from the body. A good functional immune system is, therefore, vital to the warding off of infections and the maintenance of a healthy state.

In some situations the immune responses play an undesirable role. An example is human asthma where the inhaled antigen is usually some inert substance, such as dog or cat dander, household dust, plant pollens, etc. The antibody response in this case is detrimental to health since the reaction of the antigen and antibodies in the lung leads to the release of body chemicals that contract the airways and produce the typical asthma attack. These varied responses of the lung to antigens are related to the

multiple forms in which antibodies are made in the body. Four important classes of proteins are now recognized as antibodies which perform different functions. The antibodies are called immunoglobulins (Ig), and the four classes are IgM, IgG, IgA, and IgE.

In the instance of asthma, the IgE antibodies are specifically responsible for the disease. Cells producing IgE are distributed along the respiratory mucous membrane.

A. Studies on the Threshold Level of Ozone for Enhancing the Allergic Response to Inhaled Allergen

Five previously performed experiments consistently demonstrated enhancement of allergic lung sensitization from inhaled ozone. Figure 1 shows results obtained in experiments with ozone concentrations of 0.64 ppm down to 0.16 ppm. As indicated in Table 1, all of these experiments showed a significant level of allergic enhancement.

Table 1. Statistics on Allergic Sensitization of Mice Maintained in Filtered Ambient Air Versus Mice Exposed to Ozone[1]

Experiment	Number of Mice: Ozone Exposed Groups	Number of Mice: Filtered Air Groups	Ozone Level	Probability[2]	Significance
A	14	4	0.64 ppm	P<0.018	+
B	29	19	0.64 ppm	P<0.007	+
C	20	20	0.40 ppm	P<0.001	+
D	60	55	0.24 ppm	P<0.0001	+
E	59	60	0.16 ppm	P<0.0195	+

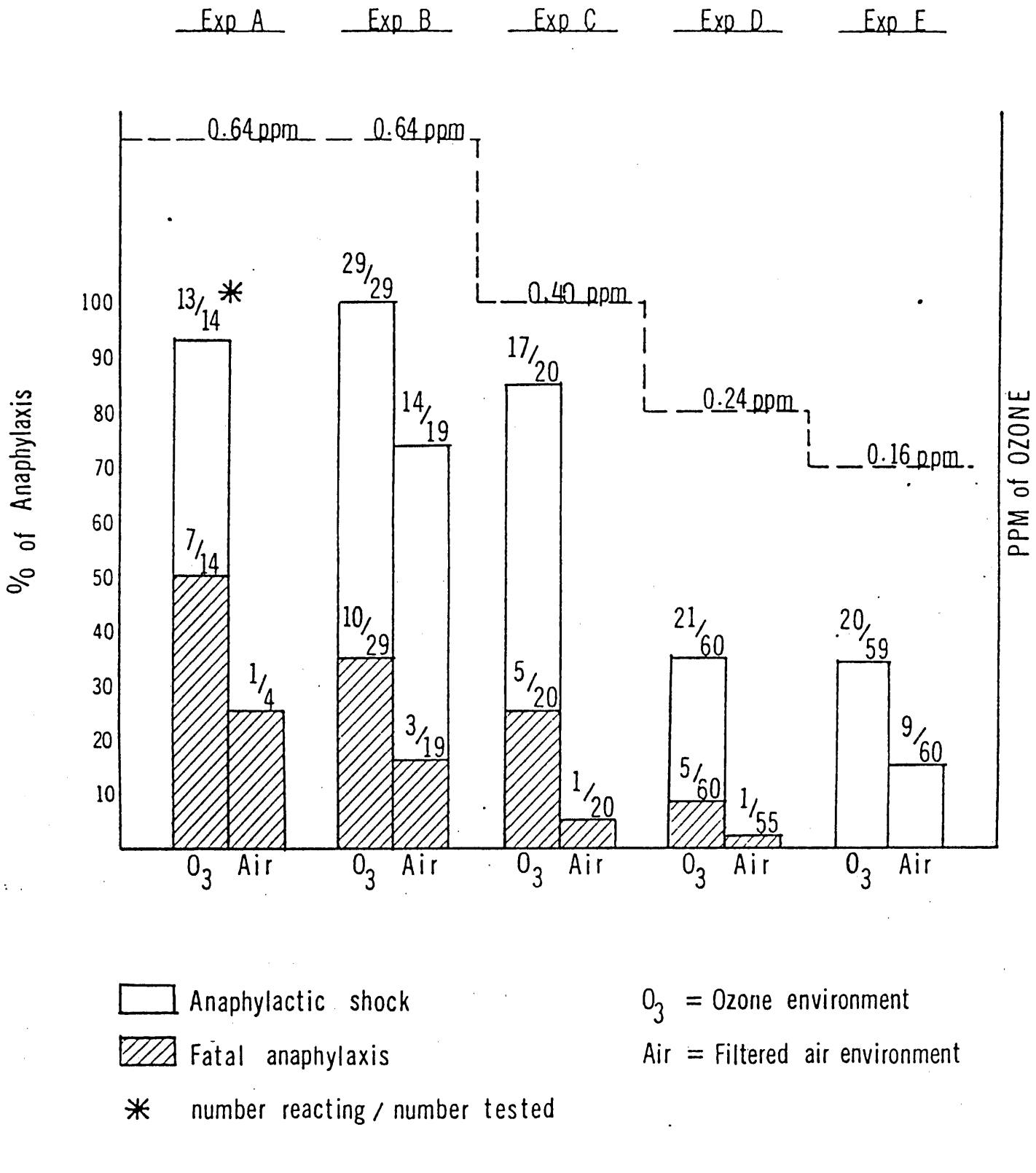
[1]In a given experiment the contact with aerosolized allergen was equal for the two compared groups. Ozone exposures were continuous for 3-4 day periods, which were repeated intermittently in 4 cycles over approximately 6 weeks.

