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Ozone-Induced Modulation of Airway Hyperresponsiveness in Guinea Pigs

Richard B Schlesinger, Mitchell Cohen, Terry Gordon, Christine Nadziejko, Judith T Zelikoff, Maureen Sisco, Jean F Regal, and Margaret G Ménache

Includes a Commentary by the Institute's Health Review Committee

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HEI STATEMENT
Synopsis of Research Report 109

Effects of Ozone on Airway Hyperresponsiveness in Guinea Pigs

INTRODUCTION

Asthma is one of the most common chronic and potentially disabling diseases among children and adults. It is characterized by three findings involving the airways: reversible airway obstruction, inflammation, and hyperresponsiveness. The last characteristic is defined as a heightened tendency of the bronchial airways to constrict. The airways of people with asthma also constrict when they inhale an allergen to which they are sensitive, and the airways constrict more when they inhale a nonspecific airway irritant such as acetylcholine. Some people without asthma develop nonspecific airway hyperresponsiveness after inhaling irritants. Controlled clinical studies have shown that exercising humans exposed to ozone (an irritant) develop airway hyperresponsiveness. Therefore, it is possible that people with asthma and others sensitive to allergens may be more susceptible to allergen-induced airway hyperresponsiveness during periods when levels of ozone are elevated.

Studies with laboratory animals have shown that short-term exposure to ozone induces airway hyperresponsiveness. However, we have little information on the effects of longer-term ozone exposures. The study by Dr Richard Schlesinger and colleagues of the New York University School of Medicine sought to determine whether long-term, intermittent exposure to ozone induces or exacerbates airway hyperresponsiveness.

APPROACH

Schlesinger and colleagues used a well-established animal model of airway hyperresponsiveness and allergic asthma to determine whether ozone can induce airway hyperresponsiveness or exacerbate existing airway hyperresponsiveness. They exposed three cohorts of male and female guinea pigs to 0.1 or 0.3 ppm ozone for 4 hours per day, 4 days per week, for 24 weeks. Control animals breathed clean air. The ozone concentrations were relevant to those encountered by humans during periods of ozone pollution. For example, levels ranging from 0.12 to 0.4 ppm have been recorded in the United States. The investigators exposed one cohort of nonsensitized animals to ozone alone. They induced hyperresponsiveness in a second cohort by sensitizing them to the allergen ovalbumin by inhalation before ozone exposure began. They induced hyperresponsiveness in a third cohort by sensitizing them to ovalbumin at the same time ozone exposure began.

Schlesinger and colleagues measured nonspecific airway hyperresponsiveness in each cohort by periodic challenge with acetylcholine. They measured specific airway hyperresponsiveness in the second and third cohorts by periodic challenge with ovalbumin. At the end of the challenge period, half of the animals in each cohort were killed for biochemical and cellular measurements of markers of inflammation in lung fluids and tissue. The remaining animals breathed clean air for 8 weeks, during which time the investigators performed similar measurements.

RESULTS AND INTERPRETATIONS

Animals exposed to ozone (but not ovalbumin) did not develop airway hyperresponsiveness. Ozone exposure exacerbated nonspecific and specific airway hyperresponsiveness in both cohorts of animals exposed to ovalbumin. The effects generally depended on the dose but were independent of gender. The levels of nonspecific and specific hyperresponsiveness were quantitatively similar and persisted for 4 weeks after exposure ceased. Ozone did not affect the levels of selected markers of inflammation; therefore, the results do not support an association of the inflammatory parameters assessed in this study with airway hyperresponsiveness.

The response of the ovalbumin-sensitized animals to ozone is consistent with studies of short-term ozone

Continued

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Dr Richard Schlesinger at New York University School of Medicine, Tuxedo NY. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

exposure on allergen-induced airway hyperresponsiveness in laboratory animals. The results of this study add to these findings by documenting the effects on hyperresponsive animals exposed to ozone for extended periods. They suggest that people with hyperresponsive airways may experience an increased response during periods of elevated ozone levels. This possibility should be evaluated in human studies.

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Research Report 109

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Department of Environmental Medicine, New York University School of Medicine, Tuxedo, New York; Department of Pharmacology, University of Minnesota, Duluth, Minnesota; and University of New Mexico, Albuquerque, New Mexico

HEI STATEMENT

This Statement is a nontechnical summary of the Investigators' Report and the Health Review Committee's Commentary.

INVESTIGATORS' REPORT

When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three ouside technical reviewers and a biostatistician. The report and the reviewers' comments are then evaluated by members of the HEI Health Review Committee, who had no role in selecting or managing the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, if necessary, revise the report.

Continued

COMMENTARY Health Review Committee

The Commentary about the Investigators' Report is prepared by the HEI Health Review Committee and staff. Its purpose is to place the study into a broader scientific context, to point out its strengths and limitations, and to discuss remaining uncertainties and implications of the findings for public health.

RELATED HEI PUBLICATIONS

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INVESTIGATORS' REPORT

Ozone-Induced Modulation of Airway Hyperresponsiveness in Guinea Pigs

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ABSTRACT

Although acute exposure to ozone (O_3*) has been shown to influence the severity and prevalence of airway hyperresponsiveness, information has been lacking on effects due to long-term exposure at relatively low exposure concentrations. The goals of this study were to determine whether long-term repeated ozone exposures could induce nonspecific hyperresponsiveness in normal, nonatopic (nonsensitized) animals, whether such exposure could exacerbate the preexisting hyperresponsive state in atopic (sensitized) animals, or both. The study was also designed to determine whether gender modulated airway responsiveness related to ozone exposure.

Airway responsiveness was measured during and after exposure to 0.1 and 0.3 ppm ozone for 4 hours/day, 4 days/week for 24 weeks in normal, nonsensitized guinea pigs, in guinea pigs sensitized to an allergen (ovalbumin) prior to initiation of ozone exposures, and in animals sensitized concurrently with ozone exposures. Both male and female animals were studied. Ozone exposure did not produce airway hyperresponsiveness in nonsensitized animals. Ozone exposure did exacerbate airway hyperresponsiveness to specific and nonspecific bronchoprovocation in both groups of sensitized animals, and this effect persisted at least 4 weeks after the end of the exposures. Although the overall degree of airway responsiveness did differ between genders (males had more responsive airways than did females), the airway response to ozone exposure did not differ between the two groups. Ozone-induced effects upon airway responsiveness were not associated with the number of pulmonary eosinophils or with any chronic pulmonary inflammatory response. Levels of antigen-specific antibodies increased in sensitized animals, and a significant correlation was observed between airway responsiveness and antibody levels. The results of this study provide support for a role of ambient ozone exposure in exacerbation of airway dysfunction in persons with atopy.

INTRODUCTION

The tendency for pulmonary airways to alter their caliber, generally by constriction, in response to a variety of antigenic or nonantigenic stimuli is termed *airway* or *bronchial responsiveness*. Under normal circumstances, this response is an essential component of respiratory tract homeostasis. The relative sensitivity to such bronchoprovocative stimuli varies widely within the general population (Weiss et al 1981), but when airways react excessively, a state of hyperresponsiveness is said to exist. This hyperresponsiveness is often associated with atopy, a hypersensitivity to certain antigens (or allergens) that is mediated by specific antibodies. Although hyperresponsiveness and atopy have been linked with asthma (Peat et al 1996; Peden 2000; Wolfe et al 2000), the pathogenetic relations among hyperresponsiveness, atopy, and asthma remain unclear (Josephs et al 1990; Smith and McFadden 1995) because nonatopic individuals without a history of asthma or other chronic lung disorder can demonstrate hyperresponsive airways (Josephs et al 1990; Morgan and Reger 1991; Paoletti et al 1995).

The etiology and expression of many respiratory tract disorders involve environmental factors, one of which may be ambient air pollution. The evidence supporting such a role of pollution is convincing, and specific links with ozone, a ubiquitous pollutant, have been made in this regard. For example, population-based studies have

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

This Investigators' Report is one part of Health Effects Institute Research Report 109, 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 Richard B Schlesinger, 32 Travano Rd, Ossining NY 10562 USA.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award R82811201 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 by it 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.

demonstrated an association between ozone and the exacerbation of airway hyperresponsiveness and other asthma-related signs and symptoms (Zwick et al 1991; US Environmental Protection Agency [EPA] 1996; Thurston and Ito 1999; McDonnell et al 1999; Peden 2000). A relation between ozone exposure and airway responsiveness is also supported by controlled studies. Acute exposures to ozone concentrations of 0.3 ppm or more have produced transient airway hyperresponsiveness in normal laboratory animals (eg, Abraham et al 1980; Holtzman et al 1983; Gordon et al 1984; Gross and Sargent 1992). In addition, clinical studies have shown airway hyperresponsiveness after exposure of healthy persons to ozone levels as low as 0.08 ppm (eg, Seltzer et al 1986; Horstmann et al 1990; Ying et al 1990; Linn et al 1994).

After analysis of both the epidemiologic and experimental exposure databases, answers to two questions remain unclear: Is ozone involved in induction of hyperresponsiveness, and are atopic individuals more susceptible to ozone-induced alterations in airway function (eg, Holtzman et al 1979; Koenig et al 1985; Kreit et al 1989; McManus et al 1990; Linn et al 1992; Peden 2000)?

Limited epidemiologic studies of long-term exposure seem to suggest that ambient ozone exposure may be involved in the development of asthma (Thurston and Ito 1999). Furthermore, some experimental evidence indicates that ozone can enhance the ability to become sensitized to inhaled antigen or at least can increase bronchial responsiveness to subsequent antigen exposure (Molfino et al 1991; Jörres et al 1996; Jenkins et al 1999). Finally, there is some indication that repeated exposures to ozone may induce airway hyperresponsiveness; for example, once weekly exposures to 1 ppm produced persistent nonspecific hyperresponsiveness in nonatopic monkeys (Johnson et al 1988).

Because much of the experimental database regarding ozone's role in the induction and exacerbation of airway dysfunction, such as hyperresponsiveness, involves acute exposures, the role of more realistic, repeated exposures at relatively low concentrations could not be determined. Further examination of the airway effects of such exposures was clearly warranted. To this end, the present study evaluated airway responsiveness during 24 weeks of 4 hours/day, 4 days/week exposure to 0.1 and 0.3 ppm ozone, as well as 8 weeks after exposure. Nonatopic and atopic guinea pigs of both genders were used as animal models.

SPECIFIC AIMS

The four specific aims of this study were as follows:

- To determine whether repeated ozone exposure over a long term could induce nonspecific hyperresponsiveness in normal, nonatopic (nonsensitized) animals.
- To determine whether such ozone exposure could exacerbate the preexisting hyperresponsive state in atopic (sensitized) animals for both specific and nonspecific bronchoprovocation.
- To evaluate the role of gender in modulating airway responsiveness related to ozone exposure.
- To evaluate the relation between other possible modulators of airway response to ozone (such as specific cell types in bronchopulmonary lavage fluid, systemic blood, and lung tissue; exhaled nitric oxide; and levels of antigen-specific antibodies in serum).

METHODS AND STUDY DESIGN

ANIMAL MODEL

The animals used in this study were normal or atopic, male and female, viral antibody–free Hartley guinea pigs (200–250 g, Charles River). Atopy was produced with use of inhaled ovalbumin as the protein allergen inducing airway sensitization (Herxheimer and West 1955; Hutson et al 1988). Although no animal model completely reproduces the entire allergic airway process found in humans, such models can target some of the common features of interest. Thus, they can provide insight into the pathological consequences of pollutant exposure. In this regard, the guinea pig is a well-established model for airway hyperresponsiveness of a type comparable with that seen in a person with asthma (Kallos and Kallos 1984; Hutson et al 1988; Thorne and Karol 1989; Turner and Martin 1997). The experimental protocol was approved by the New York University School of Medicine Committee on Animal Care and Use.

The animals were housed on corncob bedding in polycarbonate cages within a laminar flow isolator unit with a high-efficiency particulate air (HEPA) filter in a room with temperature and humidity control; they were provided food and water ad libitum. As part of a quality assurance program, the colony underwent routine clinical screening under University veterinary supervision. At killing, samples of lavage fluid were taken for microbiological analysis. Furthermore, two sentinel animals maintained in the colony were killed at half-year intervals for health surveillance. Serology was

performed for lymphocytic choriomeningitis virus, pneumonia virus, reovirus, sendai virus, paramyxovirus 5, and *Encephalitozoon cuniculi*. All new animals underwent a quarantine and adaptation period for two weeks prior to introduction into an exposure protocol. There was no evidence during the course of the study of any health problems within the colony.

SENSITIZATION PROCEDURE

Sensitization was achieved with inhalation challenge for 0.5 hours/day for 4 days using 1% ovalbumin (Grade V, Sigma Chemical, weight/volume) in pyrogen-free isotonic saline. Aerosols were produced by nebulization with a compressed air nebulizer operated at 15 psi of pressure using medical-grade breathing air. The particle size of these aerosols, as determined with a Mercer impactor, was 1.8 µm (mass median aerodynamic diameter; σ_g = 1.9). Atmosphere analysis was performed by sampling with cellulose acetate filters, followed by extraction in distilled and deionized water and measurement for total protein content with use of a commercially available kit (BioRad).

During the initial phase of this study, we needed to confirm that the ovalbumin administration regimen resulted in sensitization. The major allergic antibody class in the guinea pig is immunoglobulin G (IgG; Griffith-Johnson et al 1993); some immunoglobulin E (IgE), the major allergic antibody in humans, may also be produced.

The standard passive cutaneous anaphylaxis procedure was used to measure serum levels of IgG and IgE initially in two guinea pigs (one male and one female) that had been subjected to the ovalbumin sensitization protocol and in two naive animals. The assay was performed using serum obtained 28 days after the first of the four ovalbumin administrations. For the passive cutaneous anaphylaxis procedure, animals were injected intradermally with serial dilutions of the test serum. Subsequent titers in the sensitized female and male animals were found to be 200 and 100, respectively, indicating the presence of anti-ovalbumin IgG in the test serum. In contrast, titers in the two control (naive) animals were less than 20. No bluing was observed after 10 days, indicating the absence of serum IgE in the sensitized animals. Additional 1-day passive cutaneous anaphylaxis titers were performed with sera from other ovalbumin-challenged animals, and these initial findings were confirmed. Thus, the ovalbumin challenge procedure did result in sensitization, and this sensitization was associated with increased levels of IgG, but not IgE, in the blood. The titer of serum IgG was subsequently used in this study as the index of sensitization.

EXPERIMENTAL PLAN

Ozone Exposure Concentrations and Duration

This study involved exposures to one of three atmospheres: clean air (sham control), 0.1 ppm ozone, or 0.3 ppm ozone. The duration of exposure for each atmosphere was 4 hours/day, 4 days/week for 24 weeks. Guinea pigs show essentially continual activity throughout a 24-hour period with no prolonged periods of inactivity, as long as ambient temperature is below about 75°F. In fact, their average period of activity is about 20 hours/day (Harper 1976). Because of this pattern, exposure to the pollutant atmospheres during normal daylight hours was appropriate.

The ozone concentrations used were relevant in terms of ambient conditions. Levels of 0.3 ppm or more are frequently encountered in many regions of the United States, and over 50% of the population resides in areas where a 1-hour average of 0.1 ppm is routinely exceeded (EPA 1996). In terms of exposure duration, the dynamics of ozone formation result in a broad peak level lasting 6 to 8 hours daily, during which the maximum exposure is about 90% of the maximum 1-hour peak exposure (Rombout et al 1986; Lefohn et al 1993). Because most of the effective ozone exposure occurs over a broad time frame each day, the daily exposure duration of 4 hours, as used in the study, is reasonable.

Furthermore, multiple-day exposures to ozone are common; a pattern of 4 consecutive days/week for the exposure regimen was based upon average patterns of pollution episodes in many parts of the country (Lippmann 1992). A protocol with episodes of consecutive daily exposures better reflects ambient exposure than does either continuous exposure or exposures on random days of the week.

In addition, ozone levels in most parts of the country are highest during the period of late spring through early fall (California Air Resources Board 1988; EPA 1996). Multiple-day instances of high ozone levels occur most often during this period, so a total exposure duration of 24 weeks is also reasonable.

Study Design

This project involved three experimental protocols:

- Nonsensitized (NS) protocol: This protocol was designed to examine the effect of ozone exposure upon airway responsiveness in normal animals. Nonsensitized (nonatopic) guinea pigs were exposed to each of the three atmospheres.
- Presensitized (PS) protocol: This protocol was designed to examine the effect of ozone upon airway responsiveness in atopic animals. Animals were

completely sensitized to ovalbumin prior to entry into the exposure series. Sensitization involved a 4-day ovalbumin administration period followed by holding the animals for a total of 28 days prior to entry into the exposure series.

- Concurrently Sensitized (CS) Protocol: This protocol was designed to examine the effect of ozone on airway responsiveness in animals that were being sensitized during ozone exposure. In this case, the 4-day sensitization procedure and the ozone or air control exposures were initiated concurrently.

Because a pollutant-exposed human population would contain segments consisting of persons who were already sensitized (PS), as well as those capable of being sensitized under appropriate conditions (CS), we concluded it was reasonable to use animal models for both types of individuals. Within each protocol, exposure groups consisted of a total of ten animals per gender per exposure atmosphere. After the final exposure, ten animals from each of the three exposure groups were killed: five animals of each gender per group. The remaining animals were maintained in clean air for an additional 8 weeks (the postexposure period).

Table 1 shows the ages of the animals for each experimental protocol at the time they were first exposed to ovalbumin to initiate sensitization and at the time they were first exposed to their specific atmosphere. The age at which ozone exposures began is different for the animals in the PS protocol compared with those in the NS and CS protocols. This occurred because the ovalbumin sensitization procedure had to be initiated at the same age for the animals in the PS and CS protocols; age is a known modulating factor in the development of atopy after antigen exposure (Peden 2000).

Biological Endpoints

The main focus of this study was to assess airway responsiveness in relation to ozone exposure. Measurements of responsiveness were performed by inhaled bronchoprovocative challenge testing at approximately 4-week intervals during the course of the 24-week exposure period, as well as at 4–8 week intervals during the postexposure period. Responsiveness was assessed on days when no ozone exposure was performed in order to minimize any potential acute ozone effects on epithelial permeability to the aerosolized challenge agents. Animal body weights were obtained prior to each measurement of airway responsiveness and at time of killing.

Animals were killed 1 week after the end of the exposure or postexposure period, that is, 25 or 33 weeks after the first exposure to ozone or ozone-free air. This lag was necessary in order to allow time to perform airway responsiveness tests after the final exposures. The animals were killed with a sodium pentobarbitol overdose (150 mg/kg). This was followed by cardiac puncture to obtain blood for cell and immunoglobulin analyses, and then exsanguination. Then the trachea and upper lung were exposed, the right main bronchus was clamped just below the carina, and the left lung was lavaged through the trachea for recovery of free cells and lavage fluid. The lungs were then removed from the thorax. The left lung was fixed by airway perfusion of formalin solution, and the right lung was fixed with Carnoys solution (the latter done for mast cell analysis). Lavage fluid was examined for lactate dehydrogenase (LDH) and total soluble protein, whereas recovered cells were characterized for viability and total and differential cell counts. Table 2 provides an overview of the bioassays performed in this study.

Table 1. Age at Sensitization and Ozone Exposure

Experimental Protocol	Age (week)[a]	
	Sensitization	Ozone Exposure
Nonsensitized	—	3–4
Presensitized	3–4	7–8
Concurrently sensitized	3–4	3–4

[a] Age at the start of the ovalbumin-sensitization procedure or at the start of the ozone or control exposure series.

Table 2. Outline of Biological Assays

Physiological

Nonspecific airway responsiveness (acetylcholine)—all protocols
Specific airway responsiveness (ovalbumin)—PS and CS protocols only

Biochemical/Immunological

Lavage Fluid
- Lactate dehydrogenase (LDH)
- Total soluble protein
- Cell viability
- Cell counts (total and differential)

Systemic Blood
- Antigen-specific antibodies (IgG1/IgG2)
- Cell differential counts

Exhaled Air
- Nitric oxide

Histopathology (Lung Sections)

Mast cell number
Eosinophil number

Generation of Ozone Exposure Atmospheres

All ozone exposures were performed in 1.6-m^3 stainless steel exposure chambers maintained at 25°C (77°F) and 55% relative humidity. Ozone was generated by passing oxygen (in argon) through an ultraviolet ozone generator (OREC model 03V1-0). The concentration during exposures was measured with an ultraviolet photometer (Dasibi model 1003-PC), calibrated with use of a certified transfer standard. The fresh air used in the exposure system was passed through an air cleaning system, which included HEPA filters, activated charcoal, Purafil ($KMnO_4$-coated alumina) and lead oxide denuders, resulting in removal of ambient particles, sulfur dioxide, nitrogen oxides and ozone.

Measurement of Airway Responsiveness

Airway responsiveness was assessed by bronchoprovocative challenge testing, a procedure in which changes in specific airway conductance (sGaw) were measured after inhalation administration of increasing concentrations of bronchoconstrictive agents. These agents consisted of either a nonspecific cholinergic agonist (acetylcholine [ACH]) or a specific antigenic stimulus (ovalbumin). Both specific and nonspecific responsiveness were measured in all sensitized animals (those in the PS and CS protocols), whereas only nonspecific responsiveness was measured in nonsensitized animals (those in the NS protocol).

The provocative challenge agents were administered by inhalation because humans would be exposed to ambient antigens or nonspecific chemical stimuli by the same route. Changes in airway responsiveness during ozone exposure could reflect changes in the ability of these agents to reach airway receptors due to ozone-induced effects on the epithelium (namely, alterations in mucus secretion or epithelial permeability) rather than to an actual blunting or increased sensitivity of receptors. However, this would also likely occur in humans exposed in ambient air to ozone and to nonspecific or specific challenges. Thus, the inhalation route represents a realistic approach for assessing ozone-related effects on airway function in a manner comparable with the exposure situation for people.

Specific airway conductance was assessed in unsedated, spontaneously breathing guinea pigs with use of a noninvasive method (Agrawal 1981; Thompson et al 1987). The animal was placed within a two-piece, whole body, constant volume plethysmograph, and it breathed through a pneumotachograph (model #0, Fleisch Instruments). Conductance was based on airway driving pressure and airflow measured at the nose. Airflow and box pressure signals, which were calibrated daily, were simultaneously delivered to an oscilloscope. Conductance was calculated from the slope of the rising limb of the resulting loop, corrected for pressure and temperature.

Prior to each provocative challenge test with either ACH or ovalbumin, sGaw was measured at 5-minute intervals for 15 minutes. This was followed by measurement of sGaw after a 0.5-minute inhalation of phosphate buffered saline (PBS) generated (at 10 psi) by compressed air nebulization (DeVilbiss #45) using medical grade air; this test provided the value for baseline sGaw. After this, the animals were administered the challenge agent.

To assess nonspecific responsiveness, animals were challenged with doubling doses of ACH aerosol administered at 3-minute intervals until sGaw decreased by at least 50% from its baseline level (the value obtained after inhalation of PBS). At week 0, 0.1% ACH was the starting concentration for the nonsensitized animals, and 0.025% was used for the sensitized animals. However, with progression of the study and depending on the responsiveness of the animals, the initial concentrations were often altered. During the exposure period, nonspecific responsiveness was evaluated 24 hours after the last exposure during the week for which responsiveness was to be assessed.

To assess specific airway responsiveness, animals were challenged with ovalbumin, beginning at a concentration of 0.025% and employing the basic procedure described for ACH. Because the response to inhaled antigen in sensitized animals is less rapid than that to ACH, however, the interval between the end of each ovalbumin aerosol challenge and start of the next dose was extended to 10 minutes. The ovalbumin challenge was performed 72 hours after each ACH challenge test. This sequence of challenging with ACH prior to ovalbumin, rather than the reverse order, was used in order to avoid the possibility of any residual ovalbumin-related effects influencing the response to ACH (Finney and Forsberg 1994; Lewis and Broadley 1995).

Responsiveness was quantitated in terms of PC50, the provocation concentration that resulted in a decrease in sGaw of 50% from the PBS baseline. This was achieved by log-linear interpolation of the ACH (or ovalbumin) concentration related to the sGaw response.

Prior to the start of the ozone (or clean air control) exposure series, bronchoprovocative challenges were performed for animals of the NS and PS protocols to establish week 0 preexposure values. Two preexposure tests using ACH were performed 24 hours apart in both of these protocols, whereas a single preexposure ovalbumin challenge was performed in the PS protocol. No preexposure tests with either ACH or ovalbumin were performed for animals in the CS protocol because they were exposed to ozone while undergoing sensitization; this precluded a preexposure

ovalbumin test. The animals were too small to perform an ACH test prior to the start of the exposure series; this prevented an accurate measure of responsiveness in the plethysmographic system.

Measurement of Exhaled Nitric Oxide

Some researchers have suggested that nitric oxide (NO) may modulate airway responsiveness (Nijkamp et al 1993; Schuiling et al 1998). Levels of NO in exhaled air were measured at various time points during the course of the exposure series while the animals were in the body plethysmograph system. Levels of NO were assessed for 5 to 10 minutes through a probe placed at the exhaust port of a one-way valve (H Rudolph type), using a modified chemiluminescence nitrogen oxides analyzer (Monitor Lab model 8840) and a procedure similar to that described by Persson and Gustafsson (1993). The nitrogen oxides analyzer was calibrated with a certified NO standard. The lowest concentration of NO detectable with the analyzer was 0.2 ppb. Because of equipment problems, measurements of NO were not performed during the CS protocol.

Biochemical and Cellular Assays of Lavage Fluid and Blood

Lavage of the left lung was performed using a procedure previously described (Schlesinger et al 1992). In brief, the lung was infused in situ six times with calcium-free and magnesium-free PBS. Each withdrawal of fluid was centrifuged, and cell pellets were resuspended in Eagles' minimum essential medium and pooled. Samples of the pooled cell suspension were taken, and total recovered cell numbers and viability were determined by hemocytometer counting and trypan blue exclusion, respectively. The relative percentage of cell types was determined by differential staining with Diff-Quik (Baxter Healthcare). Lavage fluid was analyzed for levels of LDH (an index of general cytotoxicity or cell membrane damage) and total soluble protein (a measure of serum protein transudation that reflects damage to the barrier between the airways, alveoli and circulation). We employed commercially available kits (Sigma) and used the supernatant obtained from the first wash. LDH was quantitated in International Units (IU) per milliliter lavage fluid, whereas protein was quantitated as micrograms per milliliter lavage fluid.

Whole blood was collected in heparinized capillary tubes after cardiac puncture. Differential counts were determined on the basis of blood smears stained with Wright-Giemsa.

All glassware used for cellular assays was autoclaved. Prior to use, all media and cell culture reagents were screened for bacterial and fungal contamination, using standard bacteriological reagents, and for endotoxin contamination, by means of the Limulus amebocyte lysate assay (Bio-Wittaker).

