

Repeated episodes of ozone inhalation amplifies the effects of allergen sensitization and inhalation on airway immune and structural development in Rhesus monkeys

Edward S. Schelegle,^{a,*} Lisa A. Miller,^a Laurel J. Gershwin,^b Michelle V. Fanucchi,^a Laura S. Van Winkle,^a Joan E. Gerriets,^a William F. Walby,^a Valerie Mitchell,^b Brian K. Tarkington,^c Viviana J. Wong,^c Gregory L. Baker,^c Lorraine M. Pantle,^c Jesse P. Joad,^d Kent E. Pinkerton,^a Reen Wu,^a Michael J. Evans,^a Dallas M. Hyde,^a and Charles G. Plopper^a

^a Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, Davis, Davis, CA 95616, USA

^b Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, Davis, CA 95616, USA

^c California Regional Primate Research Center Exposure Facility, School of Veterinary Medicine, University of California, Davis, Davis, CA 95616, USA

^d Department of Pediatrics, School of Medicine, University of California, Davis, Davis, CA 95616, USA

Received 5 November 2002; accepted 22 April 2003

Abstract

Twenty-four infant rhesus monkeys (30 days old) were exposed to 11 episodes of filtered air (FA), house dust mite allergen aerosol (HDMA), ozone (O₃), or HDMA + O₃ (5 days each followed by 9 days of FA). Ozone was delivered for 8 h/day at 0.5 ppm. Twelve of the monkeys were sensitized to house dust mite allergen (*Dermatophagoides farinae*) at ages 14 and 28 days by subcutaneous inoculation (SQ) of HDMA in alum and intraperitoneal injection of heat-killed *Bordetella pertussis* cells. Sensitized monkeys were exposed to HDMA aerosol for 2 h/day on days 3–5 of either FA ($n = 6$) or O₃ ($n = 6$) exposure. Nonsensitized monkeys were exposed to either FA ($n = 6$) or O₃ ($n = 6$). During the exposure regimen, parameters of allergy (i.e., serum IgE, histamine, and eosinophilia), airways resistance, reactivity, and structural remodeling were evaluated. Eleven repeated 5-day cycles of inhaling 0.5 ppm ozone over a 6-month period had only mild effects on the airways of nonsensitized infant rhesus monkeys. Similarly, the repeated inhalation of HDMA by HDMA-sensitized infant monkeys resulted in only mild airway effects, with the exception of a marked increase in proximal airway and terminal bronchiole content of eosinophils. In contrast, the combined cyclic inhalation of ozone and HDMA by HDMA sensitized infants monkeys resulted in a marked increase in serum IgE, serum histamine, and airways eosinophilia. Furthermore, combined cyclic inhalation of ozone and HDMA resulted in even greater alterations in airway structure and content that were associated with a significant elevation in baseline airways resistance and reactivity. These results suggest that ozone can amplify the allergic and structural remodeling effects of HDMA sensitization and inhalation.

© 2003 Elsevier Science (USA). All rights reserved.

Introduction

Ozone is one of six air pollutants listed in the Clean Air Act. The Clean Air Act in turn directs the United States

Environmental Protection Agency to set a primary National Ambient Air Quality Standard for ozone in order to protect public health. This health protection is required to encompass the health of “sensitive” populations, such as children and asthmatics. Despite this emphasis on “sensitive” populations no studies have been conducted to examine the influence of ozone inhalation on postnatal lung development of normal infants or infants with a family history of atopic disorders and are at risk of developing allergic reactive

* Corresponding author. Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, 1 Shields Avenue, University of California, Davis, CA 95616. Fax: +1-530-752-7690.

E-mail address: esschelegle@ucdavis.edu (E.S. Schelegle).

airway diseases such as asthma. The critical need for such studies is illustrated by the findings of several recent epidemiological studies reporting that growing up in high ambient ozone environments adversely effects lung function growth (Frischer et al., 1999; Galizia and Kinney, 1999; Peters et al., 1999; Calderon-Garciduenas et al., 2000; Gauderman et al., 2000), and the alarming observation that the number of children who develop allergic asthma has increased markedly in urban areas over the past 4 decades (Weitzman et al., 1992; Newacheck and Halfon, 2000; Akinbami and Schoendorf, 2002; Smyth, 2002).