[2]Probability calculated by the exact method. Comparison groups are ozone exposed mice versus filtered air control mice.

In the current project it was proposed that the threshold concentration of ozone for enhancing allergic responses be determined. It was our expectation that two experiments would be necessary to fine-tune the ozone level which is safe, with respect to avoiding the enhancing effect on allergic lung disease. The initial experiment was to be run at an ozone concentration of 0.10 ppm.

Figure 1

Anaphylactic Reactivity of Mice Following Allergen Contact and Exposure to Ozone



B. Study to Demonstrate Allergic Enhancement from Ozone by an Asthma-like Response

A second objective of the project was to elicit allergic enhancement by ozone in an animal model where the reaction is an analogue of an asthma attack in human subjects.

The lungs of guinea pigs respond to allergic reactions with symptoms resembling those from the human lung during an asthma attack. A central feature of this is contraction of bronchiolar-smooth muscle and the accompanying effect of airway restriction [6]. When sensitized guinea pigs inhale an allergen, they appear uneasy and show labored breathing. In severe attacks, they may collapse and even die through lack of air (anoxia). Necropsy reveals lungs inflated with trapped air because the animal was unable to expire. This is similar to autopsy findings on a human subject when death occurs during an asthma attack.

The asthmatic person, or guinea pig, is in an anaphylactically sensitized state. This means that intravenous injection of the allergen could put them into shock.

At the current stage in our allergy studies, it seems appropriate to demonstrate allergic responsiveness of lung tissue in an animal model. Work with mice has given us the advantage of greater numbers for statistical treatment of the data. However, with the knowledge gained thus far, it becomes important to determine whether ozone exposure can lead to enhanced allergic sensitization in the sense of increasing lung disease *per se*.

C. Effect of Reduced Ozone Concentration on the Influenza Process

Our previous work detailed information gained on the influenza virus infection process. The most notable effect was a disease of less severity in mice that had been exposed to ozone. This was apparent in 4 experiments where ozone exposed animal groups experienced less mortality. Broad interpretations on the basis of these results are not thought to be appropriate for 2 reasons. The ozone levels were high (0.64 ppm and 0.40 ppm), and the animals were maintained continuously in the high ozone environment. Both of these factors depart from the usual environmental conditions. It was reasonable to work at higher ozone concentrations when searching for an effect. After an effect had been found, it was important to determine how it would relate to conditions more nearly approximating those of the environment. In this project an experiment was run at the ozone level of 0.16 ppm to determine if the observed effects would persist.