Antigen-Specific Immunoglobulin Assays

Levels of ovalbumin-specific antibodies (IgG1, IgG2) in the systemic blood of atopic animals were determined by enzyme-linked immunosorbent assay (ELISA) (Fraser et al 1998). Ovalbumin was placed into 96-well polystyrene ELISA plates at 2 µg/ml in 0.1 M carbonate buffer (pH 9.6) and stored overnight at room temperature. After the plates were washed, the remaining nonspecific binding sites were blocked by incubation with blocking buffer containing 0.1% bovine serum albumin in 0.17 M H_3BO_4, 0.12 M NaCl, 0.05% Tween-20, 0.05% NaN_3, and 1 mM EDTA; the components were then allowed to sit for 0.5 hour at 37°C.

After washing, the samples containing unknown amounts of ovalbumin-specific IgG were serially diluted in blocking buffer, added to the wells, and incubated for 1 hour. The plates were washed and blocking buffer was added for 10 minutes. After another washing, a 1/5,000 dilution of either rabbit anti-guinea pig IgG1 (Immunovision, Springdale AZ) or a 1/2,000 dilution of rabbit anti-guinea pig IgG2 antibody (courtesy of Dr Frank Graziano), as appropriate, was added and the plate incubated for 0.5 hour. After washing, a 1/10,000 dilution of alkaline phosphatase-labeled donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratory, West Grove PA) was added to the wells, and the plates were incubated for 0.5 hour. After washing, the alkaline phosphatase substrate, *p*-nitrophenyl phosphate (1 mg/ml, Sigma) in 10% diethanolamine, and 0.01% magnesium chloride ($MgCl_2$; pH 9.8) was added. After 0.5 hour, absorbance at 405 nm was measured on an ELISA reader. All sample and reagent volumes were 50 µL, except the substrate-diethanolamine reagent, for which 75 µL was added per well. Washing steps consisted of filling and aspirating each well with distilled water three times. All incubations were at room temperature with the plate placed on an orbital shaker set at 100 rpm.

Concentrations of ovalbumin used to coat the plate, as well as amounts of primary and secondary antibodies used in the ELISA, were determined to be optimal and to reflect relative concentrations of ovalbumin-specific antibody. A standard IgG pool was included on each ELISA plate. The IgG standard contained ovalbumin-specific IgG1 and IgG2. Because the standard also contained IgG1 and IgG2 specific for other antigens, it was purified using Protein A Sepharose affinity chromatography of serum from animals that were hyperimmunized with ovalbumin.

The concentration of ovalbumin-specific IgG1 or IgG2 in the serum was expressed as the ratio of ovalbumin-specific IgG1 or IgG2 in each sample to that in the IgG standard. To determine the relative concentration of ovalbumin-specific IgG1 or IgG2 in the different serum samples on each ELISA plate, log absorbance versus log dilution was plotted for the samples and the IgG standards. The linear portion of each curve was used in the subsequent analysis. Analysis of covariance with a common slope was performed to find intercepts of the IgG standard for each serum sample. The common slope and separate intercepts were used in inverse regression to find the dilutions of each sample giving equivalent responses as the standard. The ovalbumin-specific IgG1 or IgG2 in the IgG standard was indicated as 1, and all samples obtained from the exposed animals were expressed in relation to this standard.

Histopathology

The fixed lungs were embedded in paraffin and sectioned along the plane of the main airway axis as previously described (Schlesinger et al 1992). This sectioning method was used because longitudinal airway profiles are relatively unaffected by postmortem bronchoconstriction, which commonly occurs in guinea pigs. Sections from the formalin-fixed left lung were stained with Giemsa for identification of eosinophils, whereas sections from the right lung, fixed with Carnoys solution, were stained with 0.1% Alcian blue for identification of mast cells.

Quantitative analysis of cellular infiltration was performed on the main intrapulmonary bronchus and on small noncartilaginous bronchioles chosen at random from each section. The numbers of mast cells and eosinophils in the epithelial and subepithelial layers of each airway were quantitated per unit cross-sectional area with light microscopy, using NIH Image software. Ten fields of ×20 magnification were counted for each airway examined from each animal. Sampling sites were chosen only at points where the airway had been sectioned longitudinally. Sections were also scanned for evidence of inflammation.

DATA ANALYSIS AND STATISTICAL METHODS

This study was designed to use three distinct experimental protocols to examine effects attributable to ozone exposure over 24 weeks as well as during the 8 weeks after exposure. The study was also designed to compare the responses of male and female guinea pigs within each protocol.

Airway responsiveness quantitated as PC50, sGaw, and body weight were analyzed using multivariate profile analyses. These analyses were performed independently for each of the experimental protocols and, within each protocol, independently for the ACH and ovalbumin challenge test results. Furthermore, data for the postexposure period of each protocol were analyzed separately from the data for the exposure period. A separate analysis for each protocol was performed using the ratio of PC50 obtained with ACH to that obtained with ovalbumin; this was done to determine any differences in response between the nonspecific and specific provocative challenge tests. Results of measurements of NO in exhaled air were also analyzed using multivariate profile analysis.

Prior to the above analyses, all data were checked for homogeneity of variance using the Bartletts test. Based upon the results of this test, PC50 values, as well as the ratio of ACH to ovalbumin PC50s, were normalized using a log10 transformation. The sGaw values were squared.

As noted previously in this section, prior to the start of the ozone or clean air exposures in the NS and PS protocols, two bronchoprovocative challenge tests with ACH were performed. For the purpose of statistical analysis, the week 0 value was defined as the result of the second preexposure challenge. If this value was identified as an outlier, the result of the first challenge was then defined as the preexposure value for purposes of analysis.

To be identified as a potential outlier, the PC50 from the second preexposure challenge had to be one of the five most extreme values, and the difference between the two preexposure values had to be one of the five most extreme differences. If these conditions were met, the second preexposure value was tested as an outlier using the Nair criterion (Natrella 1963). If the second PC50 value were found to be an outlier, it was replaced for the purpose of statistical analysis with the PC50 value from the first preexposure challenge. Four of the second preexposure values from the NS protocol and one from the PS protocol were replaced based on this procedure.

The vector of dependent variables for the profile analysis consisted of measurements made during the 24-week exposure period (including the week 0 preexposure test values for NS and PS animals) or the measurements obtained at Weeks 24, 28 and 32. In the latter instance, Week 24 was represented by the last measurement obtained during the exposure period and the other two measures were obtained during the postexposure period. The independent variables in the profile analysis were ozone concentration (0, 0.1, or 0.3 ppm), animal gender, and the interaction between ozone concentration and gender. Statistical significance (at $P < 0.05$) for the profile analysis was determined by the Hotelling-Lawley trace.

If any statistically significant interaction was detected between the time vector of dependent variables and the

independent variables, univariate analysis of variance (ANOVA) for each time point was then performed. The factors in these ANOVAs were ozone concentration, gender, and interaction between gender and ozone concentration. For any ANOVA, statistical significance was evaluated by the F test ($P < 0.05$). A significant F value led to subtesting for exposure or for gender-by-exposure interactions. No subtests were required for a significant gender effect. When performed, subtesting was done with uncorrected t tests (unpaired, 2-tailed) of the least squares means.

Because a classical multivariate procedure was used to analyze the data, any animal with even a single missing measurement would be excluded from the analysis. With a small amount of missing data, the values could be interpolated based upon the animal's own trends in combination with information on the average values for the other animals. One missing body weight was interpolated in this fashion. If all animals in at least one exposure group were found to have missing information for a given time point, however, then interpolation would be an inappropriate strategy. If all animals had missing data, that specific time point was effectively excluded from the statistical analysis. This was the case in the CS protocol for Week 28 of the postexposure period.

In both the PS and CS protocols, a limited number of measurements were not performed due to technical malfunctions. In the PS protocol, the ACH challenge was not performed during exposure Week 4 for the animals to be held through the postexposure period. In the CS protocol, no ovalbumin challenges were performed at Week 4 for the animals to be held for the postexposure period. For these cases, the profile analysis was performed twice. First, all weeks were included, resulting in a sample size of half the number of animals (namely, 5 animals of each gender for each exposure group). Second, results obtained at Week 4 were excluded, resulting in a complete sample size beyond this time point (namely, 10 animals of each gender for each exposure group). The profile analysis results are reported only for the analysis based on the full sample size. The ANOVA results are reported for the full sample from the profile analysis, except for the week with the missing information. That ANOVA is treated as an independent analysis, in the sense that it was not part of the full multivariate testing. Although not reported, the full profile analysis was performed and examined to determine whether or not the results of the two analyses (that is, full sample excluding one week versus reduced sample including all weeks) were consistent. It is because the results were generally consistent that the results are reported in this fashion rather than providing full summaries for both analyses.

The results of the postmortem lavage fluid, blood cell, immunoglobulin, and airway cell assays were analyzed by three-way ANOVA. The factors were gender, ozone concentration, and time of killing (the latter, either immediately after the last exposure or after the postexposure period). All possible interactions were also tested. Statistically significant factor or interaction effects ($P < 0.05$) were subtested using uncorrected t tests (unpaired, 2-tailed). Prior to analysis, all data were tested for homogeneity of variance using the Bartletts test. Based upon this, the lavage fluid and systemic blood cell counts were normalized using an arcsin transformation, whereas IgG data were normalized using a log10 transformation. Although systemic blood cell counts were obtained for relative numbers of eosinophils, neutrophils, lymphocytes, basophils and monocytes, the basophils and monocytes were excluded from statistical analysis because their counts were low or nonexistent.

Some additional statistical analyses were performed to evaluate the strength of linear relations between particular parameters. To determine whether variability in airway responsiveness could be explained by the level of antigen-specific antibody, Pearson correlation coefficients were calculated for the relation of IgG1 and IgG2 (after log10 transformation), obtained by cardiac puncture, with the final measure of PC50 (after log10 transformation) at ovalbumin or ACH challenge, obtained before the animals were killed.

Because PC50 was measured at repeated time points, but IgG was assayed only at the time of death, and because the PS and CS protocols had different temporal relations between sensitization and ozone exposure, these analyses were performed using only the air control animals (pooled). The ratio of the PC50 value obtained for the final bronchial provocative challenge normalized (that is, divided) by that obtained at Week 0 was also examined in this manner.

To evaluate whether there was a statistical relation between eosinophils in lavage fluid and the degree of airway hyperresponsiveness, a Pearson correlation coefficient was calculated for the eosinophil fraction (arcsin transformed) and PC50 obtained after the final exposure at 24 weeks for all animals in all protocols.

Another analysis was performed to evaluate whether variability in the fraction of blood eosinophils may be associated with the degree of sensitization as measured by levels of IgG. To this end, Pearson correlation coefficients were calculated for the eosinophil fraction (arcsin-transformed) with IgG1 and IgG2 (log10-transformed) using pooled air control animals from the PS and CS protocols.

A Pearson correlation coefficient was also obtained for the relation between the total number of eosinophils in the airway sections for each animal in the PS and CS protocols and the percentage of eosinophils in lavage fluid for the

same animals. This analysis was performed to determine whether the number of these cells recovered in lavage fluid was representative of the actual number of cells in the lungs. Finally, a similar correlation analysis was performed to determine the strength of the relation between numbers of mast cells in lung tissue and PC50.

For purposes of subsequent discussion, statistical significance for all analyses is at $P < 0.05$.

RESULTS

The target and actual concentrations of ozone for each of the three experimental protocols are shown in Table 3. Target concentrations were achieved, and the exposure atmospheres varied little over the course of the study.

The various data sets obtained in this study were statistically analyzed as described. The approach employed for describing the data is to use these analyses as the basis to evaluate results for each endpoint within each protocol in terms of an overall consistency of pattern or trend. Consistency can then be related to ozone exposure, gender, or both rather than focusing on individual statistical differences at specific time points or between different time points.

BODY WEIGHT

The results of statistical analysis for body weight (BW) are shown in Table 4. Figure 1 shows the mean body weight for all animals in the experimental protocols at each time point prior to ACH challenge. Although body weight was also measured in the PS and CS protocols prior to each ovalbumin challenge, these values were similar to the ones in Figure 1 and are, therefore, not shown. The nonsensitized animals exposed to ozone showed intermittent differences from air control animals during

Table 3. Ozone Concentrations

Target Concentration (ppm)	Actual Concentration[a] (ppm)		
	NS	PS	CS
0.1	0.107 (0.0008)	0.105 (0.0006)	0.104 (0.0005)
0.3	0.291 (0.0011)	0.295 (0.0008)	0.299 (0.0006)

[a] The grand mean (± SE) of daily means obtained during exposure. The daily means were based upon readings from the ozone monitor obtained every 0.5 hour during each exposure.

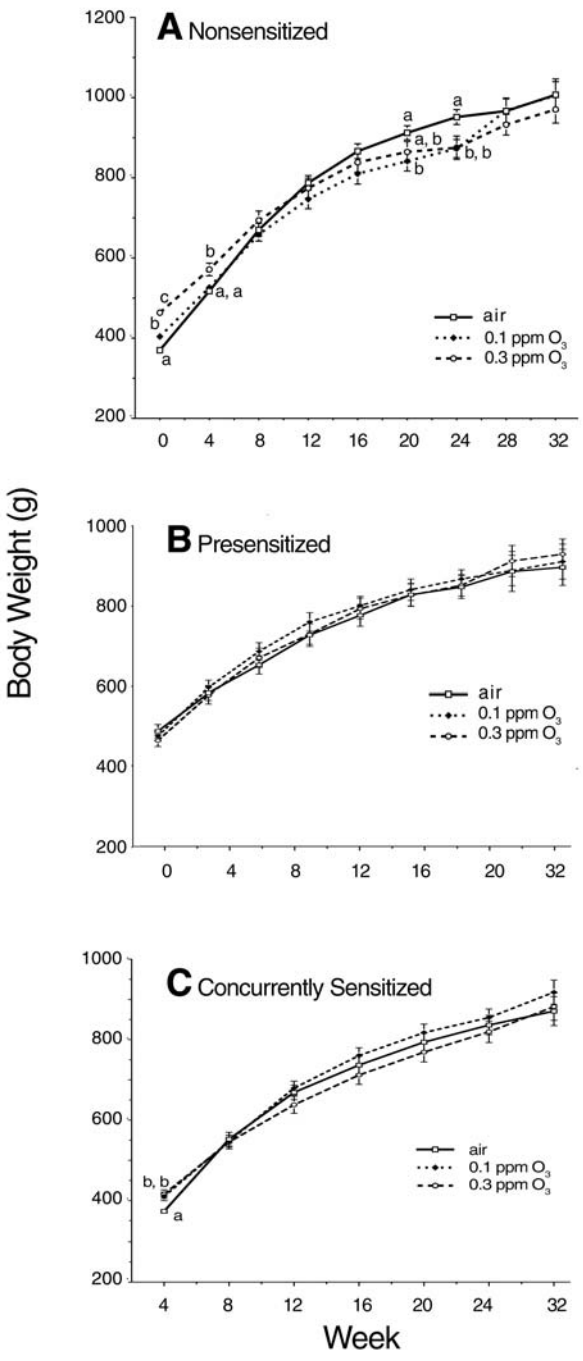

Figure 1. Body weight as function of time from start of experimental exposure. *A*, Nonsensitized animals; ***B*,** presensitized animals; and ***C*,** concurrently sensitized animals. Each point is the mean (± SE) for all animals at each time point. Statistically significant differences between exposure atmospheres at each time point are indicated by letter designations: values with the same or no letter are not significantly different. Group size is 20 animals per time point per atmosphere through Week 24 and 10 animals per time point per atmosphere for Weeks 28 and 32.

Table 4. Results of Statistical Analyses for Body Weight Among Nonsensitized, Presensitized, and Concurrently Sensitized Animals

Statistical Tests	NS	PS		CS	
		ACH	Ovalbumin	ACH	Ovalbumin
		Exposure Period			
MANOVA (3 way)					
Time	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Time • gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Time • ozone	**< 0.01**	**0.02**	**0.04**	**< 0.01**	**0.02**
Time • gender • ozone	0.50	0.92	0.98	0.25	0.06
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	0.22	0.60	0.63	0.22	0.08
Gender • ozone	0.63	0.95	0.96	0.89	0.77
ANOVA (1 way)					
Week 0					
Gender	**< 0.01**	**< 0.01**	**< 0.01**	NA[a]	NA
Ozone	**< 0.01**	0.33	0.82	NA	NA
Gender • ozone	0.20	0.93	0.75	NA	NA
Week 4					
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**[b]
Ozone	**< 0.01**	0.58	0.56	**< 0.01**	0.53
Gender • ozone	0.58	0.85	0.72	0.80	0.52
Week 8					
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	0.23	0.28	0.25	0.88	0.25
Gender • ozone	0.30	0.92	0.94	0.86	0.72
Week 12					
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	0.29	0.25	0.49	0.07	**0.05**
Gender • ozone	0.62	0.88	0.99	0.80	0.90
Week 16					
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	0.12	0.58	0.80	0.06	0.06
Gender • ozone	0.76	0.89	0.91	0.93	0.63
Week 20					
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	**0.02**	0.83	0.74	0.09	0.10
Gender • ozone	0.59	0.87	0.96	0.89	0.42
Week 24					
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	**0.01**	0.67	0.82	0.33	0.14
Gender • ozone	0.99	0.81	0.90	0.80	0.97

(Table continues next page)

[a] NA = not applicable; time = time from start of exposures; ozone = ozone concentration.

[b] Based upon analysis with reduced group size.

Table 4 (*continued*). Results of Statistical Analyses for Body Weight Among Nonsensitized, Presensitized, and Concurrently Sensitized Animals

Statistical Tests	NS	PS		CS	
		ACH	Ovalbumin	ACH	Ovalbumin
		Postexposure Period			
MANOVA (3 way)					
Time	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Time • gender	**< 0.01**	0.34	0.88	**0.02**	**< 0.01**
Time • ozone	0.58	0.43	**0.01**	0.97	0.07
Time • gender • ozone	0.14	**0.02**	0.56	0.13	**0.01**
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	0.25	0.74	0.62	0.20	0.18
Gender • ozone	0.77	0.43	0.41	0.81	0.58
ANOVA (1 way)					
Week 24					
Gender	**0.02**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	0.13	0.78	0.73	0.26	0.20
Gender • ozone	0.36	0.30	0.46	0.99	0.86
Week 28					
Gender	**< 0.01**	**< 0.01**	**< 0.01**	NA[a]	NA
Ozone	0.42	0.74	0.36	NA	NA
Gender • ozone	0.95	0.51	0.36	NA	NA
Week 32					
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	0.50	0.67	0.69	0.18	0.14
Gender • ozone	0.70	0.45	0.39	0.47	0.26

[a] NA = not applicable; time = time from start of exposures; ozone = ozone concentration.

the exposure period, but by 24 weeks, they weighed significantly less than the control animals.

The general pattern of weight change with time did not seem to differ much between the two groups of animals exposed to ozone. During the postexposure period, all three exposure cohorts (air, 0.1 ppm ozone, or 0.3 ppm ozone) had no statistically significant within-gender differences that could be ascribed to ozone, although animals exposed to 0.3 ppm ozone did seem to weigh somewhat less than animals in the other two cohorts. Furthermore, although males consistently weighed more than did females at each time point, there was no gender-ozone interaction, indicating that any effect of ozone on body weight followed a similar pattern for both genders. Figure 2 shows gender differences in the nonsensitized air control animals.

The presensitized animals exhibited no overall biologically significant consistent trend or pattern of ozone-induced effects on body weight and no gender-ozone interaction during the exposure or postexposure periods (see Figure 1B). Similarly, no consistent statistically significant pattern of ozone effect on body weight was found for the

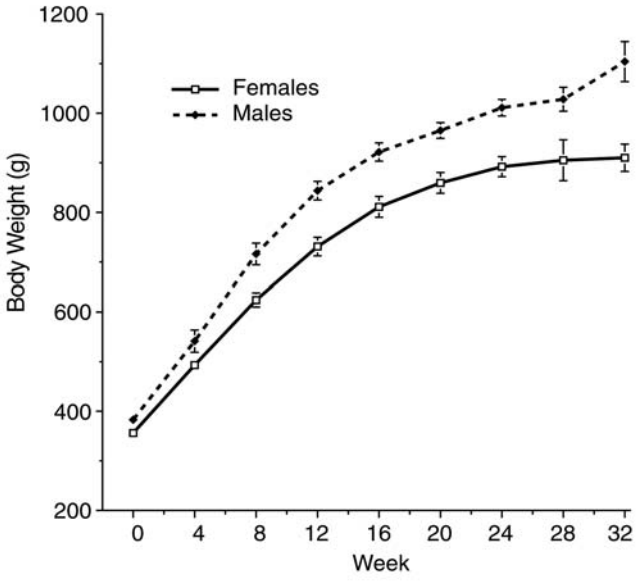

Figure 2. Body weight as function of time for nonsensitized control animals. Each value is the mean (± SE) for each gender at each time point. Group size is 10 animals per time point through Week 24 and 5 animals per time point for Weeks 28 and 32.

Table 5. Results of Statistical Analyses for sGaw Among Nonsensitized, Presensitized, and Concurrently Sensitized Animals

Statistical Tests	NS	PS		CS	
		ACH	Ovalbumin	ACH	Ovalbumin
		Exposure Period			
MANOVA (3 way)					
Time	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	0.49
Time • gender	**< 0.01**	0.58	**< 0.01**	**0.04**	0.07
Time • ozone	0.23	**0.02**	**< 0.01**	**0.04**	**0.02**
Time • gender • ozone	0.06	**0.03**	0.15	0.27	0.15
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	0.92	**< 0.01**	0.37	**0.03**	0.21
Gender • ozone	0.50	**0.01**	**< 0.01**	0.10	0.20
ANOVA (1 way)					
Week 0					
Gender	**0.05**	0.10	0.39	NA[a]	NA
Ozone	**< 0.01**	0.40	0.10	NA	NA
Gender • ozone	**< 0.01**	0.59	**0.04**	NA	NA
Week 4					
Gender	**0.02**	**0.04**[b]	0.64	0.15	0.16[b]
Ozone	**< 0.01**	**< 0.01**	**< 0.01**	**0.02**	0.26
Gender • ozone	0.52	0.16	0.71	0.48	**0.04**
Week 8					
Gender	**< 0.01**	**0.01**	**< 0.01**	0.60	**< 0.01**
Ozone	0.39	0.21	**0.01**	0.52	0.50
Gender • ozone	0.99	0.24	0.23	0.69	0.98
Week 12					
Gender	**< 0.01**	**0.03**	0.29	0.61	**0.04**
Ozone	0.18	0.20	0.63	**0.01**	0.72
Gender • ozone	0.15	**0.01**	**0.01**	0.46	**0.04**
Week 16					
Gender	**< 0.01**	**0.01**	0.06	**< 0.01**	**< 0.01**
Ozone	0.76	0.10	0.35	0.47	**0.02**
Gender • ozone	0.33	**0.01**	**0.02**	0.60	0.96
Week 20					
Gender	**< 0.01**	0.11	0.06	**< 0.01**	**< 0.01**
Ozone	0.46	**< 0.01**	**< 0.01**	**0.01**	0.11
Gender • ozone	0.23	0.29	0.30	**0.05**	**0.03**
Week 24					
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**0.03**	**0.06**
Ozone	0.73	**< 0.01**	0.59	0.71	0.23
Gender • ozone	0.91	0.09	**0.01**	0.36	0.52

(Table continues next page)

[a] NA = not applicable; time = time from start of exposure; ozone = ozone concentration.

[b] Based upon analysis with reduced group size.

Table 5 (*continued*). Results of Statistical Analyses for sGaw Among Nonsensitized, Presensitized, and Concurrently Sensitized Animals

		PS		CS	
Statistical Tests	NS	ACH	Ovalbumin	ACH	Ovalbumin
		Postexposure Period			
MANOVA (3 way)					
Time	0.11	0.84	0.33	0.95	0.21
Time • gender	0.18	0.40	0.06	0.31	**0.04**
Time • ozone	0.53	**< 0.01**	**0.01**	**0.04**	**0.05**
Time • gender • ozone	**< 0.01**	0.62	0.82	0.59	0.38
Gender	**< 0.01**	**0.01**	**< 0.01**	0.94	**0.01**
Ozone	0.53	**0.01**	**0.01**	0.42	**0.02**
Gender • ozone	0.59	**0.03**	**< 0.01**	**0.03**	0.22
ANOVA (1 way)					
Week 24					
Gender	**< 0.01**	0.10	**0.01**	0.41	0.38
Ozone	0.47	**< 0.01**	**0.01**	**0.04**	0.09
Gender • ozone	0.72	0.32	**0.04**	**0.06**	0.20
Week 28					
Gender	**< 0.01**	0.36	0.53	NA[a]	NA
Ozone	0.67	**< 0.01**	**0.05**	NA	NA
Gender • ozone	0.43	**0.01**	0.07	NA	NA
Week 32					
Gender	**< 0.01**	**0.02**	**< 0.01**	0.47	**< 0.01**
Ozone	0.42	0.65	**0.01**	0.21	**0.01**
Gender • ozone	**0.01**	0.60	**0.01**	0.22	0.37

[a] NA = not applicable; time = time from start of exposure; ozone = ozone concentration.

CS animals (see Figure 1C) although there did appear to be somewhat less weight gain during the exposure period for the cohort exposed to 0.3 ppm ozone compared with the other two cohorts. However, this was no longer evident by the end of the postexposure period. A between-gender pattern of weight difference similar to that noted for the NS animals was noted for both groups of sensitized animals (not shown).

AIRWAY CONDUCTANCE

The results of the statistical analysis for sGaw are shown in Table 5. Figure 3 shows values for baseline sGaw obtained prior to each ACH challenge. Although sGaw was also measured prior to each ovalbumin challenge in the PS and CS protocols, these values were essentially identical to the ones in Figure 3 and are, therefore, not shown. For each experimental protocol, within-gender values for sGaw remained relatively consistent throughout both the exposure and postexposure periods. There was no overall pattern or trend of ozone effect on this parameter although some differences between air-exposed and ozone-exposed animals were statistically significant at specific time points. This lack of a pattern suggests that the observed effects reflected random variability rather than any biologically significant effect on sGaw that could be related to ozone exposure. However, sGaw values in the NS animals appear to be generally lower than values in the PS and CS animals, which appear to be similar to each other.