Acute inhalation of ozone damages both proximal and distal airway epithelium, initiating a cascade of inflammatory and functional responses that subside as the airway epithelium undergoes repair. Cyclic climatic conditions results in an episodic urban exposure pattern. In adult animals, episodic exposure patterns have been shown to result in an altered response to ozone-induced epithelial damage consisting of a diminution of inflammation and epithelial cell proliferation (Schelegle et al., 2003). This diminished response to ozone-induced injury is associated with a progressive airway remodeling that is characterized by epithelial cell hypertrophy, hyperplasia, and interstitial fibrosis (Tyler et al., 1988; Barr et al., 1990; Schelegle et al., 2003). The possibility that ozone may alter the normal postnatal development of the lung is highlighted by the fact that the release of growth factors are important in both repair from injury and postnatal airway morphogenesis (Schelegle et al., 2003). As a result, infants exposed to repeated episodes of ozone would be expected to demonstrate alterations in the regional distribution and profile of growth factors within the airway, compromising both normal airway morphogenesis and differentiation.

Asthma is one of the leading causes of morbidity in childhood, affecting 5 million American children. Although the onset of airway hyperresponsiveness to allergens is typically not observed until children are of school age, epidemiologic studies suggest that the pathogenesis of allergic asthma follows a progressive course starting in the 1st year of life. In addition to family history and wheezing, general parameters of atopy such as elevated serum IgE and positive skin-prick tests have been reported to predict whether an infant will eventually develop persistent asthma (Lemanske, 2002). It is clear that genetic factors have an important role in the pathogenesis of asthma, yet inheritance does not explain why the incidence of asthma has increased in such rapid fashion within the past 40 years. Other studies point to a number of risk factors for the development of respiratory symptoms and asthma in children; these factors include low socioeconomic class, crowding, and high allergen concentrations (Lemanske, 2002). Because the rise in prevalence and severity of allergic diseases correlates with industrialized countries, it has also been postulated that ambient air pollutants such as ozone may play a role in the development of immune and respiratory symptoms associated with allergy and asthma (Bjorksten, 1999). This pos-

HDMA Sensitization and Challenge	Ozone Inhalation	
	-/-	+/-
	-/+	+/+

Fig. 1. Box diagram of experimental design. Each group was composed of six infant rhesus monkeys.

sibility is supported by studies in mice that show that ozone inhalation augments the priming of the airways to inhaled allergen and the development of an allergic response (Gershwin et al., 1981; Neter et al., 1985).

The present study was designed to determine if a cyclic regimen of ozone inhalation would amplify the allergic responses and structural remodeling associated with allergen sensitization and inhalation in infant rhesus monkeys. We hypothesize that repeated cycles of ozone inhalation alters the development of lung structure and function and amplifies airway allergic immune responses to inhaled allergen of sensitized infants. We chose to study rhesus monkeys because of their similarities in postnatal lung and immune system development with that of humans. We used house dust mite allergen (HDMA) because of our recent observation that adult rhesus monkeys can be sensitized to and subsequently develop allergic responses to inhaled HDMA (Schelegle et al., 2001). In order to examine the interaction of cyclic HDMA and ozone inhalation we examined 10 end-points that have been previously shown to be affected by repeated allergen challenge and/or cyclic ozone exposure as well as markers that would indicate growth of the airways and alveoli. Sensitized infant monkeys that inhaled HDMA and ozone in combination, although demonstrating similar airway effects to infant monkeys that inhaled HDMA alone, presented alterations in six additional end-points that were greater than the additive effects of HDMA or ozone alone. These observations, by demonstrating a synergism between ozone and allergen inhalation in sensitized infant monkeys, illustrate the need to further examine the mechanisms that underlie this synergism and the possible contribution that these mechanisms might play in the development of reactive airway disease in atopic human infants.

Methods and materials

Animals and general experimental protocol. The design of this study is illustrated in Fig. 1. Twenty-four infant rhesus monkeys (30 days old) were exposed to 11 episodes of filtered air (FA), house dust mite allergen aerosol (HDMA), ozone (O₃), or HDMA + O₃ (5 days each followed by 9 days of FA). Ozone was delivered for 8 h/day at 0.5 ppm. Twelve of the monkeys (HDMA and HDMA + O₃ groups) were sensitized to house dust mite allergen (*Dermatophagoides farinae*) at 14 days of age by subcutaneous inocula-