II. Design-Materials and Methods

A. Allergy Studies

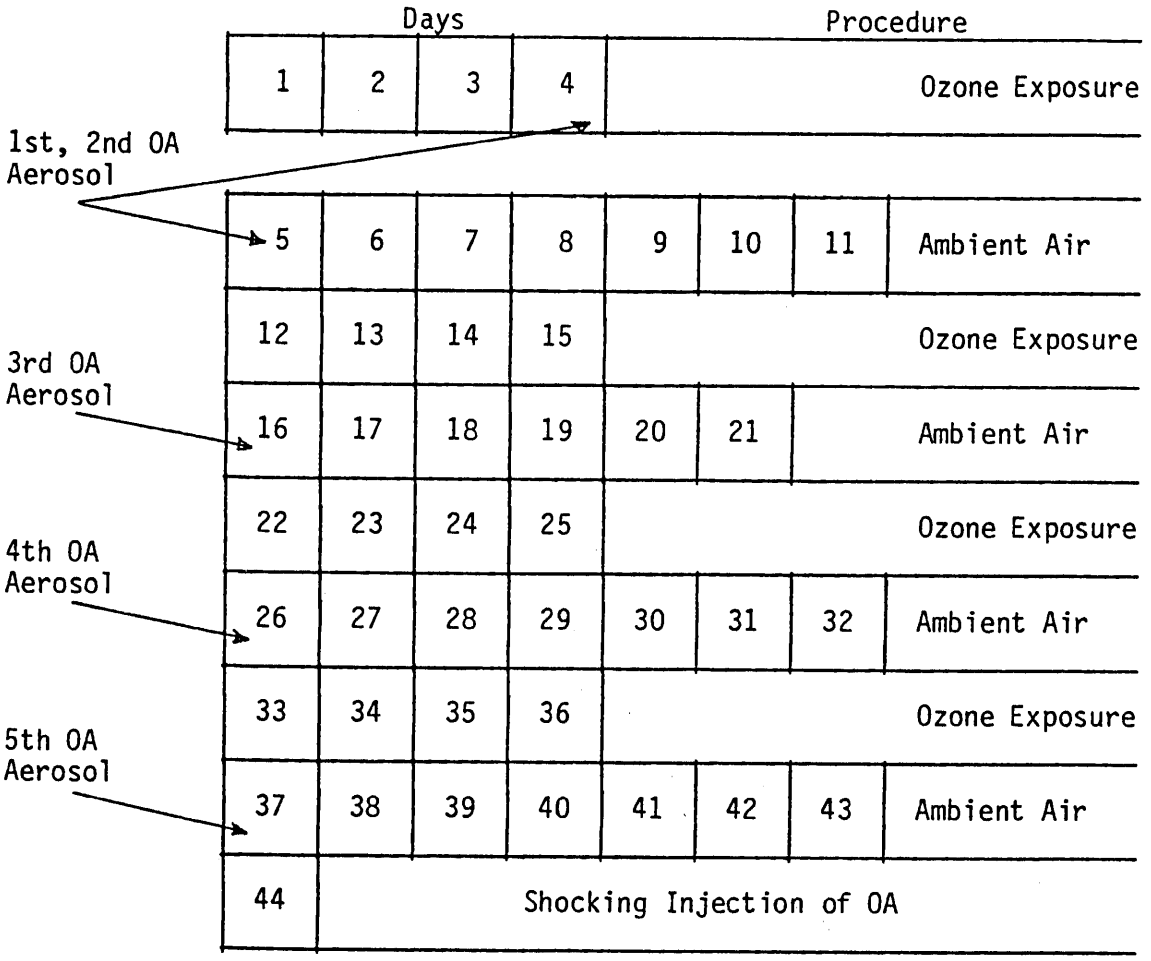
The circumstances of lung sensitization were based on findings from our previous studies. It was shown that damage to the respiratory membranes from ozone could be monitored by quantitating the serum albumin levels in respiratory secretions [7]. On day 4 of ozone exposure a peak of serum albumin in secretions indicated that this was a period of impaired membrane integrity, and a time when extrinsic antigen 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. The basic design of these experiments is shown in Figure 2.

Figure 2

Ozone Effect on Allergic Enhancement in Mice:

Experimental Design for Ozone Exposure and Allergen Contact



Ozone = Concentration was continuous over 4 day periods but varied in different experiments from 0.10 ppm to 0.64 ppm.

Allergen = Ovalbumin (OA); a 2% solution was used for aerosolization and the shocking injections.