In regard to between-gender differences, baseline sGaw values in males of all three protocols were generally lower than those in females at most time points (although these differences did not always reach statistical significance). Ozone exposure had no consistent effect on this between-gender difference. Figure 4 shows between-gender differences in baseline sGaw for the air control animals in the

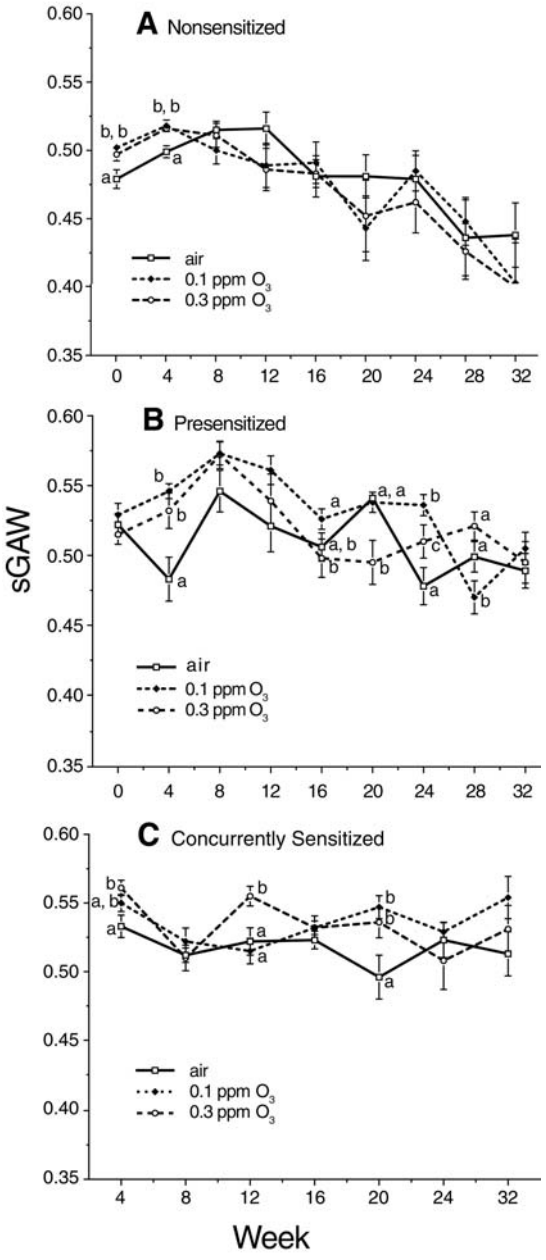

Figure 3. Baseline specific airway conductance (sGaw) as function of time from start of experimental exposure. ***A,*** Nonsensitized animals; ***B,*** presensitized animals; and ***C,*** concurrently sensitized animals. Each point is the mean (± SE) for all animals at each time point. Statistically significant differences between atmospheres at each time point are indicated by letter designations: values with the same or no letter are not significantly different. Group size is 20 animals per time point per atmosphere through Week 24 and 10 animals per time point per atmosphere for Weeks 28 and 32.

NS protocol; although not shown, a similar gender-related pattern for sGaw was noted for the other two protocols.

AIRWAY RESPONSIVENESS

Results of the statistical analysis of PC50 are shown in Table 6. Figure 5 shows PC50 values for each of the three protocols.

A comparison of PC50 for air control animals in the NS protocol with values for the controls in the PS and CS protocols shows that these latter groups of animals were indeed sensitized. As shown in Figure 6, PC50 at Week 0 in the PS animals was much lower than that for the animals in the NS protocol, indicating that the airways in the former group were indeed hyperresponsive. Similarly, at the first time point at which PC50 was measured in the CS protocol (Week 4), PC50 values for the air controls were also lower than those for the NS protocol air controls at the same point in time.

The NS animals showed no consistent pattern or trend of ozone-induced effect on PC50 obtained with ACH challenge during either the exposure or postexposure periods (see Figure 5A). However, there was a clear between-

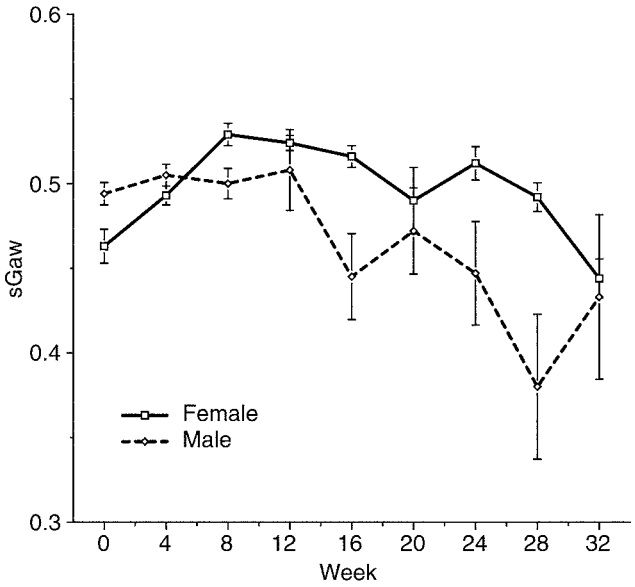

Figure 4. Specific airway conductance (sGaw) as function of time for nonsensitized air control animals. Each value is the mean (± SE) for each gender at each time point. Group size is 10 animals per time point through Week 24 and 5 animals per time point for Weeks 28 and 32.

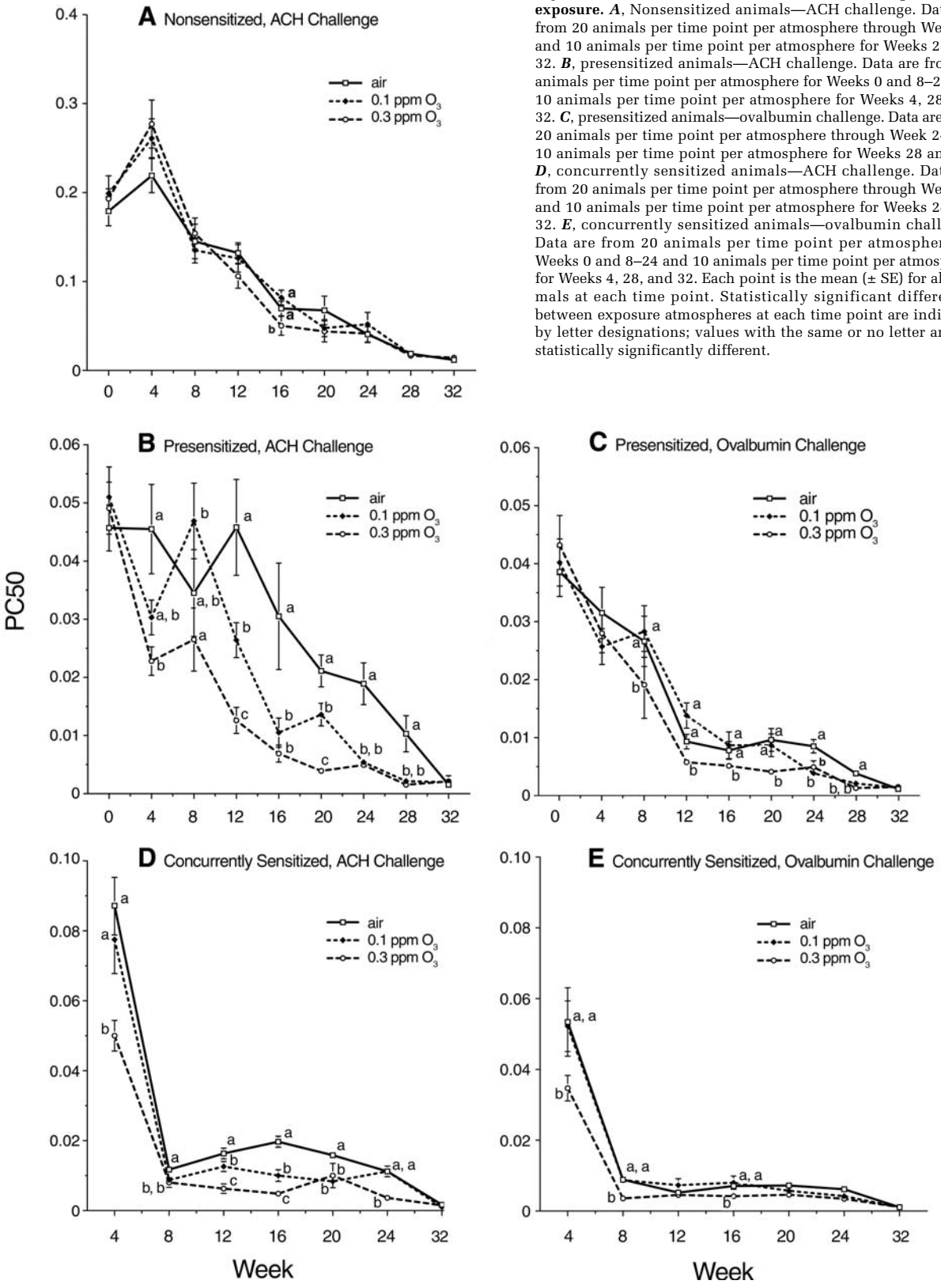

Figure 5. PC50 as function of time from start of experimental exposure. ***A***, Nonsensitized animals—ACH challenge. Data are from 20 animals per time point per atmosphere through Week 24 and 10 animals per time point per atmosphere for Weeks 28 and 32. ***B***, presensitized animals—ACH challenge. Data are from 20 animals per time point per atmosphere for Weeks 0 and 8–24 and 10 animals per time point per atmosphere for Weeks 4, 28, and 32. ***C***, presensitized animals—ovalbumin challenge. Data are from 20 animals per time point per atmosphere through Week 24 and 10 animals per time point per atmosphere for Weeks 28 and 32. ***D***, concurrently sensitized animals—ACH challenge. Data are from 20 animals per time point per atmosphere through Week 24 and 10 animals per time point per atmosphere for Weeks 28 and 32. ***E***, concurrently sensitized animals—ovalbumin challenge. Data are from 20 animals per time point per atmosphere for Weeks 0 and 8–24 and 10 animals per time point per atmosphere for Weeks 4, 28, and 32. Each point is the mean (± SE) for all animals at each time point. Statistically significant differences between exposure atmospheres at each time point are indicated by letter designations; values with the same or no letter are not statistically significantly different.

Table 6. Results of Statistical Analyses for PC50 Among Nonsensitized, Presensitized, and Concurrently Sensitized Animals

Statistical Tests	NS	PS		CS	
		ACH	Ovalbumin	ACH	Ovalbumin
		Exposure Period			
MANOVA (3 way)					
Time	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**0.01**
Time • gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**0.01**
Time • ozone	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Time • gender • ozone	0.69	**< 0.01**	0.08	**< 0.01**	**0.03**
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	0.09	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Gender • ozone	0.11	**0.05**	0.13	0.17	0.80
ANOVA (1 way)					
Week 0					
Gender	0.39	0.68	0.12	NA[a]	NA
Ozone	0.63	0.88	0.78	NA	NA
Gender • ozone	0.79	0.65	0.63	NA	NA
Week 4					
Gender	**0.01**	**0.05**[b]	0.66	0.46	**< 0.01**[b]
Ozone	0.19	**< 0.01**	0.68	**< 0.01**	**0.02**
Gender • ozone	**0.06**	**< 0.01**	**< 0.01**	0.49	**< 0.01**
Week 8					
Gender	0.09	**< 0.01**	**< 0.01**	0.81	**< 0.01**
Ozone	0.72	**0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Gender • ozone	0.57	0.24	0.60	0.06	**< 0.01**
Week 12					
Gender	**< 0.01**	**< 0.01**	**0.03**	**0.01**	**< 0.01**
Ozone	0.09	**< 0.01**	**< 0.01**	**< 0.01**	0.07
Gender • ozone	0.42	**0.02**	0.66	0.44	0.82
Week 16					
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	**< 0.01**	**0.01**	**0.03**	**< 0.01**	**0.01**
Gender • ozone	0.45	**0.02**	0.50	**0.01**	0.92
Week 20					
Gender	**< 0.01**	**< 0.01**	**< 0.01**	0.73	**< 0.01**
Ozone	0.06	**< 0.01**	**< 0.01**	**< 0.01**	0.08
Gender • ozone	0.19	0.20	0.74	0.28	0.54
Week 24					
Gender	**< 0.01**	**0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	0.57	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Gender • ozone	0.77	0.66	0.43	**0.01**	0.93

(Table continues next page)

[a] NA = not applicable; time = time from start of exposure; ozone = ozone concentration.

[b] Based upon analysis with reduced group size.

Table 6 (*continued*). Results of Statistical Analyses for PC50 Among Nonsensitized, Presensitized, and Concurrently Sensitized Animals

Statistical Tests	NS	PS		CS	
		ACH	Ovalbumin	ACH	Ovalbumin
		Postexposure Period			
MANOVA (3 way)					
Time	**0.03**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Time • gender	**< 0.01**	**0.05**	0.16	0.09	**< 0.01**
Time • ozone	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**
Time • gender • ozone	**< 0.01**	0.11	**0.02**	0.40	0.10
Gender	**< 0.01**	**0.01**	**< 0.01**	0.17	**0.01**
Ozone	**0.02**	0.06	**< 0.01**	**< 0.01**	**< 0.01**
Gender • ozone	**0.01**	**0.03**	**0.01**	0.87	0.46
ANOVA (1 way)					
Week 24					
Gender	**< 0.01**	**< 0.01**	**< 0.01**	**0.02**	**< 0.01**
Ozone	0.38	0.67	**< 0.01**	**< 0.01**	**< 0.01**
Gender • ozone	0.45	0.22	0.69	0.48	0.74
Week 28					
Gender	**0.01**	**< 0.01**	**0.03**	NA[a]	NA
Ozone	0.95	**< 0.01**	**< 0.01**	NA	NA
Gender • ozone	0.26	**0.04**	0.33	NA	NA
Week 32					
Gender	**< 0.01**	0.42	**0.01**	0.99	0.53
Ozone	**< 0.01**	0.72	0.65	0.07	0.98
Gender • ozone	**< 0.01**	**0.09**	**< 0.01**	0.76	0.10

[a] NA = not applicable; time = time from start of exposure; ozone = ozone concentration.

gender difference in airway responsiveness in these animals: Except for Week 0, PC50 values for males were generally statistically significantly lower than values in females, indicating that airways in males were normally more responsive than in females. Figure 7 shows this between-gender difference for the air control animals.

The PS animals exposed to ozone generally showed lower values for PC50 after ACH challenge than did the air controls, and the difference between the air-exposed and ozone-exposed animals reached statistical significance at most time points during the 24-week study (Figure 5B). Although the effect of ozone exposure on nonspecific responsiveness was often statistically comparable for the two ozone concentrations, there was evidence for an ozone concentration-related trend for responsiveness. There was a general overall pattern of decreasing PC50 with increasing ozone exposure concentration: that is, PC50 values for animals exposed to 0.1 ppm ozone were generally lower than values for air controls but were generally higher than values for animals exposed to 0.3 ppm ozone. The significantly increased airway responsiveness related to ozone exposure was maintained 4 weeks into the postexposure period (Week 28), but it was no longer evident by 8 weeks after exposure (Week 32).

The PS animals challenged with ovalbumin (see Figure 5C) showed somewhat less of an overall pattern of ozone concentration-related effect on PC50. Values for the air control animals and animals exposed to 0.1 ppm ozone

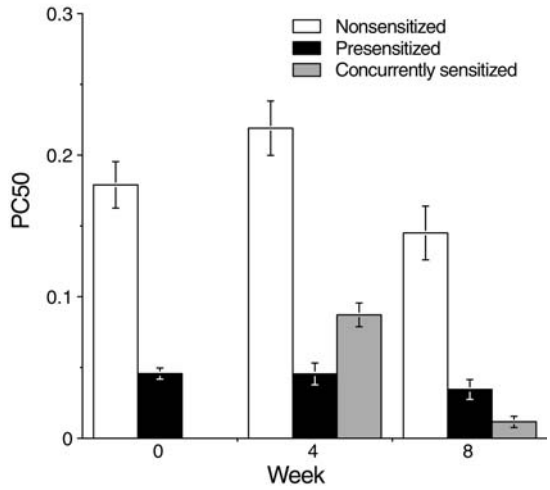

Figure 6. Comparison of PC50 with ACH challenge for air control animals in the three protocols. Each bar is the mean (± SE) for all animals at each time point. Note the reduced PC50, indicating increased airway responsiveness in the sensitized animals (PS and CS protocols) compared with values in the nonsensitized animals.

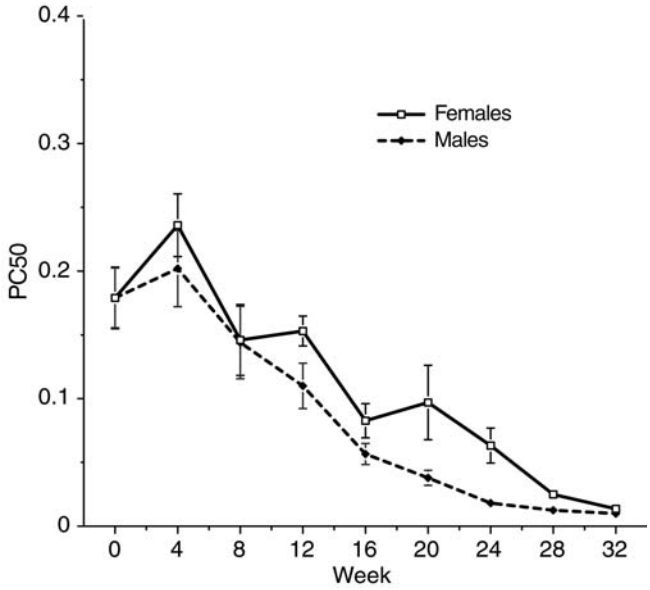

Figure 7. Gender comparison of PC50 with ACH challenge for nonsensitized air control animals. Each point is the mean (± SE) for all animals of each gender at each time point.

were not statistically significantly different until Week 24 of the exposure period. However, PC50 values for the animals exposed to 0.3 ppm ozone were generally lower than those for animals in either of the other two exposure atmospheres, and statistically significantly so, throughout most of the exposure period. Furthermore, this significant difference for animals exposed to either concentration of ozone compared with the air control animals was maintained 4 weeks into the postexposure period.

The PS animals did not show any consistent pattern of statistically significant between-gender difference in PC50 obtained with either ACH or ovalbumin and ozone exposure. However, males showed statistically significantly lower values for PC50 than did females at all times except Week 0. (This pattern was also seen in the NS animals.) This normal gender difference applied to PC50 values obtained with either ACH or ovalbumin.

The CS animals showed a pattern of generally statistically significant ozone effects on PC50 measured with either ACH or ovalbumin challenge similar to the pattern seen in PS animals (Figures 5D, 5E). A pattern of increasing responsiveness with increasing ozone concentration was also observed: At most time points during the exposure period, values for PC50 obtained with ACH for animals exposed to 0.3 ppm ozone were consistently lower than values in air control animals and in animals exposed to 0.1 ppm ozone. PC50 values with ACH for the animals exposed to 0.1 ppm ozone were generally lower than those for the air controls, and this difference reached statistical significance at a number of time points; however, this was not the case with ovalbumin challenge. On the other hand, with exposure to 0.3 ppm ozone, PC50 values with ACH challenge were statistically significantly different from values in air controls at most time points during the exposure period, as well as at early time points with ovalbumin challenge. The exposure concentration-response pattern was no longer evident by the end of the postexposure period (Week 32); there was no Week 28 measurement in this protocol. Although the values for PC50 for males were generally lower than those for females, these differences were not always statistically significant, a difference from the findings for the NS and PS protocols. In any case, however, there was no gender-related difference in response to ozone.

In order to assess whether there was any differential effect of ozone on nonspecific compared with specific airway responsiveness within the PS and CS protocols, the ratios of PC50 with ACH to PC50 with ovalbumin were evaluated. The results of the statistical analysis performed on this parameter are presented in Table 7, and Figure 8 presents the ratios. A ratio equal to 1 indicates PC50 values for both the ACH and ovalbumin challenges were the same, ie, the agonist concentration needed to produce the same degree of change in airway conductance was equivalent for both nonspecific and specific challenges. A ratio greater than 1 indicates PC50 values with ACH challenge were higher than PC50 values with OA challenge; airways were less responsive to the nonspecific provocation than they were to the specific stimulus. On the other hand, a ratio smaller than 1 indicates the opposite situation; airways were more responsive to the nonspecific than to the specific challenge.

Table 7. Results of Statistical Analyses for ACH-PC50 and Ovalbumin-PC50 Among Presensitized and Concurrently Sensitized Animals

Statistical Tests	PS	CS
Exposure Period		
MANOVA (3 way)		
Time	**< 0.01**	**< 0.01**
Time • gender	0.34	0.31
Time • ozone	**< 0.01**	**< 0.01**
Time • gender • ozone	**0.03**	**< 0.01**
Gender	0.38	**< 0.01**
Ozone	**< 0.01**	**< 0.01**
Gender • ozone	0.07	0.37
ANOVA (1 way)		
Week 0		
Gender	0.42	NA[a]
Ozone	0.96	NA
Gender • ozone	0.99	NA
Week 4		
Gender	0.62[b]	0.13[b]
Ozone	0.38	**0.04**
Gender • ozone	0.45	0.13
Week 8		
Gender	0.55	**< 0.01**
Ozone	**0.04**	**< 0.01**
Gender • ozone	0.42	**< 0.01**
Week 12		
Gender	0.25	**0.05**
Ozone	**0.03**	**< 0.01**
Gender • ozone	0.18	0.14
Week 16		
Gender	0.40	0.36
Ozone	0.06	**< 0.01**
Gender • ozone	**< 0.01**	0.07
Week 20		
Gender	0.24	**< 0.01**
Ozone	**< 0.01**	**< 0.01**
Gender • ozone	0.43	0.59
Week 24		
Gender	0.08	**< 0.01**
Ozone	0.72	**< 0.01**
Gender • ozone	0.58	0.06

(Table continues next column)

Table 7 (*continued*). Results of Statistical Analyses for ACH-PC50 and Ovalbumin-PC50 Among Presensitized and Concurrently Sensitized Animals

Statistical Tests	PS	CS
Postexposure Period		
MANOVA (3 way)		
Time	0.07	0.85
Time • gender	0.19	**0.02**
Time • ozone	**< 0.01**	**< 0.01**
Time • gender • ozone	0.11	0.42
Gender	0.19	0.19
Ozone	**0.01**	0.38
Gender • ozone	**< 0.01**	0.63
Week 24 vs Week 28		
Mean	0.07	NA[a]
Gender	0.40	NA
Ozone	**< 0.01**	NA
Gender • ozone	0.19	NA
Week 28 vs Week 32		
Mean	0.69	NA
Gender	0.07	NA
Ozone	**0.04**	NA
Gender • ozone	0.49	NA
Week 24 vs Week 32		
Mean	NA	0.85
Gender	NA	**0.02**
Ozone	NA	**< 0.01**
Gender • ozone	NA	0.42
ANOVA (1 way)		
Week 24		
Gender	0.49	**0.01**
Ozone	**< 0.01**	**< 0.01**
Gender • ozone	0.57	0.44
Week 28		
Gender	0.48	NA
Ozone	**0.02**	NA
Gender • ozone	**0.03**	NA
Week 32		
Gender	0.07	0.71
Ozone	0.06	0.27
Gender • ozone	**< 0.01**	0.64

[a] NA = not applicable; time = time from start of exposure; ozone = ozone concentration.

[b] Based upon analysis with reduced group size.

The statistical results for PS animals showed the presence of some significant ozone effects as well as one significant ozone-gender interaction. The ovalbumin PC50 values appeared to be consistently lower than ACH PC50 values; nearly all of the ratio means, including those obtained in Week 0, were greater than 1. Although the difference in the ratios between the air and ozone-exposed animals reached statistical significance during some weeks during the exposure period, there was no consistent trend of statistical significance over time or across ozone exposure groups, nor was there any effect of gender. Although ovalbumin challenge seemed to result in a generally greater degree of airway hyperresponsiveness than did ACH challenge for all exposure groups, no strong statistical evidence indicated that PC50 was decreased to a greater extent with either challenge; nor was there strong evidence that specific and nonspecific airway responsiveness were differentially affected by ozone in the PS animals.

As with the PS animals, the CS animals appeared to show a consistent pattern of lower ovalbumin PC50 than ACH PC50 values. Nearly all of the ratio means were greater than 1. The results of the statistical analysis of PC50 ratios for the CS animals also showed some significant differences related to ozone exposure, but they provided no consistent pattern indicating that ozone acted differentially on nonspecific or specific responsiveness. However, there was a consistent, and generally statistically significant, between-gender difference in sensitivity to the two challenges. This suggests that males showed a pattern of less responsiveness to the nonspecific compared with specific challenge than did females. However, this difference was not clearly related to ozone exposure.

In summary, NS animals exhibited no biologically significant ozone effect on airway responsiveness. Ozone exposure of PS or CS animals, whose airways were already hyperresponsive, resulted in a further increase in airway responsiveness to both ACH and ovalbumin, and this generally occurred in an ozone concentration-related manner. The effect of ozone was comparable for both nonspecific and specific responsiveness. Furthermore, there was no differential effect of ozone on either gender; both males and females responded to exposure in a similar fashion.

EXHALED NITRIC OXIDE

Results of the statistical analysis of exhaled NO are shown in Table 8. Figure 9 shows mean values for exhaled NO. Although some statistically significant effects were evident in both the NS and sensitized (PS and CS) animals, the lack of any consistent pattern related to ozone exposure suggested that these had no biological significance. In terms of between-gender differences, levels of NO were comparable in males and females (Figure 10).

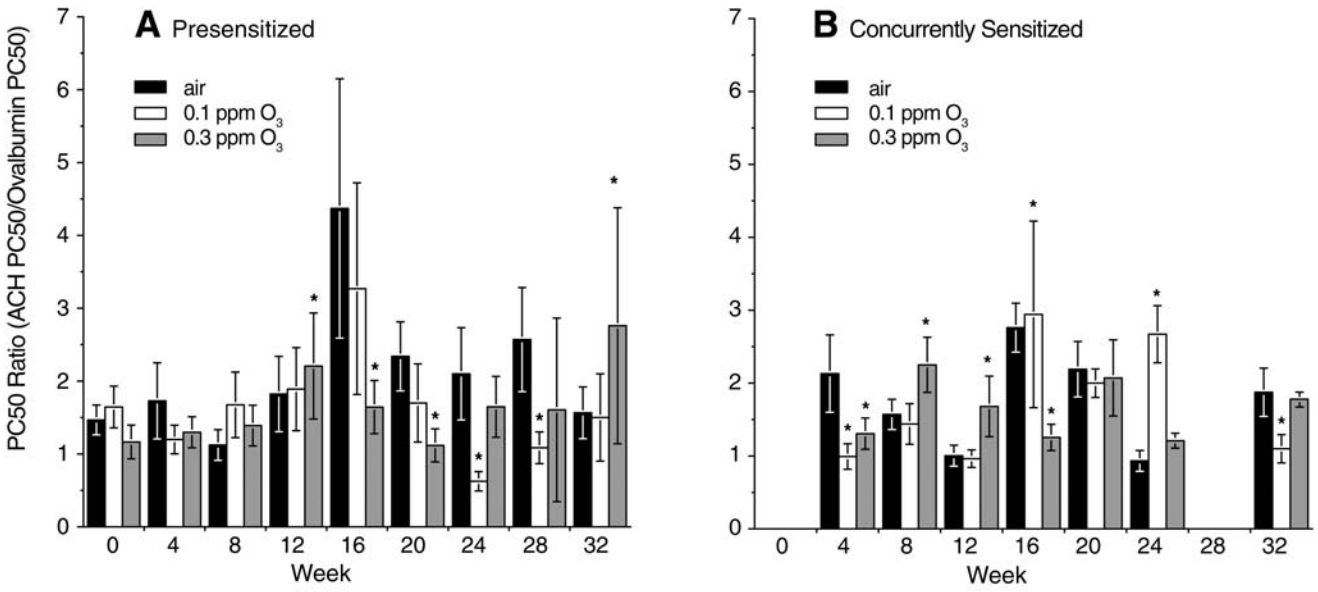

Figure 8. Comparison of nonspecific (ACH challenge) and specific (ovalbumin challenge) responsiveness for each exposure atmosphere. *A*, presensitized and ***B*,** concurrently sensitized animals. Each bar is the mean (± SE) of the ratio of PC50 with ACH challenge to PC50 with ovalbumin challenge. A ratio of 1 indicates that PC50 is identical with both challenges. A ratio greater than 1 indicates that PC50 with ACH challenge is greater than that with ovalbumin challenge. A ratio less than 1 indicates the reverse. Ratios that are significantly different from those for air control animals are indicated by an asterisk (*).