tion (SQ) of HDMA in alum and intramuscular injection of heat-killed *Bordetella pertussis* cells and again at 28 days of age by subcutaneous inoculation (SQ) of HDMA in alum. HDMA sensitization was confirmed via skin testing with intradermal HDMA on Day 38 of the exposure protocol. Sensitized monkeys were exposed to HDMA aerosol for 2 h/day on days 3–5 of either FA (HDMA, $n = 6$) or O_3 (HDMA + O_3 , $n = 6$) exposure. Nonsensitized monkeys were exposed to either FA (FA, $n = 6$) or O_3 (O_3 , $n = 6$). Airway resistance (R_{aw}), breathing pattern (BP), arterial oxygen saturation ($O_2\text{Sat}$), and breath sounds were assessed, while the monkeys were anesthetized and intubated after the 6th and 10th episodes. Raw, BP, $O_2\text{sat}$, and breath sounds were assessed during histamine dose–response and HDMA challenges.

Ozone and allergen aerosol exposures. Ozone was generated as described previously (Wilson et al., 1984) and its concentration was monitored using a Dasibi 1003-AH ozone analyzer (Dasibi Environmental Corporation, Glendale, CA). HDMA aerosol exposures were generated using house dust mite extract (Greer Laboratories, Inc. Lenoir, NC) diluted in phosphate-buffered saline (PBS) as described previously (Schelegle et al., 2001). During exposure air samples were drawn from the chamber and the mass concentration, protein content, and aerodynamic size distribution characterized as previously described (Schelegle et al., 2001).

Sedation and anesthesia. Monkeys were sedated using ketamine hydrochloride (5–10 mg/kg, im). For evaluation of airway responsiveness and bronchoscopy, the monkeys were anesthetized using Diprivan (0.1–0.2 mg/kg/min, IV) with the dose adjusted as deemed necessary by the attending veterinarian, intubated with an uncuffed endotracheal tube (2.5 mm), and placed in a head-out body plethysmograph with the intubation tube attached to a pneumatic four-way valve/pneumotachograph assembly. For necropsy, sedated animals were deeply anesthetized with intravenous sodium pentobarbital, the trachea was intubated, and the animal was maintained on positive-pressure ventilation (15.0 ml/kg, 20.0 bpm, 10–20 min) until killed by exsanguination via the systemic aorta.

Pulmonary mechanics and airways responsiveness testing. Pulmonary mechanics were measured using a transfer impedance method as previously described (Madwed and Jackson, 1997; Schelegle et al., 2001). Airway resistance (R_{aw}), airway inertance (I_{aw}), tissue resistance (R_{ti}), tissue inertance (I_{ti}), and tissue compliance (C_{ti}) were obtained by fitting the impedance data to the six-element model of the respiratory tract proposed by DuBois using a gradient optimization technique. Since only five of the six elements can be uniquely estimated using this technique, we restricted Cg to a constant value determined using a published regression equation relating functional residual capacity (FRC) and

body weight for rhesus monkeys. A goodness-of-fit statistic (GFS) for each estimate was calculated as the mean of the residual across all frequencies used for fitting the model to the data expressed as a percentage of the respiratory system resistance (sum of R_{aw} and R_{ti}). Respiratory system resistance was chosen as the denominator in this statistic because it accounts for the majority of the amplitude in the real component of impedance. Tidal volume (V_T) and breathing frequency (f_b) were recorded on a breath-by-breath basis by integrating the output of the pneumotachograph using a digital data acquisition system (PO-NE-MAH, Gould Instruments). Arterial oxygen saturation ($O_2\text{Sat}\%$) was monitored via a pulse oximeter (MOD II) and then recorded at the beginning and ending of each data collection period.

All allergen and histamine challenges were administered as aerosols at a set maximal airway pressure (10 cmH₂O) for a total of 30 breaths (1 breath/s) using a compressed air nebulizer (Vortran, Inc.) in series with a positive pressure ventilator (Bird Mark 7A respirator). Allergen challenges (0.02 mg protein/ml) were delivered as above and repeated at 4-min intervals and were terminated when airway resistance (R_{aw}) doubled or after 12 HDMA doses. R_{aw} , f_b , V_T , arterial O_2 saturation, and clinical assessment scores were expressed as the percentage of change from baseline after the 12 HDMA doses. Histamine challenges used repeated 30-s challenges separated by 240-s data collection periods, beginning with saline followed by doubling concentrations starting at 0.0625 mg/ml and ending at the concentration that doubles R_{aw} up to a maximum of 32 mg/ml or when $O_2\text{Sat}\%$ dropped below 75%. Though the data collection period was set at 240 s the next dose of histamine was not delivered until breathing frequency that was closely associated with changes in airway mechanics had returned to baseline. Data were expressed as the concentration that increases R_{aw} by 150% ($EC_{150R_{aw}}$). The $EC_{150R_{aw}}$ was determined by linear interpolation on the log-log plot of the dose–response curve with the response expressed as the percentage of baseline R_{aw} .