Mice (Specific Pathogen Free) were exposed to ozone in stainless steel chambers at the Environmental Chamber Facilities of the California Primate Research Center. Ozone concentration was continuously monitored by the ultraviolet photometric ozone analyzer (Dasibi Environmental Corporation, Glendale, CA). Control animals were housed in an environment of filtered ambient air.

Mice were allergically sensitized to an allergen by inhalation of an aerosolized allergen (lung sensitization). The allergen was ovalbumin (OA), which is a purifed crystalline form of albumin obtained from the chicken egg. Ovalbumin was used to mimic the inhalation of environmental allergens, such as plant pollen. A variety of proteins from plant, animal or microbial sources can be allergens in man.

Ovalbumin served well as an experimental allergen since its size of 44,000 daltons placed it in that range of proteins that are small enough to be absorbed through mucous membranes and large enough to be sufficiently complex to function as immunogens [8]. 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 would be effective.

After 4 days of continuous ozone exposure the mice had allergen contact from 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 μm (range of 0.5 to 3 μm). Mice were placed in the chamber and were exposed for 30 min to nebulized OA (2% solution in sterile distilled water). The relative humidity in the chamber was approximately 50% at 22°C.

The animals were then maintained in ambient air for several days before the cycle of ozone and aerosolized antigen was repeated over 4 allergen contact cycles. Times 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.

A brief account of events in this type of allergy includes the following considerations. IgE is well established as the antibody responsible for sensitizing individuals for asthmatic attacks and anaphylactic shock [9]. The B-lymphocytes synthesizing IgE are located in bronchus-associated lymphoid tissue of the lung under the epithelial lining of the airways [10]. 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 cells by receptors on the antibody molecules. Such a mast cell is sensitized. If the allergen is reintroduced, as by inhalation, the resulting antigen-antibody reaction causes the mast cell to degranulate and release pharmacologically active substances that produce the tissue responses seen in an asthma attack. An individual experiencing asthma from inhalation of the allergen 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.

Since the lung is not a dominant shock organ in allergically sensitized mice, it was necessary to reveal allergic sensitization by another means. The test procedure chosen was induction of anaphylactic shock following the intravenous injection of OA. 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, and the mice became increasingly listless and then prostrate. Within 10 min most of the severely sensitized animals were prostrate. Deaths occurred 20 to 40 min after the injections. The mice were watched carefuly over a 2-hr period, and those destined to survive the anaphylactic shock gradually increased their activity as the syndrome subsided. A few survivors gained a normal appearance within 40 min, but there were also severely affected individuals that remained cyanotic and immobile, with ruffled fur, for more than 2 hours.

Each experiment required that several animal groups be tested for anaphylactic sensitivity. The positive-control group had been sensitized by two injections of ovalbumin, and the development of shock in nearly all members of this group demonstrated that a functional allergic disease state had been established. Normal mice were injected as the negative-control group, and their failure to display any ill effects from the ovalbumin injection showed that the injected material was free from inherent toxicity. Important groups were those that had simply been exposed to aerosolized antigen, since some members of those test populations would, as expected, become allergically sensitized. Comparison of these filtered ambient air control groups with animals that had additionally been exposed to ozone were the critical test for revealing enhancement of the allergic state by ozone.

B. Effects on Influenza Virus Induced Pneumonia from the Inhalation of Ozone

It is of special interest that the influenza virus affects many of the same cells in the respiratory system that are damaged by ozone inhalation. We have investigated the pathogenesis of influenza virus infection in mice by exposing them to aerosols of influenza virus, and examining the infected respiratory tissues.

Previous work showed that fatal influenza infections were less frequent in ozone exposed animals (0.40 ppm and 0.64 ppm) than in infected mice that were breathing ambient air. This surprising observation was made in 4 separate experiments. Two factors appeared to interplay in producing this effect. First, abnormal respiratory membranes developed as a result of 15 days of continuous ozone exposure, and this appeared to change the role of lining epithelium as host cells for influenza virus. Second, ozone at the concentration used was found to inactivate influenza virus. This effect could diminish the spread of virus within the airways. Broad interpretations could not be made on the basis of these virus infection results because of the ozone concentrations used, and the continuously maintained elevation of ozone were not in accord with usual environmental conditions.

In the current project period an experiment was run at the reduced ozone concentration of 0.16 ppm to approach a more typical condition and to minimize the opportunity for ozone inactivation of virus in the lung.