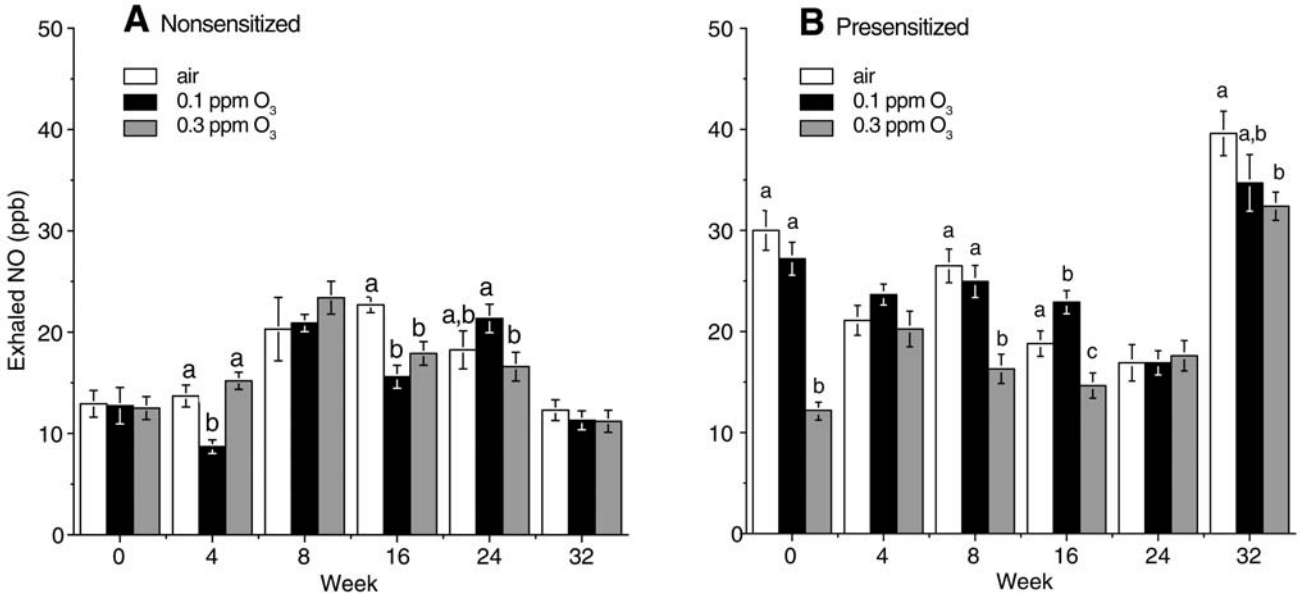

Figure 9. Exhaled nitric oxide levels. ***A*****,** Nonsensitized animals and ***B*****,** presensitized animals. Each point is the mean (± SE) for all animals at each time point. Statistically significant differences between atmospheres at each time point are indicated by letter designations: values with the same or no letter are not significantly different. Group size is 20 animals per time point per atmosphere through Week 24 and 10 animals for Week 32.

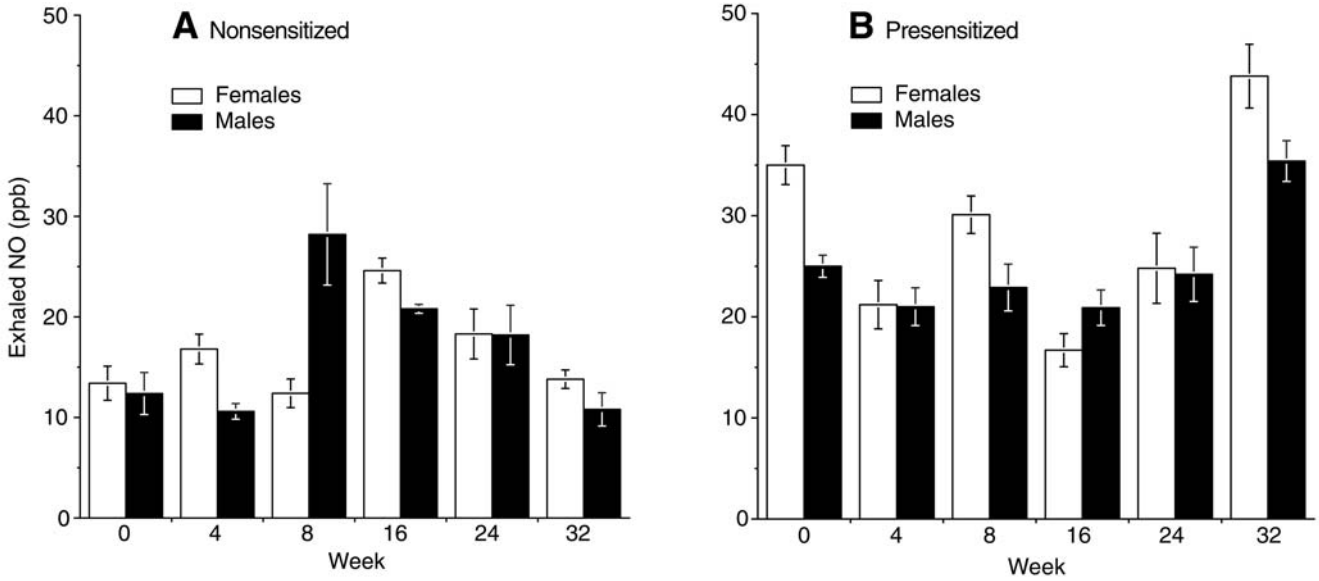

Figure 10. Exhaled nitric oxide levels in air control animals. ***A*****,** nonsensitized animals, and ***B*****,** presensitized animals. Each value is the mean (± SE) for each gender at each time point. Group size is 10 animals per time point through Week 24 and 5 animals for Week 32.

Table 8. Results of Statistical Analyses for Exhaled NO Among Nonsensitized and Presensitized Animals

Statistical Tests	NS	PS
Exposure Period		
MANOVA (3 way)		
Time[a]	**< 0.01**	**< 0.01**
Time • gender	0.06	**0.01**
Time • ozone	**< 0.01**	**0.02**
Time • gender • ozone	**< 0.01**	**< 0.01**
Gender	0.56	0.06
Ozone	0.18	**< 0.01**
Gender • ozone	0.46	0.11
ANOVA (1 way)		
Week 0		
Gender	0.41	**0.01**[b]
Ozone	0.98	**< 0.01**
Gender • ozone	0.20	**0.01**
Week 4		
Gender	**< 0.01**	0.10
Ozone	**< 0.01**	0.18
Gender • ozone	**0.01**	**< 0.01**
Week 8		
Gender	0.15	**< 0.01**
Ozone	0.45	**< 0.01**
Gender • ozone	**< 0.01**	0.48
Week 16		
Gender	**0.05**	0.18
Ozone	**< 0.01**	**< 0.01**
Gender • ozone	0.47	**0.01**
Week 24		
Gender	0.45	1.00[b]
Ozone	0.11	0.94
Gender • ozone	0.32	0.56
Postexposure Period		
MANOVA (3 way)		
Time	0.13	**< 0.01**
Time • gender	0.69	**0.04**
Time • ozone	0.07	**0.01**
Time • gender • ozone	0.32	**< 0.01**
Gender	0.58	**0.01**
Ozone	0.08	**< 0.01**
Gender • ozone	0.16	0.32
ANOVA (1 way)		
Week 24		
Gender	0.59	0.49
Ozone	**0.04**	**< 0.01**
Gender • ozone	0.23	**0.03**
Week 32		
Gender	0.91	**< 0.01**
Ozone	0.70	**0.03**
Gender • ozone	0.22	0.07

[a] Time = time from start of exposure; ozone = ozone concentration.

[b] Based upon analysis with reduced group size.

LAVAGE FLUID PARAMETERS

Results of the statistical analysis of various lavage fluid assays are shown in Table 9. Results for the three protocols demonstrate no consistent pattern for total or relative cell counts or for cell viability that could be related to ozone exposure. However, a gender-related difference was noted for eosinophils: the relative percentage in females for each experimental protocol was generally significantly higher than that in males at each time point (Figure 11). Concurrently, levels of macrophages tended to be lower in females than in males. However, the interaction was not consistent between ozone concentration and gender, suggesting that any gender differences were not related to ozone exposure.

As discussed further, a number of studies have associated eosinophils with increased airway responsiveness. Thus, simple Pearson correlation coefficients were calculated for the eosinophil fraction with PC50. The resulting correlation coefficient (0.061, $P = 0.82$) indicated no statistically significant association between eosinophils in lavage fluid and PC50.

Some statistical significance was evident for LDH (Figure 12) and total protein (Figure 13) in ozone-exposed animals compared with air controls. These instances were inconsistently related to ozone exposure, however, and we did not interpret them to have biological significance. For example, levels of LDH and protein were found in some cases to decrease with exposure to ozone, whereas lung injury induced by ozone would be expected to result in increased levels of these markers.

In summary, there were no biologically significant changes related to ozone exposure in any of the lavage fluid parameters.

SYSTEMIC BLOOD CELL DIFFERENTIALS

The results of the statistical analysis for blood cell counts are shown in Table 10. Values for individual cell types, expressed as fractions of total number of cells counted, are shown in Figure 14. In the NS animals, there was no statistically significant ozone effect on fractions of blood cells. However, a between-gender difference was seen for eosinophils, which showed a higher fraction in females than in males. This was similar to the pattern previously noted in lavage fluid although the relative fraction of eosinophils was lower in blood.

The PS animals showed statistically significant differences in cell fractions assessed after the postexposure period compared with those obtained from animals killed immediately after the exposure period. Eosinophils and neutrophils were increased in animals after the postexposure period; lymphocytes were generally decreased. Gender differences were observed in both the exposure and

Table 9. Results of Statistical Analyses for Lavage Parameters Among Nonsensitized, Presensitized, and Concurrently Sensitized Animals

	NS	PS	CS
Total cells			
Recovery[a]	**0.04**	0.10	0.64
Gender	0.28	0.29	0.22
Ozone[b]	0.85	0.48	**0.02**
Recovery • gender	0.37	0.48	**0.02**
Recovery • ozone	0.39	**< 0.01**	0.27
Gender • ozone	**0.03**	**< 0.01**	0.66
Recovery • gender • ozone	0.73	**0.05**	0.83
Viability			
Recovery	**< 0.01**	0.07	0.88
Gender	**0.05**	0.98	0.71
Ozone	0.30	**0.04**	0.21
Recovery • gender	0.36	0.72	0.56
Recovery • ozone	0.64	**0.21**	0.73
Gender • ozone	**0.04**	**0.05**	0.35
Recovery • gender • ozone	0.09	0.30	0.86
Eosinophils			
Recovery	0.06	0.92	0.09
Gender	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	**0.01**	0.20	**0.01**
Recovery • gender	0.80	0.85	0.50
Recovery • ozone	0.19	0.88	0.36
Gender • ozone	0.83	0.41	0.35
Recovery • gender • ozone	0.39	**0.01**	0.16
Macrophages			
Recovery	**< 0.01**	0.69	0.09
Gender	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	**< 0.01**	0.11	**0.01**
Recovery • gender	0.26	0.77	0.41
Recovery • ozone	**< 0.01**	0.78	0.07
Gender • ozone	0.75	0.46	0.44
Recovery • gender • ozone	0.45	**0.02**	0.17
Neutrophils			
Recovery	**< 0.01**	**0.05**	**0.01**
Gender	0.15	0.81	0.77
Ozone	**0.01**	0.43	**0.04**
Recovery • gender	**0.02**	0.91	0.11
Recovery • ozone	0.13	0.40	0.20
Gender • ozone	**0.03**	0.20	0.55
Recovery • gender • ozone	0.93	0.73	0.96

(Table continues next column)

Table 9 (*continued*). Results of Statistical Analyses for Lavage Parameters Among Nonsensitized, Presensitized, and Concurrently Sensitized Animals

	NS	PS	CS
Lymphocytes			
Recovery[a]	**< 0.01**	0.47	**0.02**
Gender	**0.02**	0.43	0.80
Ozone[b]	**< 0.01**	0.87	**< 0.01**
Recovery • gender	0.37	**0.04**	0.18
Recovery • ozone	**< 0.01**	**< 0.01**	**< 0.01**
Gender • ozone	0.25	**0.05**	0.07
Recovery • gender • ozone	0.13	**0.03**	**0.03**
Protein			
Recovery	**< 0.01**	**< 0.01**	0.30
Gender	**0.04**	0.41	0.11
Ozone	**< 0.01**	0.09	0.32
Recovery • gender	0.93	0.38	0.76
Recovery • ozone	0.39	0.11	0.26
Gender • ozone	0.15	0.19	0.66
Recovery • gender • ozone	0.64	0.96	0.18
Lactate dehydrogenase			
Recovery	**< 0.01**	**< 0.01**	0.60
Gender	**< 0.01**	**< 0.01**	**< 0.01**
Ozone	**0.01**	**< 0.01**	0.50
Recovery • gender	0.09	0.70	0.07
Recovery • ozone	**0.01**	**< 0.01**	**0.04**
Gender • ozone	0.67	**< 0.01**	0.97
Recovery • gender • ozone	0.71	0.99	0.45

[a] Recovery indicates statistical comparison of animals killed after Week 24 with those killed after the 8-week postexposure period (Week 32).

[b] Ozone = ozone concentration.

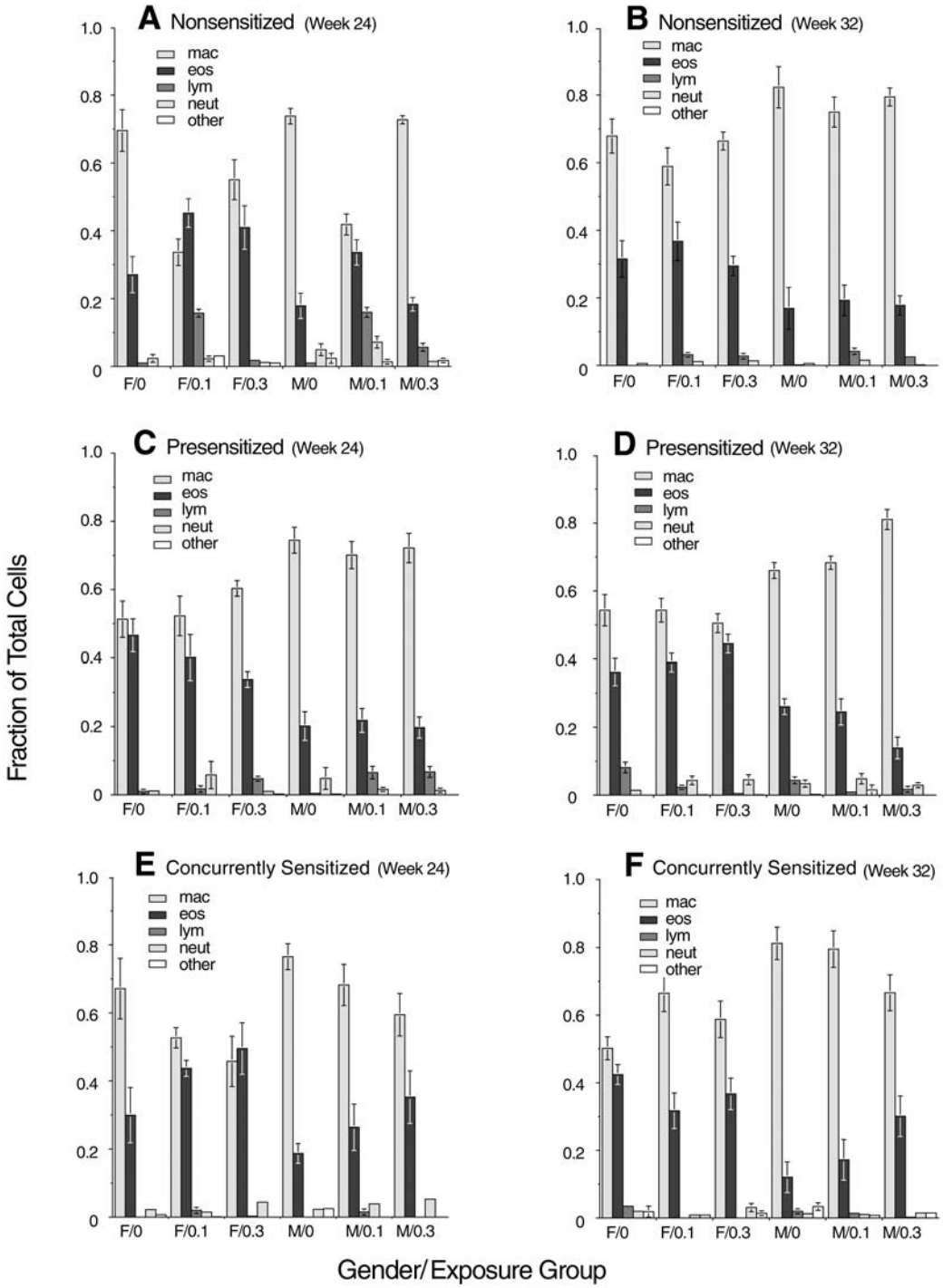

Figure 11. Cell differentials in lavage fluid. ***A***, nonsensitized animals killed immediately after the exposure series; ***B***, nonsensitized animals killed after the postexposure period; ***C,*** presensitized animals killed immediately after the exposure series; ***D***, presensitized animals killed after the postexposure period; ***E***, concurrently sensitized animals killed immediately after the exposure series; and ***F,*** concurrently sensitized animals killed after the postexposure period. Each value is the mean (± SE) obtained from 5 animals. F = female; M = male; 0 = air control; 0.1 = 0.1 ppm ozone; 0.3 = 0.3 ppm ozone; mac = macrophages; eos = eosinophils; lym = lymphocytes; neut = neutrophils.

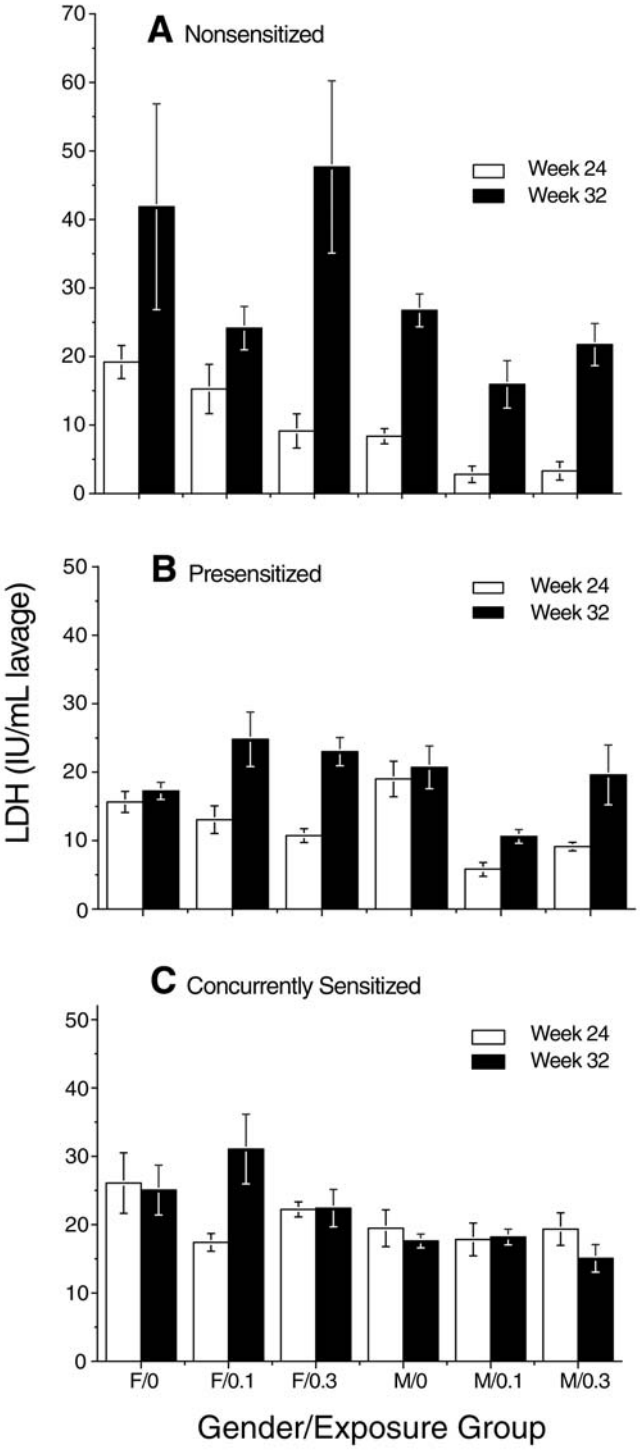

Figure 12. LDH measured in lavage fluid. ***A***, nonsensitized animals; ***B***, presensitized animals; and ***C***, concurrently sensitized animals. Values are expressed in International Units, a measure of enzyme activity, per milliliter lavage fluid. Each value is the mean (± SE) obtained from 5 animals. F = female; M = male; 0 = air control; 0.1 = 0.1 ppm ozone; 0.3 = 0.3 ppm ozone.

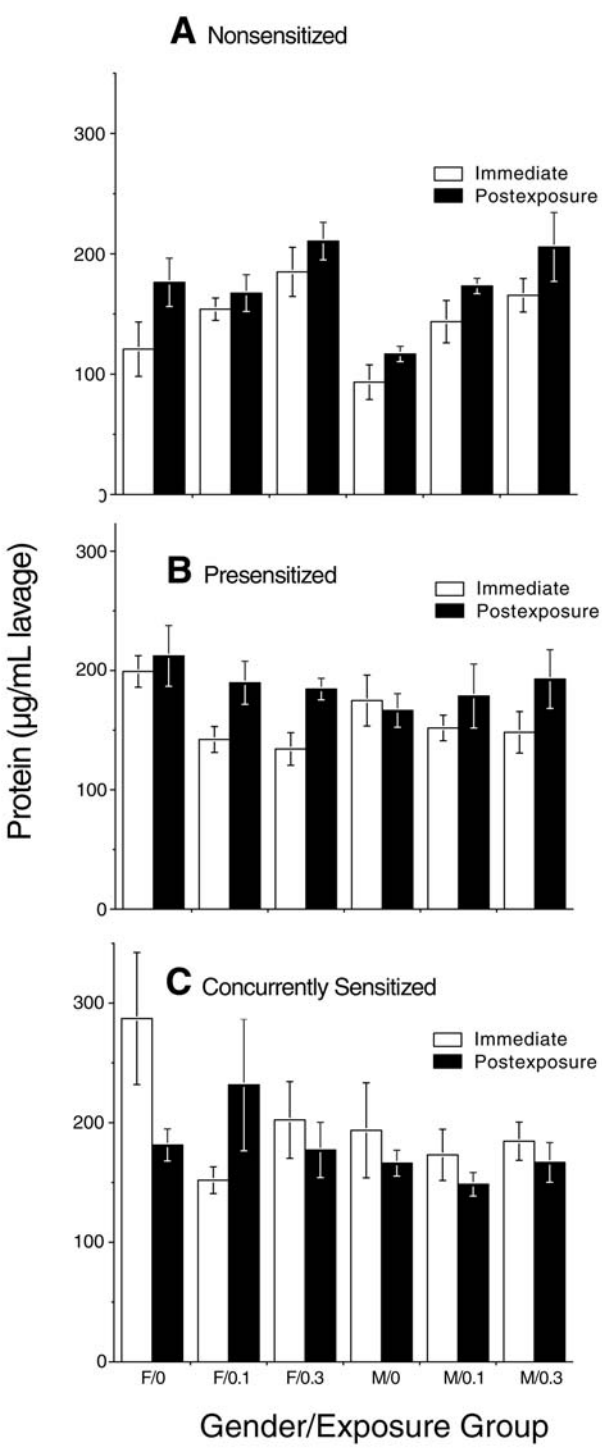

Figure 13. Protein measured in lavage fluid. ***A***, nonsensitized animals; ***B***, presensitized animals; and ***C***, concurrently sensitized animals. Values are expressed in micrograms protein per milliliter lavage fluid. Each value is the mean (± SE) obtained from 5 animals killed immediately after the exposure period or after the postexposure period. F = female; M = male; 0 = air control; 0.1 = 0.1 ppm ozone; 0.3 = 0.3 ppm ozone.

Table 10. Results of Statistical Analyses for Systemic Blood Cell Counts Among Nonsensitized, Presensitized, and Concurrently Sensitized Animals

	NS	PS	CS
Eosinophils			
Recovery[a]	—	**0.01**	0.29
Gender	**0.01**	**0.02**	0.96
Recovery • gender	—	0.43	0.82
Ozone[b]	0.94	0.33	0.69
Recovery • ozone	—	0.33	0.24
Gender • ozone	0.73	**0.02**	0.21
Recovery • ozone • gender	—	0.57	0.50
Lymphocytes			
Recovery	—	**0.04**	**0.03**
Gender	0.23	**0.04**	**<0.01**
Recovery • gender	—	0.98	0.07
Ozone	0.49	0.53	**<0.01**
Recovery • ozone	—	0.40	**<0.01**
Gender • ozone	0.49	0.21	0.22
Recovery • ozone • gender	—	0.20	0.06
Neutrophils			
Recovery	—	**0.05**	**0.02**
Gender	0.09	**0.02**	**<0.01**
Recovery • gender	—	0.89	0.06
Ozone	0.58	0.39	**<0.01**
Recovery • ozone	—	0.48	**<0.01**
Gender • ozone	0.43	0.16	0.28
Recovery • ozone • gender	—	0.24	**0.01**

[a] Recovery indicates statistical comparison of animals killed after Week 24 with those killed after the 8-week postexposure period (Week 32).

[b] Ozone = ozone concentration.

postexposure cohorts: Females had higher levels of eosinophils and lymphocytes than the males, but they had lower levels of neutrophils. A gender-by-ozone interaction was observed for eosinophils: the levels of these cells in females exposed to 0.1 or 0.3 ppm ozone were higher than those in similarly exposed males. However, this effect was not observed for other cell types. Furthermore, statistically significant increases in one cell type would be expected to be offset by similar decreases in another cell type because fractions must sum to a total of one for analysis. Therefore, the biological significance of this result was unclear.

For the CS animals, eosinophils were unaffected by any of the experimental factors. However, an ozone-related difference in lymphocyte and neutrophil response was also related to whether the animal was killed immediately after the exposure series or after the postexposure period. In the animals killed after the last exposure, lymphocytes decreased and neutrophils increased as a function of ozone concentration. In the animals held for the postexposure period, however, no changes in either cell type could be related to ozone concentration.