Breath sounds and clinical assessment of respiratory status was performed immediately after intubation, prior to HDMA aerosol challenge, and again immediately following HDMA challenge (but prior to extubation). The Clinical Assessment Score (CAS) was based on the following point system:

- +1 point for each 10% increase in ventilation rate over pre-HDMA baseline
- +1 point for each 10% decrease in pO_2 from pre-HDMA baseline
- +1 point for each abnormal lung sound at either observation time
- +2 point for evidence of dyspnea at either observation time
- +1 point for evidence of cough at either observation time

The final CAS was calculated as the difference from pre-HDMA to post-HDMA aerosol challenge.

Intradermal skin testing. Monkeys were anesthetized with ketamine hydrochloride and hair was clipped from an area of the thorax. Three separate intradermal injections (0.1 ml each) of PBS containing HDMA (1:1000 w/v), histamine (1:1000), or diluent alone were made at the site. Injection sites were observed for 30 min and wheal diameters were measured after 20 min. A skin test was considered positive if the diameter of the wheal formed in response to HDMA was greater than or equal to that halfway between the diameter of the wheal produced by the diluent (negative control) and histamine (positive control) (Scott et al., 1995).

Fluorometric determination of histamine concentration. Histamine was measured in plasma using a previously published fluorometric method (Shore et al., 1959). Histamine concentrations in samples were determined by comparison to a standard curve using known concentrations of histamine (range: 0.005–0.5 $\mu\text{g/ml}$).

Bronchoalveolar lavage and differential cell counts. At necropsy, the right caudal lobe was lavaged with approximately 50 ml of endotoxin-free PBS. For leukocyte differentials, lavage samples were cytocentrifuged, air dried, and stained with a modified Wright's stain (Diff-Quik) and 300 cells consisting of macrophages, neutrophils, eosinophils, lymphocytes, and epithelial cells were counted using light microscopy ($\times 1000$). Leukocyte differentials in lavage samples were expressed as the percentage of macrophages, neutrophils, eosinophils, lymphocytes, and epithelial cells per 300 cells counted.

House dust mite allergen-specific IgE concentrations in serum. Allergen-specific IgE was measured in serum samples using an ELISA. Briefly, 96-well microtiter plates were sensitized with 3 $\mu\text{g/well}$ HDMA in carbonate/bicarbonate buffer. Samples were precipitated with 35% saturated ammonium sulfate to remove interfering IgG, and supernatants were used as sample without further dilution. Binding of IgE was detected with a goat antihuman IgE–horseradish peroxidase-conjugated antibody (Bethyl Laboratories, Inc.). Positive controls consisted of pooled human HDMA IgE-positive sera (RAST tested high level). Data are presented as the percentage of positive control.

Necropsy and histopathology. Following exsanguination, the thorax was opened by midline incision and the entire mediastinal contents removed en bloc. The trachea and extrapulmonary bronchi were exposed and the lobar bronchi cannulated. The right middle lung lobe was fixed by airway infusion with glutaraldehyde/paraformaldehyde (1%/1%) at 30 cm hydrostatic pressure.

Distribution of mucous cells. The main axial airway of the right middle lobe was dissected and embedded in Aradite and 0.5- μm sections were stained with toluidine blue. The mass of mucous cells (V_s), as measured by volume (cubic micrometers) of mucous goblet cells per unit area (square micrometers) of basement membrane, was estimated from point and intercept counts with a cycloid grid by the following equation:

$$V_s = (2.2 \times P_o)/(I_{bl} \times 2),$$

where P_o represents the points counted for mucous goblets cells and I_{bl} represents the number of intercepts of basal lamina.