Mice were maintained for 14 days of continuous exposure to 0.16 ppm of ozone in chambers as previously described. An equal number of animals was held in a chamber receiving filtered ambient air. The mice were then exposed to nebulized influenza virus (Influenza A_0 virus (WSN) propagated in Madin-Darby bovine kidney cells). The virus aerosol was generated in the TRI-R Airborne Infection Apparatus. The animals were held for an additional 14 days in either the ozone or ambient air environment.

III. Experimental Results and Discussion

A. Threshold Study on the Role of Ozone in Allergic Enhancement - Phase I

Previous experiments had demonstrated that ozone exposure at levels of 0.64, 0.40, 0.24, and 0.16 ppm will enhance the development of an allergic state to inhaled antigen. Mice were used in these studies to determine the immunological responses of mammalian lungs to aerosolized protein antigen (ovalbumin). The allergic sensitivity of live animals was detected by anaphylactic shock following the intravenous injection of ovalbumin.

To determine the threshold concentration of ozone producing this effect, the oxidant was reduced to 0.10 ppm. The experiment required a series of control groups to reveal the appropriate responses (or lack of responses) by the mice following injection of the allergen (antigen). The critical groups for comparison purposes were animals maintained in filtered ambient air versus those exposed to 0.10 ppm of ozone. Additional comparison groups were those animals that had received an adjuvant to heighten their immunological responsiveness for the formation of the antibody class known as IgE. The adjuvant used was Bordetella pertussis cells. This bacterium is the cause of whooping cough in man, and the mice were injected with the same killed bacterial vaccine preparation that is used to immunize children. Table 2 presents a resume of the assay for *in vivo* allergic sensitivity.

Table 2 - Allergic Responses of Mice Exposed to 0.10 ppm of Ozone

Treatment Groups	Total mice	Anaphylactic Shock*		Fatal Anaphylaxis	
		No.	Percent	No.	Percent
Ozone	94	4	4.3%	0	
Ambient air	98	6	6.1%	3	3.06%
Ozone + Adjuvant**	98	88	89.8%	60	61.2%
Ambient air + Adjuvant	101	67	66.3%	47	46.5%
Controls					
Normal	10	0		0	
Adjuvant only	10	0		0	
Ozone only	10	0		0	
Ovalbumin Immunized	20	20	100%	18	90%
Ozone + OA Immunized	9	9	100%	9	100%
OA Immunized + Adjuvant	20	20	100%	20	100%

*Antigen = ovalbumin (OA).
**Adjuvant = Bordetella pertussis cells.

In this study the groups contacting aerosolized antigen, but maintained in ambient filtered air, served as the control groups for comparison with groups that were exposed to ozone. Table 3 presents statistical data indicating where significant responses did, or did not, occur when comparing those sets of animals. In addition, small groups of control animals were tested to delineate the reliability of the anaphylactic shock system and the reagents employed. The complete lack of response in the normal unmanipulated controls indicated that there was no toxic effect following the intravenous injection of the shocking dose of antigen if the animals had not been allergically sensitized. Also, a previous injection of the Bordetella pertussis adjuvant did not in itself increase reactivity if the animals had not contacted the allergen prior to the single injection of the shocking dose. Some investigators have reported increased reactivity to histamine following ozone inhalation. The "ozone only" test group indicated that the inhalation of ozone under these circumstances did not alter responses in animals that had not become sensitized by virtue of previous contact with the allergen. Three sets of animals were used to show that animals were, indeed, anaphylactically sensitized if the ovalbumin antigen had been injected prior to administration of the shocking dose. The allergic state was established when ovalbumin was injected intraperitoneally, when animals inhaled ozone in addition to receiving the sensitizing injections, and when Bordetella pertussis adjuvant was given to ovalbumin injected animals. Among these six control groups, the anaphylactic shock reaction was universally absent in animals that had not contacted ovalbumin prior to the shocking injection, and it was universally present in animals that had allergen contact prior to the intravenous

injection of the ovalbumin. In essence, these test groups were flawless in demonstrating that response to the intravenous injection of allergen was dependent upon previous sensitization and that prior allergen contact would establish a demonstrable allergic condition.

The concentration 0.10 ppm of ozone appeared to be below the threshold for enhancing allergic sensitization, with only a few animals demonstrating anaphylactic shock in either the ozone exposed group or the group held in filtered ambient air. Table 3 presents X^2 statistical analysis indicating that there was no significant difference between the responses of these two groups.