As in the PS animals, the CS females had higher levels of lymphocytes and lower levels of neutrophils than did the males, and this was independent of ozone concentration or time of killing. The between-gender differences differed for the various exposure concentration-killing time combinations for neutrophils, and they just missed significance (P = 0.06) for lymphocytes. In the animals killed immediately after ozone exposure, an exposure concentration-response gradient was observed for both males and females. In animals held through the postexposure period, however, such a gradient was not observed, and males and females had different patterns of response as a function of ozone concentration, suggesting no biologically meaningful trend.

In summary, there were no ozone exposure-related effects on blood cell differentials in NS animals or in PS animals. Among the CS animals, an ozone exposure-response gradient was observed in fractions of neutrophils and lymphocytes in the animals killed immediately after the exposure series but not in animals killed after the postexposure period.

ANTIGEN-SPECIFIC ANTIBODIES

Results of the statistical analysis of blood IgG levels are shown in Table 11. Figure 15 shows mean levels of IgG1 and IgG2 for the PS and CS animals.

Table 11. Results of Statistical Analyses for IgG Among Presensitized and Concurrently Sensitized Animals

	PS	CS
IgG1		
Recovery[a]	**0.04**	**<0.01**
Gender	0.55	**<0.01**
Recovery • gender	0.87	0.35
Ozone[b]	0.12	0.96
Recovery • ozone	0.97	0.48
Gender • ozone	0.55	0.77
Recovery • ozone • gender	0.38	0.65
IgG2		
Recovery	**< 0.01**	**<0.01**
Gender	0.30	**0.05**
Recovery • gender	**0.04**	**0.01**
Ozone	**0.04**	0.84
Recovery • ozone	0.12	0.53
Gender • ozone	0.40	0.54
Recovery • ozone • gender	0.55	0.35

[a] Recovery indicates statistical comparison of animals killed after Week 24 with those killed after the 8-week postexposure period (Week 32).

[b] Ozone = ozone concentration.

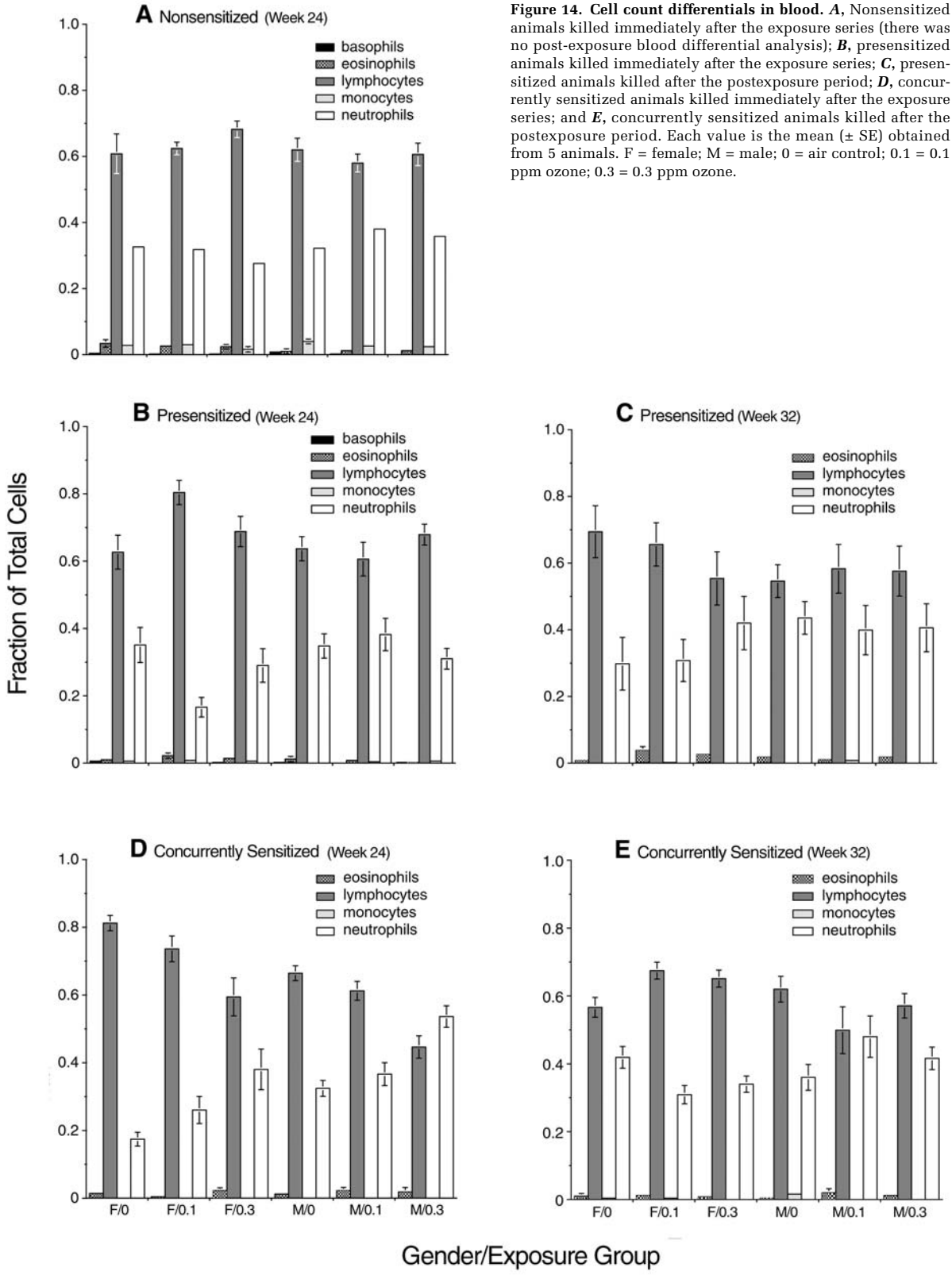

Figure 14. Cell count differentials in blood. ***A***, Nonsensitized animals killed immediately after the exposure series (there was no post-exposure blood differential analysis); ***B***, presensitized animals killed immediately after the exposure series; ***C***, presensitized animals killed after the postexposure period; ***D***, concurrently sensitized animals killed immediately after the exposure series; and ***E***, concurrently sensitized animals killed after the postexposure period. Each value is the mean (± SE) obtained from 5 animals. F = female; M = male; 0 = air control; 0.1 = 0.1 ppm ozone; 0.3 = 0.3 ppm ozone.

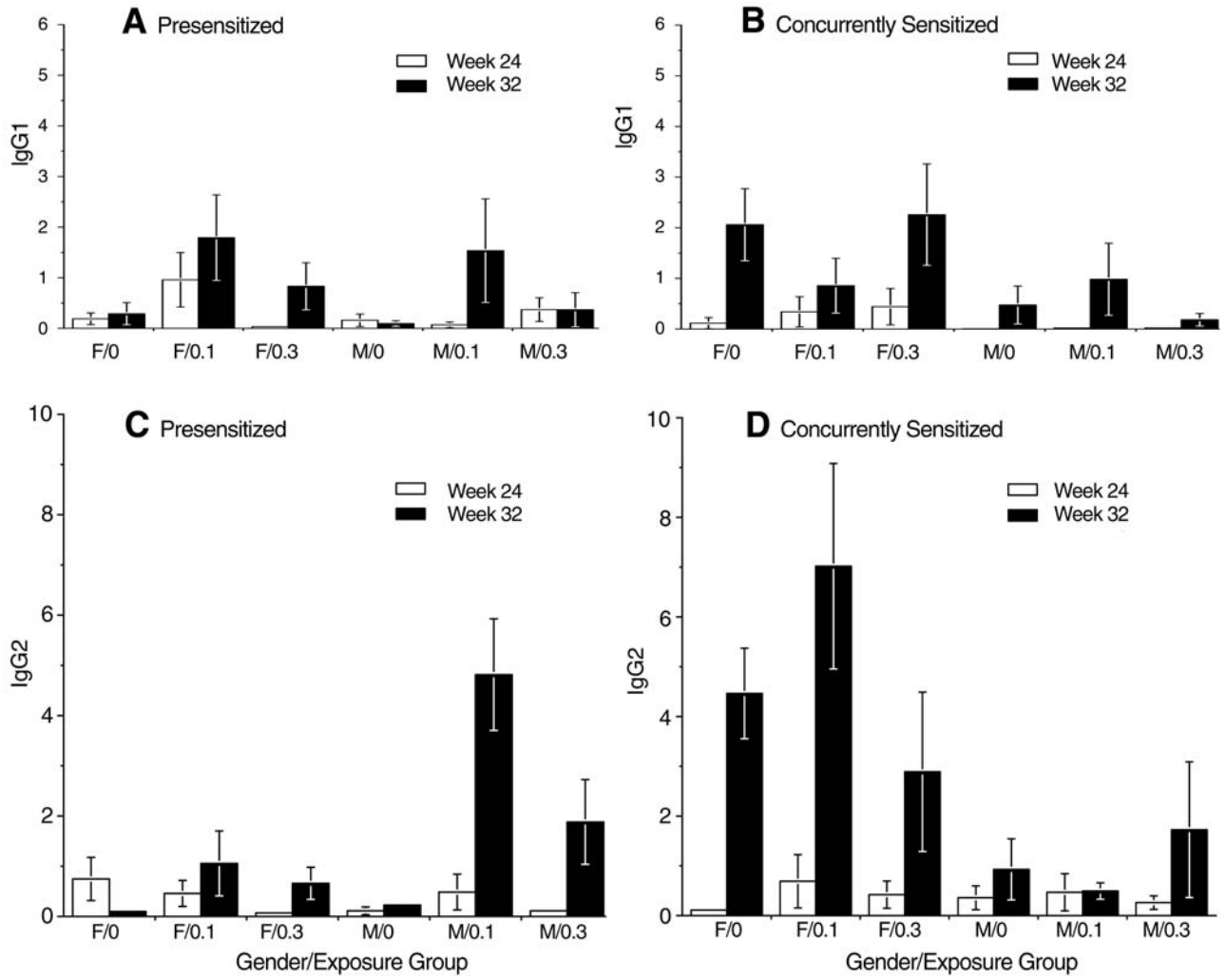

Figure 15. Antigen-specific immunoglobulin levels in blood from animals killed immediately after exposure series or after postexposure period. ***A***, IgG1 in presensitized animals; ***B***, IgG1 in concurrently sensitized animals; ***C***, IgG2 in presensitized animals; and ***D***, IgG2 in concurrently sensitized animals. The values represent the IgG concentration in blood relative to that of the IgG standard. Thus, there is no unit. Each value is the mean (± SE) obtained from 5 animals. F = female; M = male; 0 = air control; 0.1 = 0.1 ppm ozone; 0.3 = 0.3 ppm ozone.

IgG1 levels in PS and CS animals killed immediately after the end of the exposure series were unaffected by ozone. However, IgG1 levels were generally significantly higher in both PS and CS animals killed after the postexposure period compared with levels in those killed after the last exposure. Furthermore, IgG1 levels in females were generally higher than were those in males (although this difference was not statistically significant for the PS animals). A gender-killing time interaction was observed in CS animals: Regardless of ozone exposure group, males killed immediately after the last exposure had levels of IgG1 that were statistically significantly lower than were those in any of the postexposure groups. In contrast, the postexposure females showed the highest levels of IgG1.

In terms of IgG2, a statistically significant effect related to ozone exposure was observed in PS animals. This pattern was generally maintained among the CS animals, but it was not statistically significant. The highest statistically significant levels of IgG2 were observed in animals exposed to 0.1 ppm ozone. Values of IgG2 for animals exposed to 0.3 ppm ozone were intermediate between those of the control and 0.1 ppm exposure groups. Further, they were not statistically significantly different from values for either of the two groups.

As noted earlier, correlation analyses were performed to determine whether blood levels of IgG could reflect the degree of airway responsiveness. The correlation coefficients obtained from these analyses are shown in Table 12.

Table 12. Correlation of Airway Responsiveness and IgG

	IgG1		IgG2	
PC50	Correlation Coefficient	*P* Value	Correlation Coefficient	*P* Value
Ovalbumin challenge				
At week 24[a]	–0.417	**0.008**	–0.340	**0.032**
Ratio[b]	–0.496	**0.005**	–0.381	**0.034**
ACH challenge				
At week 24	–0.352	**0.026**	–0.365	**0.021**
Ratio	–0.429	**0.006**	–0.416	**0.008**

[a] Analysis using PC50 with the indicated challenge agent measured after Week 24.

[b] Analysis using ratio of PC50 at Week 24 to that at Week 0.

The correlations between PC50 with either ACH or ovalbumin challenge and IgG1 or IgG2 were all negative, indicating that increasing serum levels of IgG were associated with decreasing PC50 (ie, increasing airway responsiveness). All of the correlations were statistically significant, and they accounted for approximately 34 to 50% of the linear association between IgG and PC50. There were no substantial differences in interpretation between IgG1 and IgG2 with ACH challenge or IgG2 with ovalbumin challenge. These three sets of correlations are similar to one another, and they explain about 10% less variability than is explained by the correlation between IgG1 and ovalbumin challenge.

In an analysis designed to evaluate whether variability in the fraction of blood eosinophils might be associated with degree of sensitization as measured by levels of IgG, the correlation coefficients were found to be 0.051 ($P = 0.76$) and –0.027 ($P = 0.87$) for IgG1 and IgG2, respectively. Thus, no statistically significant relation was found between levels of IgG and blood eosinophils.

HISTOPATHOLOGY

Examination of lung tissue sections revealed no light microscopic evidence for pulmonary inflammatory response in any animal. The statistical comparison of eosinophils in the subepithelium of airways with eosinophils recovered in lavage samples resulted in a significant correlation coefficient ($r = 0.7$, $P < 0.05$), indicating that the number of cells in the lavage fluid analysis likely reflected the relative number of eosinophils in the lungs. Furthermore, ozone exposure did not influence the number of airway eosinophils, a finding consistent with the lavage fluid eosinophil analysis.

The analyses of airway mast cells indicated no statistically significant difference in cell number that could be related to ozone exposure in any of the experimental protocols. Further, there was no statistically significant correlation between mast cell number and airway responsiveness ($r = 0.12$, $P = 0.73$). As an example of mast cell numbers obtained, Figure 16 shows results from the PS group killed immediately after the exposure period.

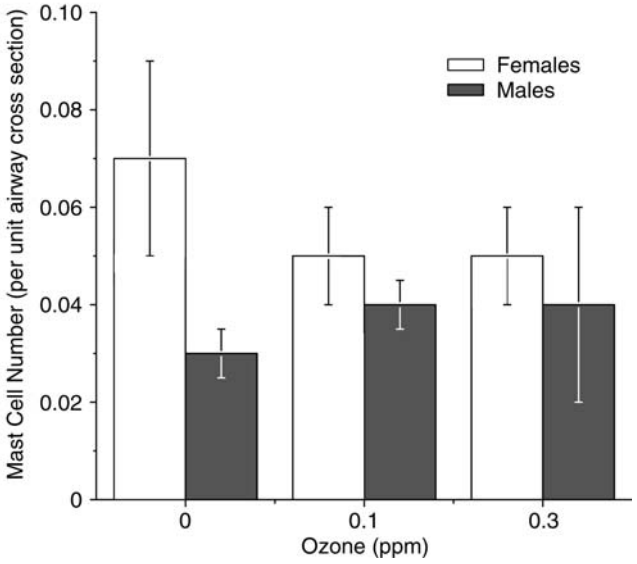

Figure 16. Mast cells per unit airway surface area in presensitized animals. Each value is the mean (± SE) obtained from 5 animals.

DISCUSSION

OZONE EXPOSURE AND AIRWAY RESPONSIVENESS

The main goal of this study was to evaluate the effect of long-term, repeated exposures to ozone on airway responsiveness in both normal, nonatopic animals and antigen-sensitized (atopic) animals. This study was the first to use relatively low exposure concentrations to evaluate both specific and nonspecific responsiveness as well as the potential modulating effect of gender. As shown in the Results section, ozone did not induce airway hyperresponsiveness in the nonatopic (NS) animals. However, in atopic animals that demonstrated hyperresponsiveness prior to any exposure (the PS animals), ozone resulted in exacerbation after either a specific or a nonspecific bronchoprovocative stimulus. This occurred in an ozone concentration-dependent but gender-independent, fashion. Furthermore, the effect of ozone was quantitatively similar for both nonspecific and specific responsiveness, and this effect persisted through at least 4 weeks after the end of the exposures. Finally, ozone did not alter baseline levels of sGaw, suggesting that normal airway caliber was not affected by exposure.

Previously reported investigations with repeated ozone exposures that examined nonspecific airway responsiveness in nonsensitized animals have shown equivocal results. For example, reduced responsiveness occurred in guinea pigs exposed to 1 ppm for 3 hours/day for 4 days (Sun and Chung 1997). However, no change was noted in guinea pigs exposed to 0.15 ppm ozone for 4 hours/day, 5 days/week for 4 months (Kagawa et al 1989) or in cynomolgus monkeys exposed to 1 ppm for 6 hours/day, 5 days/week for 12 weeks (Biagini et al 1986). On the other hand, increased nonspecific responsiveness occurred in rhesus monkeys after 19 weeks of single, 2-hour weekly exposures to 1 ppm ozone (Johnson et al 1988). The full database, which includes results from the numerous studies of acute ozone exposure (Easton and Murphy 1967; Golden et al 1978; Gordon et al 1984; Holroyde and Norris 1988; Fouke et al 1991; Yeadon et al 1992; Matsubara et al 1995; Frampton et al 1997; Folinsbee and Hazucha 2000), indicates that ozone can produce increased airway responsiveness in nonsensitized individuals of various species, including humans. However, the exposure concentrations consistently resulting in this response were higher than those used in the current investigation. Exposures at or below about 0.3 ppm have been associated with inconsistent alterations in responsiveness.

This study has shown that ozone exacerbated nonspecific hyperresponsiveness in sensitized animals. The few previously reported studies in which sensitized humans or nonhuman species were exposed to ozone, most of which involved acute exposures to levels ranging upward from 0.6 ppm, generally also showed increased responsiveness to nonspecific challenges (Holtzmann et al 1979; Thorne and Broadley 1994; Sun et al 1997).

The current investigation also showed that exposure of sensitized animals to ozone exacerbated response to an inhaled antigen (namely ovalbumin). This result is consistent with previously reported studies, all of which involved short-term exposures to ozone. In one such study (Yanai et al 1990), both sensitized and nonsensitized dogs were exposed to 3 ppm ozone for 2 hours and then challenged with antigen. Ozone did not affect airway responsiveness to antigen in nonsensitized animals, but it did increase responsiveness in sensitized hosts. Similarly, in a study of atopic adult humans with asthma, exposure to 0.12 ppm ozone for 1 hour potentiated the response to subsequently inhaled allergen (Molfino et al 1991). Other investigators noted similar effects with acute exposures to lower levels of ozone, namely 0.16 to 0.25 ppm (Jörres et al 1996; Jenkins et al 1999; Peden 1999). Thus, the current study provides evidence for an exacerbation of hyperresponsiveness with repeated exposures to ozone at a concentration below that previously shown to produce such a response.

The overall experimental design of this study did not allow for a statistical comparison of the results among the three experimental protocols. Because each of the protocols was designed to address a specific question, they differed in some experimental details in ways that precluded such a direct evaluation. However, the results can be compared in a qualitative manner.

One such comparison of interest is whether the response to provocation challenge in the ozone-exposed animals differed between the two groups of sensitized animals (PS and CS). Figure 17 shows PC50 obtained after ACH challenge in sensitized animals exposed to 0.3 ppm ozone, the concentration that produced the greatest effect on airway responsiveness, expressed as fractional change from the values obtained in air controls. The ozone-induced exacerbation of airway hyperresponsiveness was quite comparable in both protocols and, thus, appeared to be relatively independent of when the animals were sensitized.

Many biological responses evaluated with repeated ozone exposures are characterized by the phenomenon of adaptation, an attenuation of response with continued

exposure. Attenuation may occur with some endpoints and not others (Jörres et al 2000), but whether it is a factor in airway hyperresponsiveness is not clear. For example, no adaptation in responsiveness was noted in humans with 6.6 hours/day, 5-day exposures to 0.12 ppm ozone (Folinsbee et al 1994). However, 2 hours/day, 3-day exposures of humans to 0.4 ppm ozone resulted in an initial increase in airway responsiveness that returned to preexposure levels by the third day (Dimeo et al 1981). These studies involved relatively short exposure durations, and it is likely that the specific length of total exposure could affect development of adaptation. For example, nonatopic guinea pigs exposed to 0.3 ppm ozone for 4 hours/day for 1, 3 or 6 days or for 4 hours/day, 6 days/week for 12, 24 or 48 days showed increased airway responsiveness through 24 days of exposure. This change was no longer evident after 48 days of exposure, however, suggesting an adaptive phenomenon after a certain length of repeated exposures (Vargas et al 1998). Results of the current study, which had 24 weeks of exposure, did not show any evidence of adaptation in ozone-induced exacerbation of airway hyperresponsiveness.

In the current study, intragroup variability in responsiveness at various time points was often large, even within one gender. Many species, including the guinea pig, have demonstrated a large range of sensitivity to bronchoprovocative agents and variability in the resulting measures of airway responsiveness (Turner and Martin 1997). This variability may reflect interindividual differences in airway receptor numbers or sensitivity (Abraham et al 1980; Ahmed et al 1980). In addition, susceptibility to ozone-induced alterations in airway responsiveness has been shown to be quite variable within any exposure group (Douglas et al 1977; Snapper et al 1978; Habib et al 1979; Abraham et al 1980).

A potential factor underlying variability in responsiveness between individuals could be differences in the extent of sensitization as measured by blood levels of antigen-specific antibodies. We found that IgG levels correlated with PC50 but that the relation accounted for less than 50% of the linear association between these two parameters. This is consistent with results reported by others. The allergic antibody IgE in humans has also been shown to have a low correlation with increased nonspecific airway responsiveness (Palmer et al 2000). In persons with and without asthma, for example, about 30% of the individual variance in airway responsiveness has been attributed to total serum IgE levels (Burrows et al 1989; Sunyer et al 1995). Thus, to a limited extent, the degree of sensitization may be reflected in the extent of hyperresponsiveness.

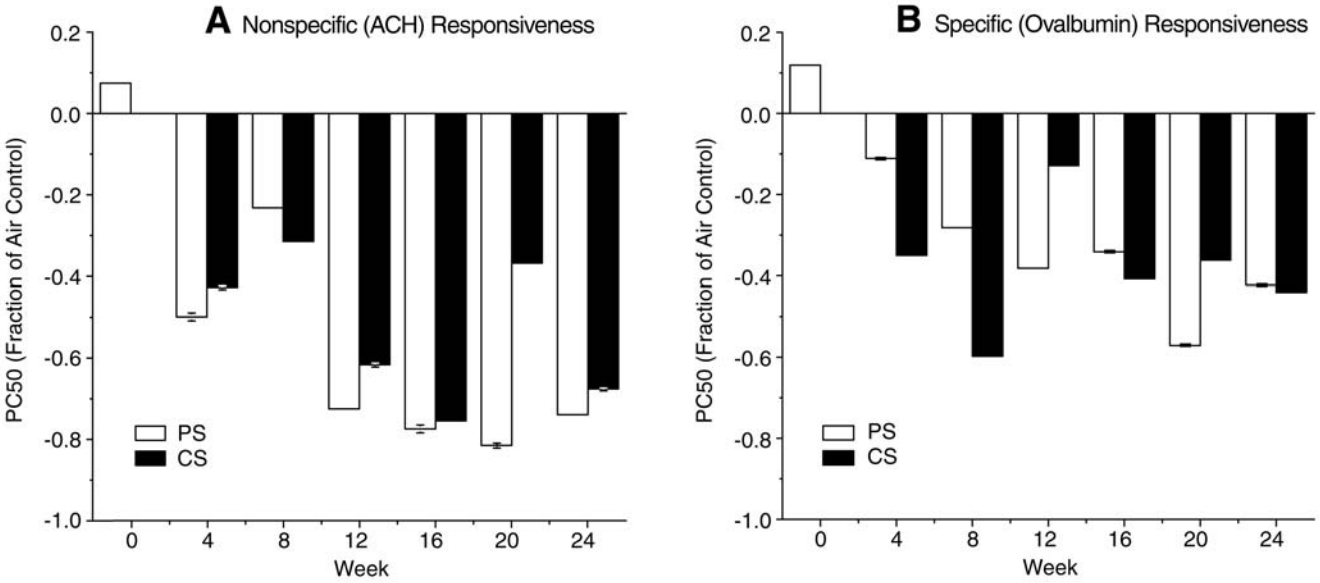

Figure 17. Comparison of presensitized and concurrently sensitized animals exposed to highest concentration of ozone. ***A***, Nonspecific responsiveness to ACH, and ***B***, specific responsiveness to ovalbumin. The PC50 is expressed as the fraction of the respective air control. Each value is the mean (± SE) obtained from 10 animals.

GENDER AS A MODULATOR OF OZONE EFFECT ON AIRWAY RESPONSIVENESS

This study was designed to allow evaluation of gender differences in airway responsiveness related to ozone exposure. The effect of ozone, when it occurred, was found to be similar for both males and females; there was no evidence of any consistent gender-based difference in the functional response to ozone exposure. Previously reported experimental studies, all involving humans, have provided conflicting results in terms of gender differences in pulmonary functional response to ozone. Some have found no differences, whereas others have suggested that females may show greater responses than do males (Adams et al 1981; Lauritzen and Adams 1985; Drechsler-Parks et al 1987). Such gender differences have been suggested to be due to differences in dose resulting from differences in lung size and ventilatory parameters between males and females as well as resulting pollutant-to-lung volume ratios (Lauritzen and Adams, 1985). However, no gender difference in pulmonary functional indices was found in humans exposed acutely to 0.35 ppm ozone when adjustments were made during exposure to assure similar ventilation (Weinmann et al 1995). In the current study, similar effects occurred in both genders in spite of differences in body size (and therefore lung size) between male and female guinea pigs.

Although the current study showed no gender dependence of ozone effect on airway responsiveness, the general degree of airway responsiveness did differ between males and females. Within each experimental protocol, males and females had a similar level of responsiveness at the start of the exposure series (Week 0), but beyond this time, female guinea pigs demonstrated less responsive airways than did males. Similarly, Wanner and colleagues (1990) noted that male and female guinea pigs were equally reactive in the early weeks of life. Gender differences began to appear at a later age. A temporal divergence of responsiveness between genders is also consistent with a human study in which no gender difference in airway responsiveness was noted below a certain age (12 years) although differences began to emerge beyond this age (Forastiere et al 1996).