Distribution of airway eosinophils. At necropsy, cross sections of the left caudal lobe from each animal were prepared for cryosectioning. The left caudal lobe was inflated with a mixture of OCT and PBS, sliced perpendicular to the long axis of the intrapulmonary airway, and then embedded into OCT. Five-micrometer frozen sections from alternate blocks were used for immunofluorescence staining. Eosinophils were detected by staining cryosections with mouse antihuman major basic protein antibody (Biodesign), followed by goat antimouse Ig ALEXA 488 (Molecular Probes). Values are the average volume of eosinophils within the combined epithelium and interstitium compartments sampled at three conducting airway sites and one terminal/respiratory bronchiolar site and are expressed relative to surface area of basal lamina in a manner similar to that used for the mass of mucous cells.

Evaluation of tracheal basement membrane thickness. The trachea was sliced perpendicular to the long axis of the airway. Tracheal slices were fixed in 1.0% paraformaldehyde for 1 h and embedded in paraffin. Five-micron sections were deparaffinized in xylene, hydrated in ethanol, and washed in PBS. The sections were next treated with pepsin (1.0 mg pepsin/ml 3.0% acetic acid) at 37°C for 2 h and blocked with bovine serum albumin. The sections were treated with antibody to collagen I (1:250)(Biogenesis, Kingston, NH), collagen III (1:1000)(Biogenetic, San Ramon, CA), or collagen V (1:500)(Biogenesis) overnight at 4°C. The sections were next washed in PBS, treated with the secondary antibody (Alexa 594, Molecular Probes, 1:1000) of 30 min, and washed in PBS and the coverslip was mounted in ELF fluorescent safe media. To determine the width of the BMZ and the height of the columnar epithelium 10 micrographs were taken in sequence around the circumference of the airway. The width of the BMZ and height of the epithelium was measured at four different points on each micrograph. The mean and standard deviation of BMZ width was determined from the combined measurements from collagens I, III, and V from each animal.

Airway diameters of the main axial path of the right middle lobe. The diameter of the first and seventh intrapulmonary airway generation were measured from 1.0- μm sections obtained from 1.0-cm thick slices cut perpendicular to the main axial path of the right middle lobe. Measurements were made on digital images obtained of the 1.0 μm sections using image analysis software (NIH Image).

Evaluation of airway, parenchyma, and blood vessel volume. The entire right middle lobe was sliced into 8–11 parallel slices perpendicular to the long axis of the main axial airway path. The volume fraction (V_v) of lung compartments (parenchyma, blood vessels, and airways) was estimated by point counting using a grid placed over print outs ($\times 15$ final magnification) of the low magnification images taken of one entire side (top) of each of the slices using standard stereologic methods (Howard and Reed, 1998). The classification of various compartments as either blood vessels or airways was confirmed using a high-power dissecting microscope on the original tissue ($> \times 20$ magnification) and the type of compartment was noted on the printout. Airways and blood vessels were defined based on composition of the wall and the size of the lumen. All other components that could not be classified as either blood vessels or airways were classified as parenchyma. The limit of detection for airways and blood vessels was 400 μm . Only profiles larger than 400 μm could be classified as blood vessels or airways. Airways and blood vessels with lumen diameters smaller than this were grouped into the parenchymal classification. Using the volume fraction, the total volume of each component was calculated based on the lung volume of the entire lobe that was determined using standard volume displacement analysis at the time of necropsy (Scherle, 1970).

Statistical analysis. All data are reported as mean $\pm$ standard error of the mean. Histamine, IgE, cellular, and airway dimensional data were initially analyzed using a two-way analysis of variance (Statview, SAS Institute, Cary, NC). Baseline airway resistance and airway responsiveness data was initially analyzed using a three-way analysis of variance with repeated measures (Statview, SAS Institute, Cary, NC). Groups were compared using Fisher's PLSD test with the significance level set at $P \leq 0.05$. Parameters for which there were partial data sets consisting of 3 per group were analyzed using the nonparametric analysis, Kruskal–Wallis tests with mean differences being determined using the Mann–Whitney test (Statview, SAS Institute, Cary, NC).