Table 3 - Statistics on Allergic Sensitization of Mice Exposed to 0.10 ppm of Ozone

Comparison Groups	Effect	X^2 Values	Significance	Probability
Ozone versus Ambient air	Anaphylactic Shock	0.3387	-	0.5 to 0.7
	Fatal Anaphylaxis	0.9303	-	0.3 to 0.5
Ozone + Adjuvant versus Ambient air + Adjuvant	Anaphylactic Shock	15.89	+	<0.001
	Fatal Anaphylaxis	4.3	+	0.025 to 0.05

The outcome for animals receiving the adjuvant injection was quite different. In this situation, wherein the ability of the animals to form the IgE class of antibody was augmented by an injection of adjuvant, the number of sensitized animals was greatly increased. Also, the low level of ozone (0.10 ppm) was still causing an enhancing effect on allergic sensitization since a significant increase in anaphylactic shock was produced in that group.

This experiment was interpreted as presenting two points of information:

1. Ozone at a concentration of 0.10 ppm was below the threshold for enhancing allergic sensitization in conventionally maintained animals under the conditions presented in the study.

2. Ozone at a concentration of 0.10 ppm had an effect for enhancing allergic sensitization if immune regulation in the body was modified to favor IgE synthesis. That modification occurred in this study when injected Bordetella pertussis cells functioned as an adjuvant. This finding raises an interesting aspect of the health effects from ozone inhalation, namely, the combined roles that inhaled ozone and routine immunization procedures for protection against whooping cough may play in establishing allergies among children.

B. Threshold study on the role of ozone in allergic enhancement - Phase II

It had been planned in the proposal for this research that the results of the ozone study at 0.10 ppm would be used to indicate the ozone level

for a second threshold experiment. Ozone at a concentration of 0.10 ppm was below the threshold for enhancing allergic sensitization in conventionally maintained animals under the conditions presented in the study. Consequently, the oxidant level for this experiment was set at 0.13 ppm. As shown in Table 4 the appropriate responses were obtained in control groups following injection of the test allergen. When the group exposed to 0.13 ppm of ozone was compared with animals maintained in filtered ambient air, a significant allergic enhancement was seen to occur from the ozone effect (probability = < 0.01).

Table 4 - Allergic Responses of Mice Exposed to 0.13 ppm of Ozone

Treatment Groups	Total Mice	Anaphylaxis*		Fatal	
		No.	Percent	No.	Percent
Ozone	102	15	14.7%	5	4.9%
Ambient air	81	3	3.7%	0	
Controls					
Normal	10	0		0	
Ozone only	10	0		0	
Ovalbumin immunized	19	19	100%	18	95%
Ozone + OA immunized	10	10	100%	9	90%

*Antigen = ovalbumin (OA)

The results of 6 experiments with ozone concentrations ranging from 0.64 down through 0.13 ppm have uniformly shown allergic enhancement. The true ozone threshold is now considered to be a concentration less than 0.13 ppm and greater than 0.10 ppm. These data can now be organized for publication.

C. Preliminary trials to demonstrate allergic enhancement from ozone in an asthma-like process

Allergic bronchoconstriction in the guinea pig is a useful model system for the study of antigen-induced bronchoconstriction in man. The asthmatic person, or guinea pig, is in an anaphylactically sensitized state. This means that injection of allergen intravenously could put them into shock with a process analogous to the allergic events that we have measured in mice. With the knowlege gained thus far, it is important to determine whether ozone exposure leads to enhanced allergic sensitization in the sense of increasing lung disease per se. Three preliminary trials have been performed to determine appropriate allergen exposure protocols. In order that the effect of ozone might be observed, it was first necessary to establish a regimen for allergic sensitization of guinea pigs breathing ambient air. A low level of allergic reactivity was needed in that animal group so that augmentation of the response in an ozone exposed group could be compared.

In the first trial 4 white-skinned guinea pigs, 250-300 g in weight, had an initial contact with the allergen from a 30 minute exposure to aerosolized ovalbumin in a 2% solution. The animals were maintained in an environment of ambient air, and the allergen contact was repeated at weekly intervals. On the third aerosolization, the 4 animals showed symptoms of airway restriction. When a fourth contact with aerosolized antigen was made, all animals responded with obvious signs of dyspnea wherein 2 collapsed. This trial demonstrated that allergic bronchoconstriction could be recognized in the test animals. However, the 100% allergic response in animals maintained in ambient air indicated that the sensitization procedure needed modification.