OZONE EXPOSURE AND ALLERGIC SENSITIZATION OF AIRWAYS

Although the ozone effect on airway responsiveness did not appear to differ between the PS and CS animals, the question of whether ozone affected sensitization remains. In other words, did exposure to ozone during the period of sensitization result in a different degree or extent of sensitization than was noted in the animals exposed to ozone after sensitization (the CS and PS protocols, respectively)? The extent of sensitization can be evaluated by comparing blood levels of antigen-specific antibody for the animals in both protocols. There was a statistically significant exposure-related effect on IgG2 in the PS animals, a pattern that was generally maintained in the CS animals, although not at a statistically significant level. Thus, it appears that increased levels of antigen-specific antibody were produced in PS and CS animals exposed to ozone compared with air-control animals, and the PS animals may have shown a somewhat stronger effect. The ability of ozone to affect allergic sensitization has been addressed previously in only one study (Osebold et al 1980). In this, mice were repeatedly exposed to ovalbumin and then to 0.5 or 0.8 ppm ozone for 24 hours/day for 3 to 4 days. The extent of sensitization in the ozone-exposed group was enhanced compared with sensitization in the air controls, a result consistent with the one reported here.

BIOLOGICAL MODULATORS OF OZONE EFFECT ON AIRWAY RESPONSIVENESS

In addition to the direct measurement of airway responsiveness, this study evaluated various biological parameters that may have modulated this physiologic endpoint. One of these was the level of endogenous NO, indirectly assessed by measuring levels of exhaled NO at various times during the exposure series. The measured NO concentrations, which ranged from 12 to 45 ppb among the sensitized animals, are quite consistent with those reported in another study. Those investigators reported that levels measured in the exhaled air of ovalbumin-sensitized guinea pigs showed stable levels of 9 ppb, rising to over 40 ppb after ovalbumin challenge (Persson and Gustafsson 1993).

NO, which is produced by various cells in the respiratory system, has a number of actions, including activity as a potent bronchodilator. Although its role in airway hyperresponsiveness remains unresolved, the inhibition of NO production or NO deficiency has been shown to contribute to airway hyperresponsiveness in guinea pigs (Nijkamp et al 1993; Schuiling et al 1998). In the current study, exhaled NO concentrations showed large variability within each group, and there was little indication of any differences that could be related to ozone exposure.

Pulmonary inflammatory cells are another potential modulator of airway responsiveness related to ozone exposure. Studies of atopic animals and humans with asthma suggest that airway hyperresponsiveness may be associated with increased numbers of eosinophils, monocytes, macrophages, neutrophils, mast cells, and lymphocytes in

the airway subepithelium (Larsen 1991). In bronchial lavage studies conducted after allergen inhalation challenge, an influx of eosinophils and neutrophils into the airways of patients with asthma was associated with the development of airway hyperresponsiveness (DeMonchy et al 1985; Beasley et al 1989). Nonatopic guinea pigs exposed to 1 ppm ozone for 3 hours/day for 4 days were found to have increased numbers of macrophages, eosinophils, and neutrophils in lavage fluid even at time points when ozone-induced airway hyperresponsiveness was returning to control levels (Sun and Chung 1997).

One cell type that has been particularly associated with airway dysfunction is the eosinophil. For example, increased numbers of eosinophils in peripheral blood have been associated with bronchial hyperresponsiveness in humans (Rijcken et al 1993). In the current investigation, however, there was no statistical relation between levels of eosinophils in lavage fluid and degree of airway responsiveness measured after the last ozone exposure (Week 24).

In general, a causal association between eosinophils and airway hyperresponsiveness appears to be equivocal. Some studies have found a relation between numbers of eosinophils in lavage fluid and airway hyperresponsiveness after antigen challenge in sensitized guinea pigs (Dunn et al 1988; Hutson et al 1988; Sanjar et al 1990b; Santing et al 1994). Other studies have noted, however, that eosinophilia is not accompanied by hyperresponsiveness, suggesting that there may be no causal relation between the two phenomena (Sanjar et al 1990a,b; Chapman et al 1991; Kips et al 1992; Pretolani et al 1994; Sun et al 1997). To explain these disparate results, it has been suggested that animals sensitized with low doses of allergen exhibit airway eosinophilia in the absence of hyperresponsiveness, whereas animals sensitized using higher doses of allergen exhibit both eosinophilia and hyperresponsiveness (Chapman et al 1991; Sanjar et al 1990b). When data from individual animals were examined in this regard, however, no correlation between these two parameters could be detected (Sanjar et al 1990a), a finding consistent with results in the current study.

Thus, available evidence, including results from the current study, seems to indicate that, although eosinophilia may occur in association with airway hyperresponsiveness, there is no definitive evidence for a causal relation (Sanjar et al 1990b). In fact, guinea pigs have been shown to demonstrate airway hyperresponsiveness despite low eosinophil numbers in the airways or with eosinophilia in the absence of attendant hyperresponsiveness. Humans with asthma may have eosinophilia without airway hyperresponsiveness (Chapman et al 1991), and hyperresponsiveness in humans has been shown to occur independently of levels of eosinophils and total amount of IgE (Desjardins et al 1988). Some researchers have suggested that eosinophil activation rather than accumulation is the constitutive factor in the development of airway hyperresponsiveness in the sensitized guinea pig (Pretolani et al 1994). Cell activation was not measured in the current study.

Although a consistent temporal association between the onset of ozone-induced airway hyperresponsiveness and neutrophil influx into the airways has been found in some studies in other species (Holtzman et al 1983; Seltzer et al 1986), no such temporal effect was seen in guinea pigs in the current study. Thus, this may reflect differences in the relation between these responses and ozone exposure in the different species examined. Ozone exposure may be linked with both hyperresponsiveness and neutrophilic inflammation. However, current indications are that, as is the case with eosinophilia, the two processes are not necessarily correlated with each other (Peden 1999).

Other investigators have suggested that mast cells may play a role in airway responsiveness, as well as in atopic asthma (Wardlaw et al 1988; van den Toorn et al 2000). Increased number of such cells could result in enhanced responsiveness due to increased release of histamine, a bronchoconstrictor. In the current study, there was no increase in such cells with ozone exposure. This is consistent with other reports showing no relation between numbers of mast cells and airway responsiveness (Smith and McFadden 1995).

As noted earlier, the animals in the present study were killed about 5 to 7 days after their final test of airway responsiveness (after the 24-week ozone exposure period or after the 8-week postexposure period). This time frame may have had an impact on some of the results. For example, guinea pigs that were sensitized to ovalbumin and subsequently challenged by ovalbumin inhalation showed a peak response of eosinophilia in lavage fluid 24 hours after challenge (Underwood et al 1992). It is possible that any early post-ovalbumin exposure eosinophila, neutrophilia, or other measure of inflammation was resolved by the time of lavage in the present investigation (Hutson et al 1988). However, the available evidence indicates that a relation between degree of bronchial responsiveness and intensity of airway inflammatory markers is not certain, and there may actually be no correlation between airway responsiveness and specific cellular infiltrates or epithelial loss (Smith and McFadden 1995). This lack of consistent correlation between intensity of inflammation, number of inflammatory cells, mediators or both, and degree of airway responsiveness undermines any assumption that hyperresponsiveness is driven by inflam-

mation (Smith and McFadden 1995). This reasoning is supported by results of the current study, including the lack of evidence of any pulmonary inflammatory response in the light-microscopy examination of airway sections.

Some researchers have suggested that ozone-induced airway hyperresponsiveness may be due to a loss of normal epithelial function rather than infiltration of inflammatory cells into the airways (Matsubara et al 1995). In a study of ovalbumin-sensitized guinea pigs, increased response to ACH challenge was found without any light-microscopy evidence of epithelial damage (Masaki et al 1994). Such increased nonspecific airway responsiveness may be due to altered epithelial function, which included changes in neural reflexes and in the regulation of mediators of airway caliber. The results of the current study provide support for this possible mechanism.

RELEVANCE OF THE ANIMAL MODEL

Animal models are essential to test hypotheses about the pathogenesis of airway dysfunction that are not testable in human studies, such as effects of controlled long-term exposures to air pollutants. In attempting to relate results of this study to ozone-related human airway functional alterations, two caveats need to be mentioned. One is that the animals were exposed to ozone and had airway responsiveness evaluated over a time during which they matured from prepuberty (about 4 weeks of age) through adolescence and into adulthood (beginning at about 16 weeks of age) (Gaultier et al 1984). The timing of exposure to environmental stresses (such as allergens and toxicants) during respiratory tract development is important in the subsequent expression of atopy as well as other airway functional phenotypes (Dietert et al 2000; Peden 2000; Pinkerton and Joad 2000). In humans, for example, the critical window for such exposure in terms of potential impact of environmental agents is the first year of life (Peden 2000). This concept likely applies to the guinea pig as well, although immune development differs between humans and those mammals with shorter gestational periods. The results of the current study might have differed if the ozone exposures, antigen challenges, or both had begun with older guinea pigs (Pinkerton and Joad 2000). For example, age-related differences between younger and older adult humans have been noted in pulmonary functional responses to ozone (Drechsler-Parks et al 1987), with a greater response among younger adults. The effect of pollutant exposure upon airway responsiveness and the interaction between such exposure and atopy likely depend upon the age range within which such effects are measured, and trends and relations may differ at different stages of life.

Regarding age, due to the need to initiate sensitization at the same age in the PS and CS protocols, there was a 3 to 4 week difference in the age at which ozone exposures began between the NS and CS protocols and the PS protocol (Table 1). It cannot be determined whether this age difference affected the response to ozone between the two sensitized protocols or between the PS and NS protocols, but the effects of ozone on airway responsiveness in the PS and CS protocols appear to be similar.

A second caveat in evaluating the relevance to humans of results from a study using an animal model is that such models cannot mimic all aspects of human disease. They should be selected for particular aspects of interest. In this case, the guinea pig was selected to mimic two features of human airway disease, sensitization to antigen (atopy) and nonspecific hyperresponsiveness to spasmogenic agents. Given this, an assessment of relevance also requires consideration of the site of action of inhaled materials in terms of the response that was evaluated. Unlike humans, guinea pigs are obligate nasal breathers. Because the noninvasive measure of airway function performed in this study was composed of a combination of nasal airway (upper respiratory tract) and tracheobronchial (lower respiratory tract) responses, two possibilities exist. First, ozone-induced changes in airway responsiveness could reflect functional alterations within the upper respiratory tract rather than the lower airways. Second, changes in the upper respiratory tract could contribute significantly to overall measurements (Johns et al 1990). For example, this might be due to an enhanced deposition of inhaled bronchoprovocative agents within the nasal passages. However, lower airway responses dominate measures of respiratory resistance, and therefore conductance, in guinea pigs. Although nasal resistance constitutes the majority of total respiratory system resistance measured with use of a plethysmograph, the nasal passages likely only add a constant resistance to the measured values. Therefore, any changes in resistance are dominated by effects in responsiveness occurring within the pulmonary airways (Holroyde and Norris 1988; Finney and Forsberg 1994). Thus, it is likely that the ozone-induced alterations in airway responsiveness observed in this study reflected effects within the lungs and not solely within the upper respiratory tract, which is similar to what would be likely to occur in ozone-exposed humans.

CONCLUSIONS

Airway hyperresponsiveness is an exaggerated response to a bronchoconstrictive stimulus. Although it has been traditionally considered to be a hallmark of asthma and measurement of airway responsiveness is often used to

identify individuals who may be at risk for asthma or related conditions, responsiveness demonstrates a distribution in the population. Persons with asthma occupy the sensitive end (Smith and McFadden 1995; Peden 2000). In any case, exacerbation of hyperresponsiveness after exposure to an inhaled pollutant has a practical health impact, which can range from minor airway constriction to a severe asthma attack.

This study has shown that long-term repeated exposures to ozone did not result in induction of airway hyperresponsiveness in nonsensitized animals. However, exposures in sensitized animals did result in a persistent exacerbation of existing hyperresponsiveness to both nonspecific and specific stimuli. This suggests that individuals with hyperresponsive airways may be at increased risk from exposure to air pollutants, supporting evidence that ambient ozone is associated with aggravation of preexisting pulmonary disease such as asthma (Thurston and Ito 1999) and that ozone adversely affects atopic people (Peden 2000). On the other hand, although a limited number of long-term exposure epidemiologic studies may suggest that ozone is involved in induction of asthma (Thurston and Ito 1999), the current study does not support this, at least in terms of the development of airway hyperresponsiveness in nonatopic individuals.

The nature of the ozone effect upon airway hyperresponsiveness did not seem to depend upon the temporal relation between development of atopy and ozone exposure. Furthermore, although there was a clear gender difference in baseline responsiveness, there was no gender-associated difference in the effect of ozone on this parameter. Finally, ozone-induced changes in responsiveness were not associated with the number of eosinophils in lavage fluid or with other inflammatory cells in the lungs.

The prevalence and severity of airway diseases such as asthma are increasing worldwide (Sears 1997). Although the factors involved are likely to be complex, various environmental influences, including air pollution, are recognized as potentially playing a role in genesis, exacerbation, or both (Bascom 1996; Becklake and Ernst 1997; von Mutius 1997; Platts-Mills et al 2000). Because persons with asthma generally have lower levels of lung function and increased airway responsiveness compared with normal individuals, the clinical consequences of any effects induced by ozone exposure may be quite serious (Bascom 1996).

Animal models of disease are not exactly the same as their human disease counterpart, and airway dysfunction in such models exhibits both similarities and dissimilarities to that in humans. Thus, it can often be difficult to relate information obtained in these models to the prototypical human manifestation of airway hyperresponsiveness. Despite this drawback, it is possible to compare general features of an animal model to human airway hyperresponsiveness and to define its relevance to the human situation. Various stimuli can evoke airway narrowing in animals that is accompanied in some cases by hyperresponsiveness, thus modeling potentially relevant triggers of asthma attack in humans. Results of the current investigation suggest that ozone may be one of these triggers, at least in one fairly large susceptible human population, namely atopic individuals.

REFERENCES

Abraham WM, Januszkiewicz AJ, Mingle M, Welker M, Wanner A, Sackner A. 1980. Sensitivity of bronchoprovocation and tracheal mucous velocity in detecting airway response to O_3. J Appl Physiol 48:789–793.

Adams WC, Savin W, Christo AE. 1981. Detection of ozone toxicity during continuous exercise via the effective dose concept. J Appl Physiol 51:415–422.

Agrawal KP. 1981. Specific airway conductance in guinea pigs: Normal values and histamine induced fall. Respir Physiol 43:23–30.

Ahmed T, Eyre P, Januszkiewicz J, Wanner A. 1980. Role of H1- and H2-receptors in airway reactions to histamine in conscious sheep. J Appl Physiol 49:826–833.

Bascom R. 1996. Environmental factors and respiratory hypersensitivity: The Americas. Toxicol Lett 86:115–130.

Beasley R, Roche WM, Roberts JA, Holgate ST. 1989. Cellular events in the bronchi in mild asthma and after bronchial provocation. Am Rev Respir Dis 139:806–817.

Becklake MR, Ernst P. 1997. Environmental factors. Lancet 350 (Suppl 11):10–13.

Biagini RE, Moorman WJ, Lewis TR, Bernstein IL. 1986. Ozone enhancement of platinum asthma in a primate model. Am Rev Respir Dis 134:719–725.

Burrows B, Martinez F, Halonen, Barbee R, Cline M. 1989. Association of asthma with serum IgE levels and skin-test reactivity to allergens. N Engl J Med 320:271–277.

California Air Resources Board. 1988. Summary of 1988 Air Quality Data: Gaseous and Particulate Pollutants. Air Qual Data 20:51–59.

Chapman ID, Boubekeur K, Morley J. 1991. Airway eosinophilia and airway hyperreactivity are parallel rather than

sequential events in the guinea pig. In: New Drugs for Asthma Therapy, pp 445–456. Birkhauser Verlag, Basel, Switzerland.

DeMonchy JGR, Kauffman HF, Venge P, Koeter GH, Janse HM, Sluiter HJ, De Vries K. 1985. Bronchoalveolar eosinophila during allergen-induced late asthmatic reactions. Am Rev Respir Dis 131:373–376.

Desjardins A, de Luca S, Cartier P. 1988. Nonspecific bronchial hyperresponsiveness to inhaled histamine and hyperventilation of cold dry air in subjects with respiratory symptoms of uncertain etiology. Am Rev Respir Dis 137:1020–1025.

Dietert RR, Etzel RA, Chen D, Halonen M, Holladay SD, Jarabek AM, Landreth K, Peden DB, Pinkerton K, Smialowicz RJ, Zoetis T. 2000. Workshop to identify critical windows of exposure for children's health: Immune and respiratory systems work group summary. Environ Health Perspect (Suppl) 108(3):483–490.

Dimeo MJ, Glenn MG, Holtzman MJ, Sheller JR, Nadel JA, Boushey HA. 1981. Threshold concentration of ozone causing an increase in bronchial reactivity in humans and adaptation with repeated exposures. Am Rev Respir Dis 124:245–248.

Douglas JS, Ridgway P, Brink C. 1977. Airway response of the guinea pig in vivo and in vitro. J Pharmacol Exp Ther 202:116–124.

Drechsler-Parks DM, Bedi JF, Horvath SM. 1987. Pulmonary function responses of older men and women to ozone exposure. Exp Gerontol 22:91–101.

Dunn CJ, Elliott GA, Oostveen JA, Richards IM. 1988. Development of a prolonged eosinophil-rich inflammatory leukocyte infiltration in the guinea-pig asthmatic response to ovalbumin inhalation. Am Rev Respir Dis 137:541–547.

Easton RE, Murphy SD. 1967. Experimental ozone preexposure and histamine: Effect on the acute toxicity and respiratory function effects of histamine in guinea pigs. Arch Environ Health 15:160–166.

Environmental Protection Agency (US). 1996. Air Quality Criteria for Ozone and Related Photochemical Pollutants. Document EPA/600/P-93/004aF. EPA Office of Research and Development, Washington DC.

Finney MJB, Forsberg KIH. 1994. Quantification of nasal involvement in a guinea pig plethysmograph. J Appl Physiol 76:1432–1438.

Folinsbee LJ, Hazucha MJ. 2000. Time course of response to ozone exposure I. Healthy adult females. Inhalation Toxicol 12:151–167.

Folinsbee LJ, Horstman DH, Kehrl HR, Harder S, Abdul-Salaan S, Ives PJ. 1994. Respiratory responses to repeated prolonged exposure to 0.12 ppm ozone. Am J Respir Crit Care Med 149:98–105.

Forastiere F, Corbo GM, Dell'orco V, Pistelli R, Agabiti N, Kriebel D. 1996. A longitudinal evaluation of bronchial responsiveness to methacholine in children: Role of baseline lung function, gender, and change in atopic status. Am J Respir Crit Care Med 153:1098–1104.

Fouke JM, DeLemos RA, Dunn MJ, McFadden Jr ER. 1991. Effects of ozone on lung mechanics and cyclooxygenase metabolites in dogs. Prostaglandins 42:343–353.

Frampton MW, Morrow PE, Torres A, Cox C, Voter KZ, Utell MJ. 1997. Ozone responsiveness in smokers and nonsmokers. Am J Respir Crit Care Med 155:116–121.

Fraser DG, Graziano FM, Larsen CP, Regal JF. 1998. The role of IgG1 and IgG2 in trimellitic anhydride-induced allergic response in the guinea pig lung. Toxicol Appl Pharmacol 150:218–227.

Gaultier CL, Harf A, Lorino AM, Atlan G. 1984. Lung mechanics in growing guinea pigs. Respir Physiol 56:217–228.

Golden JA, Nadel JA, Boushey HA. 1978. Bronchial hyperreactivity in healthy subjects after exposure to ozone. Am Rev Respir Dis 118:287–293.

Gordon T, Drazen JM, Amdur MO, Venugopalan CS. 1984. Ozone induced airway hyperreactivity in guinea pigs. J Appl Physiol 57:1034–1038.

Griffith-Johnson DA, Jin R, Karol MH. 1993. The role of purified IgG1 in pulmonary hypersensitivity responses of the guinea pigs. J Toxicol Environ Health 40:117–127.

Gross KB, Sargent NE. 1992. Increases in bronchial responsiveness induced by ozone inhalation and the effects of selected non-steroidal antiinflammatory agents. Am Rev Respir Dis 145:A428–A432.

Habib MP, Pare PD, Engel LA. 1979. Variability of airway responses to inhaled histamine in normal subjects. J Appl Physiol 47:51–58.

Harper LV. 1976. Behavior. In: The Biology of the Guinea Pig (Wagner JE, Manning PJ, eds), pp 31–52. Academic Press, Orlando FL.

Herxheimer H, West T. 1955. Sensitization of guinea pigs by inhalation. J Physiol (London) 127:564–571.

Holroyde MC, Norris AA. 1988. The effect of ozone on reactivity of upper and lower airways in guinea-pigs. Br J Pharmacol 94:938–946.

Holtzman MJ, Cunningham JH, Sheller JR, Irsigler GB, Nadel JA, Boushey HA. 1979. Effect of ozone on bronchial reactivity in atopic and nonatopic subjects. Am Rev Respir Dis 120:1059–1067.

Holtzman MJ, Fabbri LM, O'Byrne PM, Gold BD, Aizawa H, Walters EH, Alpert SE, Nadel JA. 1983. Importance of airway inflammation for hyperresponsiveness induced by ozone. Am Rev Respir Dis 127:686–690.

Horstmann DH, Folinsbee LJ, Ives PJ, Abdul-Salaam S, McDonnell WF. 1990. Ozone concentration and pulmonary response relationships for 6.6-hour exposures with five hours of moderate exercise to 0.08, 0.10, and 0.12 ppm. Am Rev Respir Dis 142:1158–1163.

Hutson PA, Church MK, Clay TP, Miller P, Holgate SP. 1988. Early and late-phase bronchoconstriction after allergen challenge of nonanesthetized guinea pigs: I. The association of disordered airway physiology to leukocyte infiltration. Am Rev Respir Dis 137:548–557.

Jenkins HS, Devalia JL, Mister RL, Bevan AM, Rusznak C, Davies RJ. 1999. The effect of exposure to ozone and nitrogen dioxide on the airway response of atopic asthmatics to inhaled allergen: Dose- and time-dependent effects. Am J Respir Crit Care Med 160:33–39.

Johns K, Sorkness R, Graziano F, Castleman W, Lemanske Jr RF. 1990. Contribution of upper airways to antigen-induced late airway obstructive responses in guinea pigs. Am Rev Respir Dis 142:138–142.

Johnson HG, Stout BK, Ruppel PL. 1988. Inhibition of the 5-lipoxygenase pathway with piripost (U-60,257) protects normal primates from ozone-induced methacholine hyperresponsive small airways. Prostaglandins 35:459–466.

Jörres RA, Holz O, Zachgo W, Timm P, Koschyk S, Muller B, Frimminger F, Seeger W, Kelly FJ, Dunster C, Frischer T, Lubec G, Waschewski M, Niendorf A, Mugnussen H. 2000. The effect of repeated ozone exposures on inflammatory markers in bronchoalveolar lavage fluid and mucosal biopsies. Am J Crit Care Med 161:1855–1861.

Jörres R, Nowak D, Magnussen H. 1996. The effect of ozone exposure on allergen responsiveness in subjects with asthma or rhinitis. Am J Respir Crit Care Med 153:56–64.

Josephs LK, Gregg I, Holgate ST. 1990. Does non-specific bronchial responsiveness indicate the severity of asthma? Eur Respir J 3:220–227.

Kagawa J, Haga M, Miyazaki M. 1989. Effects of repeated exposure to 0.15 ppm O_3 for four months on bronchial reactivity in guinea pigs (4 hrs/d, 5 days/wk). In: Atmospheric Ozone Research and Its Policy Implications: Proceedings of the 3rd US-Dutch International Symposium (Schneider T, Lee SD, Wolters GJR, Grant LD, eds), pp 545–552. Elsevier Science Publishing Co, New York, NY.

Kallos P, Kallos L. 1984. Experimental asthma in guinea pigs revisited. Int Arch Allergy Appl Immunol 73:77–85.

Kips JC, Cuvelier CA, Pauwels RA. 1992. Effect of acute and chronic antigen inhalation on airway morphology and responsiveness in actively sensitized rats. Am Rev Respir Dis 145:1306–1310.

Koenig JQ, Covert DS, Morgan MS. 1985. Acute effects of 0.12 ppm ozone or 0.12 ppm nitrogen dioxide on pulmonary function in healthy and asthmatic adolescents. Am Rev Respir Dis 132:648–651.

Kreit JW, Gross KB, Moore TB, Lorenzen TJ, D'Arcy J, Eschembacher WL. 1989. Ozone-induced changes in pulmonary function and bronchial responsiveness in asthmatics. J Appl Physiol 66:217–222.

Larsen GL. 1991. Experimental models of reversible airway obstruction. In: The Lung: Scientific Foundations (Crystal RG, West JB eds), pp 913–966. Raven Press, New York NY.

Lauritzen SK, Adams WC. 1985. Ozone inhalation effects consequent to continuous exercise in females: Comparison to males. J Appl Physiol 59:1601–1606.

Lefohn AS, Foley JK, Shadwick DS, Tilton BE. 1993. Changes in diurnal patterns related to changes in ozone levels. J Air Waste Manag Assoc 43:1472–1478.

Lewis CA, Boradley KJ. 1995. Airway hyper- or hyporeactivity to inhaled spasmogens 24 h after ovalbumin challenge of sensitized guinea-pigs. Br J Pharmacol 116:2351–2358.

Linn WS, Shamoo DA, Anderson KR, Peng RC, Avol EL, Hackney JD. 1992. Responses of asthmatics in prolonged, repeated exposures to ozone and sulfuric acid. Am Rev Respir Dis 145:A428–A438.

Linn WS, Shamoo DA, Anderson KR, Peng RC, Avol EL, Hackney JD. 1994. Effects of prolonged, repeated exposure to ozone, sulfuric acid and the combination in healthy and

asthmatic volunteers. Am J Respir Crit Care Med 150:431–440.

Lippmann M. 1992. Ozone. In: Environmental Toxicants: Human Exposures and Their Health Effects (Lippmann M, ed), pp. 465–519. Van Nostrand Reinhold, New York NY.

Masaki Y, Munakata M, Amishima M, Homma Y, Kawakami Y. 1994. In vivo, in vitro correlation of acetylcholine airway responsiveness in sensitized guinea pigs: The role of modified epithelial functions. Am J Respir Crit Care Med 149:1494–1498.

Matsubara S, Fushimi K, Kaminuma O, Kikkawa H, Shimazu N, Iwasaki H, Ikezawa K. 1995. Importance of impairment of the airway epithelium for ozone-induced airway hyperresponsiveness in guinea pigs. Jap J Pharmacol 67:375–382.

McDonnell WF, Abbey DE, Nishino N, Lebowitz MD. 1999. Long-term ambient ozone concentration and the incidence of asthma in nonsmoking adults: The Ahsmog study. Environ Res 80(Sect A):110–121.

McManus MS, Gong H, Clark JJ. 1990. Tolerance to repeated daily ozone exposures in asthmatic subjects. Am Rev Respir Dis 141:A70–A75.

Molfino NA, Wright SC, Katz I, Tarlo S, Silverman F, McClean PA, Szalai JP, Raizenne M, Slutsky AS, Zamel N. 1991. Effect of low concentrations of ozone on inhaled allergen responses in asthmatic subjects. Lancet 338:199–203.

Morgan WKC, Reger RB. 1991. Chronic airflow limitation and occupation. In: Chronic Obstructive Pulmonary Disease (Cherniack NS, ed), WB Saunders Co, Philadelphia PA.

Natrella MG. 1963. Experimental Statistics. National Bureau of Standards Handbook 91. US Government Printing Office, Washington DC.

Nijkamp FP, van der Linde J, Folketts G. 1993. Nitric oxide synthesis inhibitors induce airway hyperresponsiveness in the guinea pig in vivo and in vitro: Role of the epithelium. Am Rev Respir Dis 148:727–734.

Osebold JW, Gershwin LJ, Zee YC. 1980. Studies on the enhancement of allergic lung sensitization by inhalation of ozone and sulfuric acid aerosol. J Environ Pathol Toxicol Oncol 3:221–234.

Palmer LJ, Burton PR, Faux JA, James AL, Musk AW, Coodson WOCM. 2000. Independent inheritance of serum immunoglobulin E concentrations and airway responsiveness. Am J Crit Care Med 161:1836–1843.

Paoletti P, Carrozi L, Viegi G, Modena P, Ballerin L, Pede F, Grado L, Baldacci S, Pedreschi M, Vellutini M, Paggiaro P, Mammini U, Fabbri L, Giuntini C. 1995. Distribution of bronchial responsiveness in a general population: Effect of sex, age, smoking and level of pulmonary function. Am J Respir Crit Care Med 151:1770–1777.

Peat JK, Salome CM, Xuan W. 1996. On adjusting measurements of airway responsiveness for lung size and airway caliber. Am J Respir Crit Care Med 154: 870–875.

Peden D. 1999. Controlled exposures of asthmatics to air pollutants. In: Air Pollution and Health (Holgate ST, Samet JM, Koren HS, Maynard RL, eds), pp 865–880. Academic Press, Orlando FL.

Peden DB. 2000. Development of atopy and asthma: Candidate environmental influences and important periods of exposure. Environ Health Perspect (Suppl) 108(3):475–482.

Persson MG, Gustafsson LE. 1993. Allergen-induced airway obstruction in guinea-pigs is associated with changes in nitric oxide levels in exhaled air. Acta Physiol Scand 149:461–466.

Pinkerton KE, Joad JP. 2000. The mammalian respiratory system and critical windows of exposure for children's health. Environ Health Perspect (Suppl) 108(3):457–462.

Platts-Mills TAE, Carter MC, Heymann PW. 2000. Specific and nonspecific obstructive lung disease in children: Causes of changes in the prevalence of asthma. Environ Health Perspect (Suppl) 108(4):725–731.

Pretolani M, Ruffié C, Joseph D, Campos MG, Church MK, Lefort J, Vargaftig BB. 1994. Role of eosinophil activation in the bronchial reactivity of allergic guinea pigs. Am J Respir Crit Care Med 149:L1167–L1174.

Rijcken B, Schouten JP, Mensinga TT, Weiss ST, De Vries K, van der Lende R. 1993. Factors associated with bronchial responsiveness to histamine in a population sample of adults. Am Rev Respir Dis 147:1447–1453.

Rombout PJA, Lioy PJ, Goldstein BD. 1986. Rationale for an eight-hour ozone standard. J Air Pollut Control Assoc 36:913–917.

Sanjar S, Aoki S, Boubekeur K, Chapman ID, Smith D, Kings MA, Morley J. 1990a. Eosinophil accumulation in pulmonary airways of guinea-pigs induced by exposure to

an aerosol of platelet activating factor: Effect of anti-asthma drugs. Br J Pharmacol 99:267–272.

Sanjar S, Aoki S, Kristersson A, Smith D, Morley J. 1990b. Antigen challenge induces pulmonary airway eosinophil accumulation in sensitized guinea pigs: The effects of anti-asthma drugs. Br J Pharmacol 99:679–686.

Santing RE, Hoekstra Y, Pasman Y, Zaagsma J, Meurs H. 1994. The importance of eosinophil activation for the development of allergen-induced bronchial hyperreactivity in conscious, unrestrained guinea-pigs. Clin Exp Allergy 24:1157–1163.

Schlesinger RB, Gorczynski JE, Dennison J, Richards L, Kinney PL, Bosland MC. 1992. Long-term intermittent exposures to sulfuric acid aerosol, ozone, and their combination: Alterations in tracheobronchial mucociliary clearance and epithelial secretory cells. Exp Lung Res 18:505–534.

Schuiling M, Meurs H, Zuidhof AB, Venema N, Zaagsma J. 1998. Dual action of iNOS-derived nitric oxide in allergen-induced airway hyperreactivity in conscious, unrestrained guinea pigs. Am J Respir Crit Care Med 158:1442–1449.

Sears MR. 1997. Descriptive epidemiology of asthma. Lancet 350(Suppl 11):1–4.

Seltzer J, Bigby BG, Stulbarg M, Holtzman MJ, Nadel JA, Ueki IF, Leikauf GD, Goetzl EJ, Boushey HA. 1986. O_3-induced change in bronchial reactivity to methacholine and airway inflammation in humans. J Appl Physiol 60:1321–1326.

Smith L, McFadden ER Jr. 1995. Bronchial hyperreactivity revisited. Allergy Asthma Immunol 74:454–469.

Snapper JR, Drazen JM, Loring SH, Schneider W, Ingram RH Jr. 1978. Distribution of pulmonary responsiveness to aerosol histamine in dogs. J Appl Physiol 44:738–742.

Sun J, Chung F. 1997. Airway inflammation despite loss of bronchial hyperresponsiveness after multiple ozone exposures. Respir Med 91:47–55.

Sun J, Koto H, Chung KF. 1997. Interaction of ozone and allergen challenges, bronchial responsiveness and inflammation in sensitized guinea pigs. Int Arch Allergy Immunol 112:191–195.

Sunyer J, Anto JM, Sabria J, Roca J, Morell F, Rodriguez-Roisin R, Rodrigo MJ. 1995. Relationship between serum IgE and airway responsiveness in adults with asthma. J Allergy Clin Immunol 95:699–706.

Thompson JE, Gordon T, Scypinski LA, Sheppard D. 1987. Tachykinins mediate toluene diisocyanate-induced airway hyperresponsiveness. Am Rev Respir Dis 136:1118–1121.

Thorne JR, Broadley KJ. 1994. Adenosine-induced bronchoconstriction in conscious hyperresponsive and sensitized guinea pigs. Am J Respir Crit Care Med 149:392–399.

Thorne PS, Karol MH. 1989. Association of fever with late-onset pulmonary hypersensitivity in guinea pig. Toxicol Appl Pharmacol 100:247–258.

Thurston GD, Ito K. 1999. Epidemiological studies of ozone exposure effects. In: Air Pollution and Health (Holgate ST, Samet JM, Koren HS, Maynard RL, eds), pp 485–510. Academic Press, Orlando FL.

Turner DJ, Martin JG. 1997. Animal models. In: Asthma (Barnes PJ, Grunstein MM, Leff AR, Woolcock AJ, eds), pp 261–274. Lippincott-Raven, Philadelphia PA.

Underwood DC, Osborn RR, Hand JM. 1992. Lack of late-phase airway responses in conscious guinea pigs after a variety of antigen challenges. Agents Actions 37:191–194.

van den Toorn LM, Prins JB, Overbeek SE, Hoogsteden HC, de Jongste JC. 2000. Adolescents in clinical remission of atopic asthma have elevated exhaled nitric oxide levels and bronchial hyperresponsivenes. Am J Respir Crit Care Med 162:953–957.

Vargas MH, Romero L, Sommer B, Zamudio P, Gustin P, Montano LM. 1998. Chronic exposure to ozone causes tolerance to airway hyperresponsiveness in guinea pigs: Lack of SOD role. J Appl Physiol 84:1749–1755.

von Mutius E. 1997. Towards prevention. Lancet 350 (Suppl 11):14–17.

Wanner A, Abraham WM, Douglas JS, Drazen JM, Richerson HB, Ram JS. 1990. Models of airway hyperresponsiveness. Am Rev Respir Dis 142:253–257.

Wardlaw AJ, Dunnette S, Gleich GH, Collins JV, Kay AB. 1988. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Am Rev Respir Dis 137:62–69.

Weinmann GG, Weidenbach-Gerbase M, Foster WM, Zacur H, Frank R. 1995. Evidence for ozone-induced small-airway dysfunction: Lack of menstrual-cycle and gender effects. Am J Respir Crit Care Med 152:988–996.

Weiss ST, Tager IB, Weiss JW, Speizer FE, Ingram RH Jr. 1981. The distribution of airways responsiveness in a population sample of adults and children. Am Rev Respir Dis 123(Suppl):130–136.

Wolfe R, Carlin JB, Oswald H, Olinsky A, Phelan PD, Robertson CF. 2000. Association between allergy and asthma from childhood to middle adulthood in an Australian cohort study. Am J Respir Crit Care Med 162:2177–2181.

Yanai M, Ohrui T, Aikawa T, Okayama H, Sekizawa K, Maeyama K, Sasaki H, Takishima T. 1990. Ozone increases susceptibility to antigen inhalation in allergic dogs. J Appl Physiol 68:2267–2273.

Yeadon M, Wilkinson D, Darley-Usmar V, O'Leary VJ, Payne AN. 1992. Mechanisms contribution to ozone-induced bronchial hyperreactivity in guinea-pigs. Pulm Pharmacol 5:39–50.

Ying RL, Gross KB, Terzo TS, Eschenbacher WL. 1990. Indomethacin does not inhibit the ozone-induced increase in bronchial responsiveness in human subjects. Am Rev Respir Dis 142:817–821.

Zwick H, Popp W, Wagner C, Reiser K, Schmoger J, Bock A, Herkner K, Hadunsky K. 1991. Effects of ozone on the respiratory health, allergic sensitization, and cellular immune system in children. Am Rev Respir Dis 144:1075–1079.

OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

Schlesinger RB, Cohen M, Gordon T, Nadziejko C, Zelikoff JT, Sisco M, Regal JF, Ménache MG. Ozone alters airway responsiveness in atopic but not nonatopic guinea pigs. Inhalation Toxicol. In press.

ABBREVIATIONS AND OTHER TERMS

ACH	acetylcholine
ANOVA	analysis of variance
CS	concurrently sensitized (protocol or animal associated with protocol)
ELISA	enzyme linked immunosorbent assay
EPA	Environmental Protection Agency (US)
HEPA	high-efficiency particulate air (filter)
IgE	immunoglobulin E
IgG	immunoglobulin G
IU	international unit
LDH	lactate dehydrogenase
NAAQS	National Ambient Air Quality Standard (US)
NO	nitric oxide
NS	nonsensitized (protocol or animal associated with protocol)
O_3	ozone
PBS	phosphate-buffered saline
PC50	provocation concentration resulting in a 50% decline in sGaw
PS	presensitized (protocol or animal associated with protocol)
RFA	Request for Application
RFPAs	Requests for Preliminary Applications
sGaw	specific airway conductance

COMMENTARY
Health Review Committee

INTRODUCTION

Since its inception, the Health Effects Institute has supported studies on oxidants, primarily nitrogen dioxide and ozone. A variety of experimental approaches have been used, including in vitro, animal, controlled human exposure, and epidemiologic studies. One goal of HEI's ozone research program has been to provide scientific information to aid the US Environmental Protection Agency (EPA*) deliberations of whether the US National Ambient Air Quality Standard (NAAQS) for ozone adequately protects human health. After its most recent review of the scientific basis for the ozone NAAQS (EPA 1996), the EPA lowered its standard from 0.12 ppm ozone (a level not to be exceeded for more than 1 hour, once per year) to 0.08 ppm ozone. The lower level was based on an eight-hour average to protect against longer exposure periods (EPA 1997). The EPA's decision was based on evidence that ozone causes reversible adverse effects on the respiratory system after short-term (6.6-hour) exposure to 0.08 ppm ozone or higher together with moderate exercise (EPA 1996). In 1999, after industry and state litigants challenged the new ozone (and particulate matter) standard, the US Court of Appeals for the District of Columbia Circuit rejected EPA's standards (EPA 2000), sending the regulations back to the agency. The EPA reinstated the previous ozone standard but appealed the decision to the United States Supreme Court (EPA 2000). In November 2000, the Supreme Court listened to arguments from each side. In February 2001 the Court upheld EPA's authority under the Clean Air Act to set NAAQSs that protect the American public from the harmful effects of air pollution. In March 2002, the US Court of Appeals for the District of Columbia ruled that the EPA acted within its legal jurisdiction in promulgating stricter regulations for ozone and particulate matter (EPA 2002).

HEI's Requests for Applications (RFAs) have targeted research on air toxics, diesel exhaust, particulate matter, and oxygenates in fuel. In addition to RFAs, HEI issues Requests for Preliminary Applications (RFPAs), which encourage investigators with interests outside the current RFAs but compatible with HEI's mission, to submit short preliminary applications describing a proposed study. In response to RFPA 93-2, *Health Effects of Exposure to Motor Vehicle Emissions*, Dr Richard Schlesinger of New York University's Institute of Environmental Medicine submitted a preliminary application entitled *Ozone-Induced Airway Hyperresponsiveness*. The HEI Health Research Committee requested a full application because the members believed that Schlesinger's proposal to study the relation between ozone exposure and allergen inhalation was an important issue that would complement human studies. External reviewers of the full application noted Schlesinger's expertise as an inhalation toxicologist and the quality of the experimental design. The Research Committee concurred with the positive evaluation of the external reviewers and recommended that the study be funded.[†]

The following Commentary is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study and by placing the Investigators' Report into scientific and regulatory perspective.

SCIENTIFIC BACKGROUND

Asthma is one of the most common chronic and potentially disabling diseases to occur among children and adults. Asthma is characterized by three findings involving the airways: reversible obstruction, inflammation, and airway hyperresponsiveness, a heightened tendency of the bronchial airways to constrict. During bronchoconstriction, airway resistance (defined as the driving pressure of air through the airways divided by the airflow rate) increases and its reciprocal function, airway conductance, decreases. Because airway resistance and airway conductance vary with lung volume, both measures are divided by lung volume and expressed as specific airway resistance (sRaw) and specific airway conductance (sGaw). Both sRaw and sGaw change based on airway smooth muscle tone. For example, resistance decreases when smooth muscle relaxes and increases when smooth muscle contracts. Airway hyperresponsiveness is considered specific when it involves immune pathways evoked by an antigen (such as an allergen) binding to an immunoglobulin and nonspecific when it is mediated by mechanisms that do not involve the immune system (such as in response to a chemical irritant). Several studies have confirmed that allergen inhalation

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

[†] Dr Schlesinger's 3-year study, Ozone-Induced Airway Hyperresponsiveness, began in December 1994. Total expenditures were $360,536. The draft Investigators' Report from Schlesinger and colleagues was received for review in May 1999. A revised report, received in May 2001, was accepted for publication in June 2001. During the review process, the HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Report and in the Review Committee's Commentary.

causes specific airway hyperresponsiveness in humans (Nelson 2000; O'Byrne and Inman 2000). Controlled clinical studies have shown that exercising humans exposed to ozone at concentrations of 0.4 ppm (Ying et al 1990; Devlin et al 1996), 0.22 or 0.20 ppm (Balmes et al 1996, 1997; Frampton et al 1997a,b), 0.12 ppm (Linn et al 1994; Gong et al 1986; Folinsbee et al 1988), or 0.08 ppm (Horstman et al 1990) develop nonspecific airway hyperresponsiveness. Therefore, people with asthma may be more susceptible to allergen-induced airway hyperresponsiveness when ozone levels are elevated.

OZONE AND ALLERGEN RESPONSE

Ozone may exacerbate airway dysfunction in people with asthma by sensitizing the airway mucosa, by enhancing cellular responses to allergen, or by exerting a direct effect on airway inflammation (Peden et al 1995). Some clinical studies suggest that subjects with asthma can experience increased airway hyperresponsiveness to inhaled allergens (such as ragweed, animal dander, or dust mite allergen) after exposure to 0.12 ppm or 0.25 ppm ozone for 1 to 3 hours (Molfino et al 1991; Jörres et al 1996). In contrast, other subjects with mild asthma exposed to 0.12 ppm ozone for 1 hour at rest did not show increased responsiveness to ragweed or grass allergen (Hanania et al 1998; Ball et al 1996). Jenkins and colleagues (1999) reported that ozone-induced changes in airway response to allergen might depend on threshold concentration rather than the total amount of ozone inhaled over time. They found that the airway response of subjects with asthma to inhaled *Dermatophagoides pteronyssinus* (house dust mite) allergen was unchanged after exposure to 0.10 ppm ozone for 6 hours. However, airway response increased when the subjects were exposed for only 3 hours to 0.20 ppm ozone.

OZONE IN NONSENSITIZED ANIMALS

A number of studies have assessed the effect of long- and short-term exposure to ozone in nonsensitized animals. For the most part, these animal studies involved higher levels of exposure than human controlled exposure studies and the animal study conducted by Schlesinger and colleagues. Exposure to high (1 to 3 ppm) levels of ozone for 30 minutes to 2 hours produced transient airway hyperresponsiveness in dogs (Holtzman et al 1983; Fabbri et al 1984, 1985; O'Byrne et al 1984a,b; Aizawa et al 1985; Stevens et al 1995) and rats (Koto et al 1997). Dogs exposed to 0.5 ppm ozone for 2 hours also developed airway hyperresponsiveness (Fouke et al 1991), and guinea pigs developed airway hyperresponsiveness after exposure to 1 to 3 ppm ozone for 15 minutes to 3 hours (Gordon et al 1984; Murlas and Roum 1985; Yeadon et al 1992; Sun and Chung 1997). Guinea pigs exposed to 0.3 ppm ozone for 4 hours per day developed airway hyperresponsiveness after 1, 3, 6, 12, or 24 days of exposure. After 48 days of exposure, airway hyperresponsiveness decreased to control levels, suggesting that long-term exposure to ozone led to tolerance to ozone-induced airway hyperresponsiveness (Vargas et al 1998). Two studies in monkeys produced conflicting results. Johnson and coworkers (1988) exposed rhesus monkeys to 1 ppm ozone for 2 hours once per week. Airway hyperresponsiveness developed after 19 weeks of exposure and persisted for 15 weeks after cessation of exposure. Biagini and colleagues (1986) reported that cynomolgus monkeys exposed to 1 ppm ozone for 6 hours per day, 5 days per week, for 12 weeks did not develop airway hyperresponsiveness. Differences in ozone susceptibility between the two strains of monkeys may have been responsible for the conflicting results.

In addition to inducing airway hyperresponsiveness, ozone exposure causes transient lung inflammation, but whether ozone-induced inflammation is responsible for airway hyperresponsiveness is unclear. Several studies implicate ozone-induced airway inflammation in airway hyperresponsiveness (Holtzman et al 1983; Fabbri et al 1984, 1985; O'Byrne et al 1984b; Stevens et al 1995; Vargas et al 1998). In contrast, the results of Murlas and Roum (1985), Sun and Chung (1997), and Koto et al (1997) failed to identify an association between inflammation and airway hyperresponsiveness in guinea pigs or rats exposed to ozone. Crimi and coworkers (1998) concluded that the relation between chronic airway hyperresponsiveness and inflammation in asthma is unclear because long-term treatment with anti-inflammatory agents does not consistently reduce airway hyperresponsiveness. In a related report, Brusasco and colleagues (1998) examined 26 studies conducted between 1987 and 1998 on the relation between airway inflammation and airway hyperresponsiveness in humans; they found an almost equal number showing significant or nonsignificant correlations. They concluded that no clear correlation was evident but that the two may be loosely related.

OZONE IN ANTIGEN-SENSITIZED (ATOPIC) ANIMALS

Atopy refers to allergic-type responses that develop in antigen-sensitized humans and other species. Yanai and coworkers (1990) reported that exposure to 3 ppm ozone for 1 hour increased airway hyperresponsiveness in response to *Ascaris suum* (pig intestinal roundworm) antigen in dogs presensitized with the same antigen. Sun and colleagues (1997) reported that exposure to 1 ppm ozone for 1 hour augmented ovalbumin-induced airway

hyperresponsiveness in ovalbumin-sensitized guinea pigs. In contrast, Vargas and coworkers (1994) reported that antigen challenge with ovalbumin induced airway hyperresponsiveness in ovalbumin-sensitized guinea pigs, but ozone exposure (3 ppm for 1 hour) did not increase antigen-induced airway hyperresponsiveness.

In summary, there is evidence for the induction of specific and nonspecific airway hyperresponsiveness after short-term exposure to ozone; however, we have less information on the effects of long-term ozone exposure on airway hyperresponsiveness. In addition, because people can be exposed to both allergens and ozone-polluted air for extended periods, understanding the effects of combined exposures on airway hyperresponsiveness is critical. The study of Schlesinger and colleagues provides information on both issues using a well-established animal model of airway hyperresponsiveness and allergic asthma.

TECHNICAL EVALUATION

SPECIFIC AIMS

Schlesinger and coworkers addressed four questions regarding the relation between ozone exposure and airway hyperresponsiveness.

1. Does long-term, repeated exposure to low concentrations of ozone (0.1 and 0.3 ppm) induce nonspecific airway hyperresponsiveness in nonsensitized animals?
2. Does such ozone exposure exacerbate specific and nonspecific airway hyperresponsiveness in sensitized (atopic) animals?
3. Does gender play a role in modulating airway hyperresponsiveness in relation to ozone exposure?
4. Is there a relation between airway hyperresponsiveness and other possible modulators of airway response to ozone?

STUDY DESIGN AND METHODS

Animal Model and Exposure Protocols

Guinea pigs have long been used as a model for studies on the immune and inflammatory mechanisms of asthma and on the effect of pollutant exposures on asthma. These animals exhibit allergic responses that have some homology to human atopic responses. Guinea pigs also possess well-developed lung smooth muscle, which contracts extensively in response to antigen exposure and is a prerequisite for bronchoconstriction (Ratner et al 1927 reviewed by Pretolani and Vargaftig 1993; Karol 1994; Bice et al 2000). Schlesinger and coworkers used three exposure protocols to explore the effect of extended exposure to ozone alone or to ozone plus antigen (ovalbumin) on airway hyperresponsiveness, on cellular and chemical measures of inflammation in lung fluids, lung tissue and blood, and on antibody levels.

The nonsensitized (NS) protocol addressed the effect of ozone exposure on nonspecific airway hyperresponsiveness in nonsensitized animals. The presensitized (PS) protocol examined the effect of ozone on nonspecific and specific airway hyperresponsiveness in animals presensitized by inhaling ovalbumin for 0.5 hours per day for 4 days. Ozone or clean air exposures began approximately 28 days after the ovalbumin sensitization period. The concurrently sensitized (CS) protocol examined the effect of ozone on airway hyperresponsiveness in animals inhaling ovalbumin (0.5 hours per day for 4 days) at the same time ozone exposures began. The guinea pigs were 3 to 4 weeks old when sensitization to ovalbumin began. Because the CS animals' exposure to ozone began at the same time as the sensitization procedure, the NS and CS animals were 3 to 4 weeks old when ozone exposures began. However, the PS animals were 7 to 8 weeks old when ozone exposures began because of the 28-day presensitization period. Age modulates the development of atopy after antigen exposure (Peden 2000). Therefore, the investigators stressed the importance of beginning the sensitization procedure for the PS and CS groups at the same age.

Schlesinger and coworkers exposed male and female guinea pigs in each protocol to 0.1 or 0.3 ppm ozone for 4 hours per day, 4 days per week, for 24 weeks. Control animals breathed clean air. Each exposure group in each protocol contained 10 animals per gender.

Measurement of Airway Hyperresponsiveness

The investigators measured sGaw by a noninvasive method called constant volume plethysmography (described in detail in the Investigators' Report) that allowed them to observe the breathing pattern of animals and detect airway constriction, the measure of airway hyperresponsiveness. Initially they measured baseline sGaw by administering an aerosol of phosphate-buffered saline. The investigators determined changes in sGaw as a measure of airway hyperresponsiveness by bronchial provocation challenge with a bronchoconstrictor agent. Hyperresponsive airways require significantly less bronchoconstrictor to decrease sGaw than do nonresponsive airways. They determined nonspecific airway hyperresponsiveness by administering aerosols of increasing concentrations of acetylcholine (ACH); specific airway hyperresponsiveness was determined by administering aerosols of increasing concentrations of ovalbumin.

At approximately 4-week intervals during the 24-week exposure period, the investigators measured airway hyperresponsiveness on a day without ozone exposure to minimize any ozone effect on lung epithelial permeability that might affect the challenge response. The concentration of ACH or ovalbumin that reduced sGaw to 50% of the baseline level was designated as the provocative challenge 50 (PC50). The investigators administered ovalbumin 3 days after ACH challenge in the PS and CS protocols to avoid a pulmonary reaction to ovalbumin during the ACH challenge. One week after the exposure period ended, half the animals were killed for measurement of cellular, biochemical, and immunologic endpoints. The remaining animals breathed clean air for an additional 8 weeks (postexposure period). The investigators measured nonspecific and specific airway hyperresponsiveness at 4 and 8 weeks after exposure ended to determine whether responses to ozone attenuated. One week after the postexposure period ended, the animals were killed for biochemical, cellular, and immunologic measurements. The 1-week lag between the end of the exposure or postexposure period and animal killing was necessary to allow time to measure airway hyperresponsiveness.

Biochemical, Cellular, and Immunologic Analyses

Schlesinger and coworkers measured nitric oxide (NO) levels in exhaled air during the ozone exposures because NO is a potent bronchodilator and guinea pigs exposed to inhibitors of NO production by inhalation develop airway hyperresponsiveness (Nijkamp et al 1993). In addition, alveolar macrophages from rats exposed to high levels of ozone (1 to 2 ppm for 3 hours) produce more NO than cells from controls exposed to clean air (Pendino et al 1993).

After the animals were killed, Schlesinger and colleagues obtained peripheral blood and counted lymphocytes, neutrophils, monocytes, eosinophils, and basophils in all animal groups and measured the levels of immunuglobulin G (IgG) 1 and IgG2 (as indicators of ovalbumin-induced atopy) in PS and CS animals. IgG is the major class of allergic antibodies in guinea pigs and the primary indicator of atopy. They performed bronchoalveolar lavage and measured (1) the total number of cells, (2) the number of macrophages, eosinophils, neutrophils and lymphocytes, (3) cell viability, (4) lactate dehydrogenase (LDH) activity (an indicator of cell injury), and (5) total soluble protein (an indicator of increased cell permeability). The investigators also measured the number of mast cells and eosinophils in lung tissue by histopathology.

Statistical Analyses

The dependent variables in this study were the PC50 and sGaw determinations made over the course of the exposure and postexposure periods (the time vector). The independent variables were ozone concentration (0.1 or 0.3 ppm), gender, and the interaction between ozone concentration and gender. Statistically significant interactions ($P < 0.05$) were determined by the Hotelling-Lawley trace. If the investigators detected statistically significant interactions between the time vector of dependent variables and the independent variables, they performed univariate analyses of variance (ANOVA) for each time point. The factors in the ANOVA were also ozone concentration, gender, and the interaction between gender and ozone concentration. For any ANOVA, statistical significance ($P < 0.05$) was evaluated with the F test. If the F test indicated statistical significance, the investigators conducted subtesting for exposure or gender by exposure interactions.