In a second trial the experiment was repeated except that the dose of aerosolized allergen was reduced to a 0.1% solution. This was 1/20th the amount used in the first trial. The animals were given 3 sensitizing contacts with the allergen. The fourth aerosol exposure was a provoking allergen concentration of 2% solution. One animal displayed severe dyspnea, collapsed, and died. A second animal developed moderate dyspnea, while the remaining 2 guinea pigs appeared unaffected. A third trial was performed wherein the dose of aerosolized allergen was again used at the concentration of a 0.1% solution. Four guinea pigs were maintained in ambient air. Four additional guinea pigs were held in a chamber containing 0.20 ppm of ozone for four days. They were then exposed to aerosolized allergen along with the ambient air control guinea pigs. All animals were held in ambient air for a week and then the cycle was repeated for a total of three 30 minute sensitizing exposures to the allergen. The animals were again rested a week in ambient air. All of the guinea pigs were then tested for asthma-like responses by inhaling a 2% solution of aerosolized ovalbumin. Table 5 presents results from the third trial.

Table 5 - Preliminary Trial for Observing Asthma-like Responses in Ozone Exposed Guinea Pigs

Environment	Guinea Pig	Symptoms from third allergen contact*	Symptoms from provoking dose of allergen**	Test for skin sensitizing antibodies
Ozone Exposed	#1	None	None	Negative
Ozone Exposed	#2	None	None	Negative
Ozone Exposed	#3	None	None	Weak positive
Ozone Exposed	#4	Dyspnea	Dyspnea and collapse	Strong positive
Ambient Air	#5	None	None	Negative
Ambient Air	#6	None	None	Negative
Ambient Air	#7	None	Dyspnea and collapse	Strong positive
Ambient Air	#8	None	None	Negative

*Aerosolized ovalbumin solution (0.1%).
**Aerosolized ovalbumin solution (2.0%).

One of the ozone exposed guinea pigs displayed signs of airway restriction from inhaling ovalbumin at the time of the third sensitizing exposure (animal #4). This indicated that the animal had been allergically sensitized by only two exposures to the allergen. This same animal developed severe dyspnea and collapsed from anoxia when the provoking dose (2% ovalbumin solution) was aerosolized. One of the animals in the control group maintained in ambient air (animal #7) displayed a similar level of dyspnea and collapsed. Three days later all of the animals were tested for atopic sensitivity by intradermal injections of ovalbumin in 4 graded doses. The 2 animals that had shown severe airway restriction gave strongly positive wheal and flare skin reactions, which were fully developed within 30 minutes and had disappeared after 2 hours. In addition, a second ozone exposed animal (#3) showed sensitization on the skin test.

The protocol used in the third trial appears appropriate for a larger scale experiment. It was seen that 1 out of 4 animals maintained in ambient air became allergically sensitized. The ozone exposed animals presented suggestive evidence of greater responsiveness to the allergen. One animal had become sensitized by only 2 contacts with the allergen, and 2 of 4 animals formed skin-sensitizing antibodies to the allergen. During the ensuing project period work will continue on this phase of the study.

D. Effect of reduced ozone concentration (0.16 ppm) on the influenza process

Previous studies on influenza virus infection in mice had revealed that the disease process was less severe in animals that were held in a raised ozone environment before and after exposure to the virus. In those experiments the ozone concentration was quite high (0.64 and 0.40 ppm). Our *in vitro* investigations have revealed that ozone at those levels can readily inactivate influenza virus. Ozone reduced to a level of 0.16 ppm did not adversely affect the virus during the first 8 hours of contact, and it was considered necessary to perform an experiment at this reduced level to determine whether the mortality sparing effect of ozone would disappear. The experiment has been run and mortality data were obtained as shown on Table 6.