The investigators also used ANOVA to analyze the results of the lavage, blood cell, immunoglobulin (IgG1, IgG2), and airway cell assays. The factors were gender, ozone concentration, and time of death, either immediately after the last exposure or after the postexposure period.

RESULTS AND INTERPRETATIONS

Table 6 in the Investigators' Report presents the key statistical analyses of PC50 values for animals in each protocol for the exposure and postexposure periods. The results of this section of the study are summarized in Commentary Table 1.

Nonsensitized Animals

Ozone did not induce airway hyperresponsiveness in NS guinea pigs during the exposure or postexposure periods. The investigators do report that PC50 values were significantly lower after ACH challenge for male guinea pigs than for females, indicating that the airways of males were more responsive.

Presensitized Animals

In PS animals challenged with ACH, ozone exposure caused statistically significant increases in airway hyperresponsiveness at most time points during the exposure period. Schlesinger and coworkers report that the effect on airway hyperresponsiveness was often comparable for both ozone concentrations; however, they suggest that there was a general, overall pattern of decreasing PC50 at the higher ozone concentration. The increase in airway hyperresponsiveness remained statistically significant

Commentary Table 1. Airway Hyperresponsiveness in Presensitized and Concurrently Sensitized Guinea Pigs Exposed to Ozone[a]

Nonspecific Challenge with Acetylcholine	Specific Challenge with Ovalbumin
• Significantly increased airway hyperresponsiveness began during ozone exposure period and persisted 4 weeks after exposure • Hyperresponsiveness to 0.3 ppm ozone was somewhat stronger than to 0.1 ppm ozone • No hyperresponsiveness was evident at 8 weeks after ozone exposure at either dose level	• Significantly increased airway hyperresponsiveness to 0.1 ppm ozone did not appear until Week 24 but persisted 4 weeks after exposure • Significantly increased airway hyperresponsiveness to 0.3 ppm ozone began early in exposure period and persisted 4 weeks after exposure • No hyperresponsiveness was evident at 8 weeks after ozone exposure at either dose level

[a] The nonsensitized animals showed no changes as measured by the nonspecific challenge with ACH.

after 4 weeks of the postexposure period but was no longer evident after 8 weeks.

In PS animals challenged with ovalbumin, airway hyperresponsiveness did not show a statistically significant increase in animals exposed to 0.1 ppm ozone compared with airway hyperresponsiveness in controls until Week 24, the last week of exposure. In contrast, the increase in airway hyperresponsiveness between control animals and animals exposed to 0.3 ppm ozone was statistically significant throughout most of the exposure period. The increased hyperresponsiveness for animals exposed to either concentration of ozone remained elevated 4 weeks into the postexposure period.

PS animals showed no consistent statistically significant gender effect with respect to ozone-induced increased nonspecific or specific airway hyperresponsiveness. However, as with NS animals, male PS guinea pigs had lower PC50 values after both ACH and ovalbumin challenges than did females, but the differences were unrelated to ozone exposure level.

Concurrently Sensitized Animals

In CS animals challenged with ovalbumin or ACH, the pattern of increased airway hyperresponsiveness in response to ozone exposures over time was similar to that seen in the PS animals. As with the PS animals, the CS guinea pigs were more responsive to 0.3 ppm ozone than to 0.1 ppm ozone. There were no gender-related differences in response to ozone and, in contrast to the NS and PS animals, male CS guinea pigs did not show increased responsiveness to ACH or ovalbumin challenges.

Differential Sensitivity to Nonspecific or Specific Bronchoconstrictor Challenge

The investigators assessed the degree to which ozone exposure differentially affected the PS and CS guinea pigs' responses to bronchoconstrictor challenge by determining the ACH-PC50/ovalbumin-PC50 ratio. A ratio of one would indicate a similar response to both challenges, a ratio greater than one would indicate that the response to the specific ovalbumin challenge was more than that to the nonspecific ACH challenge, and a ratio less than one would indicate the reverse. Although Schlesinger and coworkers found a somewhat greater response to ovalbumin than to ACH in the PS animals, the differences between the ratios for ozone-exposed and control animals were statistically significant at only a few time points and ozone concentrations. Because there were no consistent trends over time, ozone exposure group, or gender, the investigators concluded that ozone exposure did not differentially affect nonspecific or specific airway hyperresponsiveness in this cohort. A generally similar pattern was observed with the CS animals with respect to time and ozone exposure group. However, in contrast to the PS group, there was a statistically significant increase in the male response to ovalbumin challenge, but this was not related to the concentration of ozone.

Biochemical and Cellular Analyses

The results of these analyses are summarized in Commentary Table 2. There was no consistent pattern of ozone-induced changes in exhaled NO, and NO levels were comparable in males and females in all groups. There were no ozone-induced changes among groups in the total number of cells in bronchoalveolar lavage fluid, in the numbers of macrophages, eosinophils, neutrophils, or lymphocytes, in the levels of LDH activity or total protein, or in cell viability. There were no statistically significant relations between gender and ozone exposure. Ozone exposure had no effect on the levels of eosinophils or mast cells in lung tissue compared with levels in controls. Thus, the results of lavage and histopathologic analyses indicated that airway inflammation was not a factor in the airway hyperresponsiveness observed in this study.

There were no statistically significant ozone effects on the numbers of blood cells (namely, lymphocytes, neutrophils, monocytes, eosinophils, or basophils) in the NS or PS animals. Ozone exposure reduced the number of lymphocytes and increased the number of neutrophils in the

Commentary Table 2. Biochemical and Cellular Analyses of Guinea Pigs Exposed to Ozone

Nitric Oxide in Breath	Bronchoalveolar Lavage	Lung Tissue	Blood Cells
Nonsensitized Animals			
No change from controls	No changes from controls in total numbers of cells, in macrophages, eosinophils, neutrophils, lymphocytes, LDH activity, total protein, or cell viability	No changes from controls in the numbers of eosinophils or mast cells	No changes from controls in the numbers of lymphocytes, neutrophils, monocytes, eosinophils, or basophils
Presensitized Animals			
No changes from controls			
Concurrently Sensitized Animals			
No changes from controls			Lymphocytes decreased and neutrophils increased at the end (Week 24) of ozone exposure compared with controls

blood of CS animals, but only at the end of the 24-week exposure period.

The investigators noted several gender differences in the levels of blood cells, but they were unrelated to ozone exposure. For example, the numbers of eosinophils were higher in NS and PS females, the numbers of lymphocytes were higher in PS and CS females, and the numbers of neutrophils were higher in PS and CS males.

Immunologic Analyses

Commentary Table 3 presents the key results of these analyses. Ozone exposure did not affect blood IgG1 levels in PS or CS animals killed immediately after the end of the exposure period compared with levels in controls. The IgG1 level in ozone-exposed PS and CS animals killed at the end of the postexposure period was significantly higher than levels in the cohorts killed at the end of the exposure period. The level of IgG1 was higher in CS females killed at the end of the postexposure period compared with males killed at the same time, and this difference was statistically significant. The level of IgG2 was also significantly higher in PS animals exposed to 0.1 ppm ozone and killed at the end of the postexposure period than in the cohort killed at the end of the exposure period. CS animals showed a somewhat similar effect, but the difference was not statistically significant. The level of IgG2 in animals exposed to 0.3 ppm ozone fell between the levels seen in the control and 0.1 ppm ozone cohorts, but these differences were not statistically significant. Schlesinger and coworkers performed correlation analyses

Commentary Table 3. Immunologic Analyses on Sensitized Guinea Pigs Exposed to Ozone

IgG1	IgG2
Presensitized Animals	
No change from controls at end of exposure (Week 24)	Higher in animals exposed to 0.1 ppm ozone at 8 weeks after exposures (Week 32) compared with same exposure cohort at end of exposure (Week 24)
Elevated level at 8 weeks after exposure compared with same exposure cohort at end of exposure (Week 32)	Exposure to 0.3 ppm ozone produced a level between controls and animals exposed to 0.1 ppm (Week 24), but differences were not statistically significant
Concurrently Sensitized Animals	
Similar to presensitized animals, but significantly increased in females compared with males at 8 weeks after exposure (Week 32)	Similar trend as in presensitized animals exposed to 0.1 ppm ozone
	Similar results as in presensitized animals exposed to 0.3 ppm ozone

to determine whether blood IgG levels reflected the degree of airway hyperresponsiveness. The statistically significant negative correlations between PC50 and ACH, PC50 and ovalbumin, IgG1, and IgG2 indicated that an increased blood level of IgG was associated with decreased PC50, the measure of airway hyperresponsiveness.

DISCUSSION

This important study sought to answer a key question concerning the effect of ozone on the lungs: does long-term, intermittent exposure to ozone exacerbate airway hyperresponsiveness? The study was well designed to answer this question. The investigators made repeated measurements on three cohorts of guinea pigs exposed to two levels of ozone (0.1 and 0.3 ppm) for approximately six months, and they also followed changes that developed during a recovery time in clean air. The first cohort comprised nonsensitized animals exposed to ozone and periodically challenged with a bronchoconstrictor, ACH, that induces nonspecific airway hyperresponsiveness. The second cohort comprised animals whose airways were made hyperresponsive by inhalation of the allergen ovalbumin before ozone exposure began. The third cohort comprised animals whose airways were made hyperresponsive by ovalbumin inhalation concurrently with the beginning of ozone exposure. Specific airway hyperresponsiveness in the second and third cohorts was assessed by periodic ovalbumin challenge, which causes bronchoconstriction; nonspecific airway hyperresponsiveness was assessed by ACH challenge.

Ozone did not induce airway hyperresponsiveness in normal guinea pigs. Ozone exposure did exacerbate nonspecific and specific airway hyperresponsiveness in animals whose airways were made hyperresponsive either before ozone exposure began or concurrently with ozone exposure. These effects generally depended on ozone dose but were independent of gender. The levels of nonspecific and specific airway hyperresponsiveness induced by ozone exposure were quantitatively similar and persisted for 4 weeks after exposure ended.

There was no consistent pattern of ozone-induced effects on NO exhalation, and NO levels were similar in males and females. There were no statistically significant ozone-induced changes in cell viability or in the levels of cellular or molecular markers of inflammation in lavage fluid, nor were interactions observed between ozone exposure and gender. Ozone exposure had no effect on the numbers of cellular markers of inflammation (eosinophils and mast cells) in lung tissue. Thus, the results of this study do not support an association of inflammation with the appearance or exacerbation of nonspecific or specific airway hyperresponsiveness. Ozone exposure had no effect on the numbers of specific blood cells in the NS or PS animals. Ozone exposure reduced lymphocyte numbers and increased neutrophil numbers in the blood of CS guinea pigs only at the end of the 24-week exposure period.

There were a few gender differences in blood cell numbers, but they were unrelated to ozone exposure. For example, the numbers of eosinophils were higher in NS and PS females, the numbers of lymphocytes were higher in PS and CS females, and the numbers of lymphocytes were higher in PS and CS males.

IgG1 and IgG2 were produced in response to ovalbumin. Ozone had no effect on the production of IgG1 in PS or CS animals killed at the end of the exposure period compared with IgG1 in control animals. However, the levels of IgG1 in both cohorts were elevated in animals killed at the end of the postexposure period compared with the levels seen at the end of exposure. IgG1 levels were elevated in females of both cohorts, compared with males, killed at the end of the postexposure period. IgG2 levels were elevated in PS animals exposed to 0.1 ppm ozone and killed at the end of the postexposure period compared with the levels seen in animals killed at the end of the exposure period. The negative correlations between PC50 and ACH, ovalbumin, IgG1, and IgG2 indicated that increasing levels of IgG were associated with decreasing PC50, the measure of increased airway hyperresponsiveness.

The ozone concentrations used in this study (0.1 and 0.3 ppm) were relevant to those encountered by humans. In 1997, 48 million people in 77 counties within the United States were exposed repeatedly to ozone concentrations greater than 0.12 ppm; people in sections of California and the northeastern United States were exposed to ozone concentrations reaching 0.204 ppm. Residents of Houston, Texas, were exposed to ozone levels ranging from 0.205 to 0.404 ppm ozone (EPA 1998). Daily peak ozone concentrations last 6 to 8 hours, and the likely human exposure is approximately 90% of the maximum 1-hour peak exposure (Rombout et al 1986; Lefohn et al 1993); therefore, the experimental exposure of 4 hours per day was reasonable. Multiple-day exposures to ozone are common. The investigators' experimental design of 4 days of exposure per week was based on the average patterns of ozone pollution episodes in many parts of the US (Lippmann 1992). In addition, the exposure duration of 24 weeks was appropriate because ozone levels in most parts of the country are highest from late spring through early fall (EPA 1996).

Further strengths of the study were the well-characterized guinea pig model of airway hyperresponsiveness, analysis of effects between genders, the attempt to correlate physiologic airway hyperresponsiveness data with markers of inflammation in lavage fluid and lung tissue, and the comparison of ozone effects with specific and nonspecific airway challenge. As mentioned by the authors, no animal model completely reproduces the entire allergic airway process found in humans; however, guinea pigs develop a type of airway hyperresponsiveness that is comparable to asthmatic humans (Kallos and Kallos 1984; Hutson et al 1988; Thorne and Karol 1989; Turner and Martin 1997). Unlike humans, however, their allergic response is mediated predominantly by IgG2 antibodies rather than immunoglobulin E (IgE) antibodies. The response of the ovalbumin-sensitized animals to extended periods of ozone exposure in this study is consistent with the results of studies of airway hyperresponsiveness after short-term exposure of guinea pigs to much higher levels of ozone (Yanai et al 1990; Vargas et al 1994). Because the ozone concentrations were relevant to those encountered by humans during periods of elevated ozone air pollution, the results presented by Schlesinger and coworkers suggest that people with hyperresponsive airways may experience an increased response during periods of elevated ozone levels.

SUMMARY

The study by Schlesinger and colleagues sought to answer a key question concerning the effect of ozone on the lungs: does long-term, intermittent exposure to ozone exacerbate airway hyperresponsiveness? Ozone levels of 0.1 ppm and 0.3 ppm did not induce airway hyperresponsiveness in normal guinea pigs. Ozone exposure did exacerbate nonspecific and specific airway hyperresponsiveness in animals whose airways were made hyperresponsive with ovalbumin, either before ozone exposure began or concurrently with ozone exposure. These effects were generally dose dependent but independent of gender. The levels of nonspecific or specific airway hyperresponsiveness induced by ozone exposure were quantitatively similar and persisted for 4 weeks after the end of exposure. The investigators obtained evidence that increased IgG was associated with decreasing PC50, the measure of increased airway hyperresponsiveness. Measures of markers of inflammation in lavage fluid and lung tissue did not correlate with ozone exposure; thus, the results of this study do not support an association of inflammation with the appearance or exacerbation of nonspecific or specific airway hyperresponsiveness in guinea pigs exposed to ozone.

The response of the ovalbumin-sensitized animals to extended periods of ozone exposure in this study is consistent with the results of studies of airway hyperresponsiveness after short-term exposure of guinea pigs to much higher levels of ozone (Yanai et al 1990; Vargas et al 1994). A major strength of this study is that the ozone concentrations were relevant to those encountered by humans during periods of elevated ozone air pollution. The results presented by Schlesinger and coworkers suggest that people with hyperresponsive airways may experience an increased response during periods of elevated ozone levels. This possibility should be evaluated in human studies.

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REFERENCES

Aizawa H, Chung KF, Leikauf GD, Ueki I, Bethel RA, O'Byrne PM, Hirose T, Nadel JA. 1985. Significance of thromboxane generation in ozone-induced airway hyperresponsiveness in dogs. J Appl Physiol 59:1918–1923.

Ball BA, Folinsbee LJ, Peden DB, Kehrl HR. 1996. Allergen bronchoprovocation of patients with mild allergic asthma after ozone exposure. J Allergy Clin Immunol 98:563–572.

Balmes JR, Chen LL, Scannell C, Tager I, Christian D, Hearne PQ, Kelly T, Aris RM. 1996. Ozone-induced decrements in FEV_1 and FVC do not correlate with measures of inflammation. Am J Respir Crit Care Med 153:904–909.

Balmes JR, Aris RM, Chen LL, Scannell C, Tager IB, Finkbeiner W, Christian D, Kelly T, Hearne PQ, Ferrando R, Welch B. 1997. Effects of Ozone on Normal and Potentially Sensitive Human Subjects: Part I. Airway Inflammation and Responsiveness to Ozone in Normal and Asthmatic Subjects. Research Report 78. Health Effects Institute, Cambridge MA.

Biagini RE, Moorman WJ, Lewis TR, Bernstein IL. 1986. Ozone enhancement of platinum asthma in a primate model. Am Rev Respir Dis 134:719–725.

Bice DE, Seagrave JC, Green FHY. 2000. Animal models of asthma: Potential usefulness for studying health effects of inhaled particles. Inhalation Toxicol 12:829–862.

Brusasco V, Crimi E, Pellegrino R. 1998. Airway hyperresponsiveness in asthma: Not just a matter of airway inflammation. Thorax 53:992–998.

Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, Brusasco V. 1998. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. Am J Respir Crit Care Med 157:1–3.

Devlin RB, McDonnell WF, Becker S, Madden MC, McGee MP, Perez R, Hatch G, House DE, Koren HS. 1996. Time-dependent changes of inflammatory mediators in the lungs of humans exposed to 0.4 ppm ozone for 2 hr: A comparison of mediators found in bronchoalveolar lavage fluid 1 and 18 hr after exposure. Toxicol Appl Pharmacol 138:176–185.

Environmental Protection Agency (US). 1996. Air Quality Criteria for Ozone and Related Photochemical Oxidants. EPA/600/P-93/004aF. Office of Research and Development, Washington DC.

Environmental Protection Agency (US). 1997. National Ambient Air Quality Standards for Ozone: Final Rule. 40 CFR Part 50. Fed Regist 62:38855–38896.

Environmental Protection Agency (US). 1998. National Air Quality and Emissions Trends Report, 1997. EPA 454/R-98-016. Office of Air Quality Planning and Standards, Research Triangle Park NC.

Environmental Protection Agency (US). 2000. One-hour smog standard reinstated to protect public health. Headquarters Press Release July 6, 2000. EPA, Washington DC.

Environmental Protection Agency (US). 2002. DC circuit court upholds clean air standards. Headquarters Press Release March 26, 2002. EPA, Washington DC.

Fabbri LM, Aizawa H, Alpert SE, Walters EH, O'Byrne PM, Gold BD, Nadel JA, Holtzman MJ. 1984. Airway hyperresponsiveness and changes in cell counts in bronchoalveolar lavage after ozone exposure in dogs. Am Rev Respir Dis 129:288–291.

Fabbri LM, Aizawa H, O'Byrne PM, Bethel RA, Walters EH, Holtzman MJ, Nadel JA. 1985. An anti-inflammatory drug (BW755C) inhibits airway hyperresponsiveness induced by ozone in dogs. J Allergy Clin Immunol 76:162–166.

Folinsbee LJ, McDonnell WF, Horstman DH. 1988. Pulmonary function and symptom responses after 6.6-hour exposure to 0.12 ppm ozone with moderate exercise. J Air Pollut Control Assoc 38:28–35.

Fouke JM, Wolin AD, McFadden ER Jr. 1991. Effects of ozone on lung mechanics and cyclooxygenase metabolites in dogs. Prostaglandins 42:343–353.

Frampton MW, Morrow PE, Torres A, Cox C, Voter KZ, Utell MJ. 1997a. Ozone responsiveness in smokers and nonsmokers. Am J Respir Crit Care Med 155:116–121.

Frampton MW, Morrow PE, Torres A, Voter KZ, Whitin JC, Cox C, Speers DM, Tsai Y, Utell MJ. 1997b. Effects of Ozone on Normal and Potentially Sensitive Human Subjects: Part II. Airway Inflammation and Responsiveness to Ozone in Nonsmokers and Smokers. Research Report 78. Health Effects Institute, Cambridge MA.

Gong H Jr, Bradley PW, Simmons MS, Tashkin DP. 1986. Impaired exercise performance and pulmonary function in elite cyclists during low-level ozone exposure in a hot environment. Am Rev Respir Dis 134:726–733.

Gordon T, Venugopalan CS, Amdur MO, Drazen JM. 1984. Ozone-induced airway hyperreactivity in the guinea pig. J Appl Physiol 57:1034–1038.

Hanania NA, Tarlo SM, Silverman F, Urch B, Senathirajah N, Zamel N, Corey P. 1998. Effect of exposure to low levels of ozone on the response to inhaled allergen in allergic asthmatic patients. Chest 114:752–756.

Holtzman MJ, Fabbri LM, O'Byrne PM, Gold BD, Aizawa H, Walters EH, Alpert SE, Nadel JA. 1983. Importance of airway inflammation for hyperresponsiveness induced by ozone. Am Rev Respir Dis 127:686–690.

Horstman DH, Folinsbee LJ, Ives PJ, Abdul-Salaam S, McDonnell WF. 1990. Ozone concentration and pulmonary response relationships for 6.6-hour exposures with five hours of moderate exercise to 0.08, 0.10, and 0.12 ppm. Am Rev Respir Dis 142:1158–1163.

Hutson PA, Church MK, Clay TP, Miller P, Holgate SP. 1988. Early and late-phase bronchoconstriction after allergen challenge of nonanesthetized guinea pigs: I. The association of disordered airway physiology to leukocyte infiltration. Am Rev Respir Dis 137:548–557.

Jenkins HS, Devalia JL, Mister RL, Bevan AM, Rusznak C, Davies RJ. 1999. The effect of exposure to ozone and nitrogen dioxide on the airway response of atopic asthmatics to inhaled allergen: Dose- and time-dependent effects. Am J Respir Crit Care Med 160:33–39.

Johnson HG, Stout BK, Ruppel PL. 1988. Inhibition of the 5-lipoxygenase pathway with piriprost (U-60,257) protects normal primates from ozone-induced methacholine hyperresponsive small airways. Prostaglandins 35:459–466.

Jörres R, Nowak D, Magnussen H. 1996. The effect of ozone exposure on allergen responsiveness in subjects with asthma or rhinitis. Am J Respir Crit Care Med 153:56–64.

Kallos P, Kallos L. 1984. Experimental asthma in guinea pigs revisited. Int Arch Allergy Appl Immunol 73:77–85.

Karol MH. 1994. Animal models of occupational asthma. Eur Respir J 7:555–568.

Koto H, Salmon M, Haddad el-B, Huang TJ, Zagorski J, Chung KF. 1997. Role of cytokine-induced neutrophil chemoattractant (CINC) in ozone-induced airway inflammation and hyperresponsiveness. Am J Respir Crit Care Med 156:234–239.

Lefohn AS, Foley JK, Shadwick DS, Tilton BE. 1993. Changes in diurnal patterns related to changes in ozone levels. J Air Waste Manag Assoc 43:1472–1478.

Linn WS, Shamoo DA, Anderson KR, Peng RC, Avol EL, Hackney JD. 1994. Effects of prolonged, repeated exposure to ozone, sulfuric acid, and their combination in healthy and asthmatic volunteers. Am J Respir Crit Care Med 150:431–440.

Lippmann M. 1992. Ozone. In: Environmental Toxicants: Human Exposures and Their Health Effects (Lippmann M, ed). Van Nostrand Reinhold, New York NY, pp 465–519.

Molfino NA, Wright SC, Katz I, Tarlo S, Silverman F, McClean PA, Szalai JP, Raizenne M, Slutsky AS, Zamel N. 1991. Effect of low concentrations of ozone on inhaled allergen responses in asthmatic subjects. Lancet 338:199–203.

Murlas CG, Roum JH. 1985. Sequence of pathologic changes in the airway mucosa of guinea pigs during ozone-induced bronchial hyperreactivity. Am Rev Respir Dis 131:314–320.

Nelson HS. 2000. The importance of allergens in the development of asthma and the persistence of symptoms. J Allergy Clin Immunol 105:S628–S632.

Nijkamp FP, van der Linde HJ, Folkerts G. 1993. Nitric oxide synthesis inhibitors induce airway hyperresponsiveness in the guinea pig in vitro and in vivo: Role of the epithelium. Am Rev Respir Dis 148:727–734.

O'Byrne PM, Inman MD. 2000. New considerations about measuring airway hyperresponsiveness. J Asthma 37:293–302.

O'Byrne PM, Walters EH, Aizawa H, Fabbri LM, Holtzman MJ, Nadel JA. 1984a. Indomethacin inhibits the airway hyperresponsiveness but not the neutrophil influx induced by ozone in dogs. Am Rev Respir Dis 130:220–224.

O'Byrne PM, Walters EH, Gold BD, Aizawa HA, Fabbri LM, Alpert SE, Nadel JA, Holtzman MJ. 1984b. Neutrophil depletion inhibits airway hyperresponsiveness induced by ozone exposure. Am Rev Respir Dis 130:214–219.

Peden DB. 2000. Development of atopy and asthma: Candidate environmental influences and important periods of exposure. Environ Health Perspect 108(Suppl 3):475–482.

Peden DB, Setzer RW Jr, Devlin RB. 1995. Ozone exposure has both a priming effect on allergen-induced responses and an intrinsic inflammatory action in the nasal airways of perennially allergic asthmatics. Am J Respir Crit Care Med 151:1336–1345.

Pendino KJ, Laskin JD, Shuler RL, Punjabi CJ, Laskin DL. 1993. Enhanced production of nitric oxide by rat alveolar macrophages after inhalation of a pulmonary irritant is associated with increased expression of nitric oxide synthase. J Immunol 151:7196–7205.

Pretolani M, Vargaftig BB. 1993. From lung hypersensitivity to bronchial hyperreactivity: What can we learn from studies on animal models? Biochem Pharmacol 45:791–800.

Ratner B, Jackson HC, Gruehl HL. 1927. Respiratory anaphylaxis: Sensitization, shock, bronchial asthma and death induced in the guinea pig by the nasal inhalation of dry horse dander. Am J Dis Child 34:23–47.

Rombout PJA, Lioy PJ, Goldstein BD. 1986. Rationale for an eight-hour ozone standard. J Air Pollut Control Assoc 36:913–917.

Stevens WH, Vanderheyden C, Wattie J, Lane CG, Smith W, O'Byrne PM. 1995. Effect of a leukotriene B4 receptor antagonist SC-53228 on ozone-induced airway hyperresponsiveness and inflammation in dogs. Am J Respir Crit Care Med 152:1443–1448.

Sun J, Chung KF. 1997. Airway inflammation despite loss of bronchial hyper-responsiveness after multiple ozone exposures. Respir Med 91:47–55.

Sun J, Koto H, Chung KF. 1997. Interaction of ozone and allergen challenges on bronchial responsiveness and inflammation in sensitized guinea pigs. Int Arch Allergy Immunol 112:191–195.

Thorne PS, Karol MH. 1989. Association of fever with late-onset pulmonary hypersensitivity in guinea pig. Toxicol Appl Pharmacol 100:247–258.

Turner DJ, Martin JG. 1997. Animal models. In: Asthma (Barnes PJ, Grunstein MM, Leff AR, Woolcock AJ, eds), pp 261–274. Lippincott-Raven, Philadelphia PA.

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