Table 6. Mortality Pattern of Influenza Virus Infection in Mice

	Day of Death Following Infection								
Animal Group	7	8	9	10	11	12	13	14	Total
Filtered ambient air	0	4	8	2	2	1	2	2	21
Ozone exposed (0.16 ppm)	0	0	3	1	1	0	0	0	5

Death occurred in 5 animals out of 60 in the ozone group and 21 animals out of 60 in the ambient air group. Chi square analysis indicated a significant difference in the animal responses with a probability of <0.001. An effect from ozone was seen again, even at this reduced concentration of the

oxidant. Tissues and body fluids will be analyzed from this experiment and deliberations will be made about the underlying mechanisms leading to this effect.

IV. Publications that have appeared during this project period

A. "Immunoglobulin E-containing cells in mouse lung following allergen inhalation and ozone exposure." L. J. Gershwin, J. W. Osebold, Y. C. Zee. International Archives of Allergy and Applied Immunology 65: 266-277, 1981.

Cells containing IgE were enumerated and their location in mouse lungs was determined by direct immunofluorescence. Lungs were studied from mice that had been immunized with aerosolized ovalbumin as well as from normal mice and from mice that were exposed to ozone (0.40 or 0.64 ppm) prior to receiving aerosolized antigen. Total IgE cells increased 9.4-fold in mice that received aerosolized ovalbumin as compared to normal mice. When ozone exposure was added to the effects of aerosolized ovalbumin, the increase of IgE cells over normal was 34.2-fold. IgE cell counts correlated well with anaphylactic sensitivity to intravenous challenge with ovalbumin.

B. "Immunogenic collagen in induced ascitic fluid: Concurrent preparation of anti-murine immunoglobulin E." L. J. Gershwin, J. W. Osebold, Y. C. Zee. American Journal of Veterinary Research 42: 1306-1309, 1981.

Antiserum to murine IgE was induced by inoculation of goats with a pool of partially purified IgE from serum and adjuvant-induced ascitic fluid. Antibodies to collagen were found to be present in the antiserum when the latter was conjugated with fluorescein isothiocyanate applied to mouse pulmonic tissue. The connective tissue fluorescence was eliminated following absorption with mouse collagen. A high quality anti-IgE reagent was produced.

C. "Dynamics of B-lymphocytes in the lungs of mice exposed to aerosolized influenza virus." S. L. Owens, J. W. Osebold, and Y. C. Zee. Infection and Immunity 33: 231-238, 1981.

Immunoglobulin-containing cells were revealed by immunofluorescence in lung sections from mice infected with influenza virus by the aerosol route. The responding B-cell populations appeared in two principal locations: along major airways and in consolidated lesions within lung parenchyma. IgA-containing cells were the most numerous isotype occurring along the airways.

D. "Exposure to ozone reduces influenza disease severity and alters distribution of influenza viral antigens in murine lungs." J. A. Wolcott, Y. C. Zee, and J. W. Osebold. Applied and Environmental Microbiology 44: 723-731, 1982.

Exposure of mice to ozone at a concentration of 0.40 ppm was shown to alter the pathogenesis of respiratory infection after aerosol infection of mice with influenza A virus. Findings included prolonged survival time and reduced mortality in the ozone exposed animals. Direct immunofluorescence studies indicated that exposure to ozone reduced the involvement of

respiratory epithelium in the infectious process and resulted in a less widespread infection of the alveolar parenchyma. The study suggested that the distribution of influenza virus in the murine lung was a key factor in disease severity.

E. "An in vitro system for studying the effects of ozone on mammalian cell cultures and viruses." D. C. Bolton, B. K. Tarkington, Y. C. Zee, and J. W. Osebold. Environmental Research 27: 466-475, 1982.

A unique in vitro system was developed for exposing mammalian cell cultures, viruses, or both to ozone under conditions mimicking those of the respiratory tract. The system was designed to allow 2 test cultures and 1 control culture to be simultaneously exposed to different, precisely defined, concentrations of ozone. The input and exhaust concentrations of ozone were sequentially monitored with an ultraviolet photometric ozone analyzer.

F. "The biological effects of ozone on representative members of five groups of animal viruses." D. C. Bolton, Y. C. Zee, and J. W. Osebold. Environmental Research 27: 476-484, 1982.

In an effort to establish the biological relevance of the reactions of ozone with soluble proteins and lipid bilayer membrane systems, representative viruses from 5 major virus groups were exposed to moderate concentrations of ozone. The 3 enveloped viruses tested exhibited susceptibilities to ozone inactivation which correlated with their thermal lability, in the absence of ozone. The two non-enveloped viruses examined were relatively resistant to ozone inactivation.

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