Results

Response to HDMA challenge

Sensitization with house dust mite allergen resulted in positive skin reactivity in 11 of 12 treated monkeys. The 1

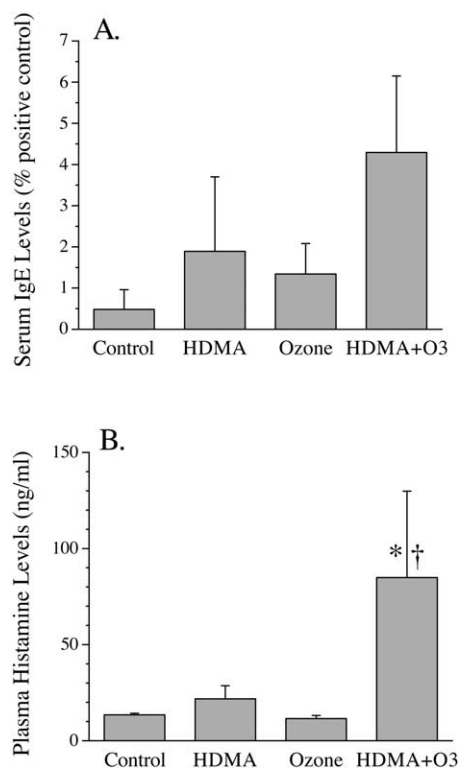


Fig. 2. Serum IgE levels average over weeks 11–19 of the exposure protocol (A) and plasma histamine levels average over weeks 14–22 of the exposure protocol (B) for filtered air control, house dust mite allergen sensitized and challenged (HDMA), ozone exposed, and HDMA in combination with ozone exposure (HDMA + O₃) infant rhesus monkeys. Data are reported as mean $\pm$ standard error of the mean. (*) A significant difference from filtered air control. (†) A significant difference from ozone exposed.

monkey that was skin test negative was in the HDMA + O₃ group. All nonsensitized monkeys in the filtered air and ozone groups had negative skin tests to HDMA.

Serum HDMA-specific IgE following 10 cycles of exposure was not significantly affected in any group (Fig. 2A), though there was a trend ($P = 0.082$) toward an elevated serum HDMA-specific IgE in the HDMA + O₃ group compared to the filtered air and ozone alone groups that were not exposed to HDMA aerosol.

The average plasma histamine levels following allergen challenge from exposure cycles 5 through 10 was significantly elevated in the HDMA + O₃ group compared to the filtered air and ozone alone groups that were not exposed to HDMA aerosol (Fig. 2B).

Aerosol challenge with HDMA aerosol at the end of the 10th episode did not significantly affect R_{aw} , f_B , V_T , arterial O₂ saturation, and clinical assessment scores in any group.

Baseline R_{aw} and airway responsiveness

Group mean baseline R_{aw} , I_{aw} , R_{ti} , I_{ti} , and C_{ti} , as well as the goodness-of-fit statistic (GFS) for the cycle 6 and 10 data are given in Table 1. The GFS was less than 1% for all

Table 1

Estimated parameters and goodness-of-fit statistic for fit of transfer impedance data to the six-parameter model of the respiratory system of De Bois

	R_{aw}	I_{aw}	R_{ti}	I_{ti}	C_{ti}	GFS
Cycle 6						
FA	41.0 ± 3.7	0.264 ± 0.093	1.79 ± 1.30	0.002 ± 0.001	0.557 ± 0.225	1.08 ± 0.161
HDMA	49.2 ± 7.9	0.262 ± 0.088	1.96 ± 0.87	0.002 ± 0.001	0.503 ± 0.201	1.17 ± 0.14
O ₃	66.5 ± 23.5	0.221 ± 0.070	3.93 ± 1.77	0.002 ± 0.001	0.499 ± 0.202	0.97 ± 0.27
HDMA + O ₃	144.0 ± 44.7*†	0.272 ± 0.096	3.50 ± 1.44	0.003 ± 0.001	0.462 ± 0.184	1.42 ± 0.10
Cycle 10						
FA	43.6 ± 5.7	0.445 ± 0.013	5.00 ± 2.23	0.005 ± 0.001	0.520 ± 0.210	0.72 ± 0.08
HDMA	72.3 ± 13.3	0.452 ± 0.012	4.83 ± 2.14	0.003 ± 0.001	0.447 ± 0.180	0.91 ± 0.04
O ₃	66.6 ± 10.0	0.425 ± 0.016	4.85 ± 2.16	0.003 ± 0.000	0.519 ± 0.210	0.72 ± 0.08
HDMA + O ₃	123.8 ± 28.5*††	0.221 ± 0.098	4.76 ± 1.67	0.003 ± 0.001	0.478 ± 0.190	0.95 ± 0.09

Note. Data were obtained from infant rhesus monkeys that were cyclically exposed to filtered air (FA), house dust mite allergen (HDMA), ozone (O₃), and the combination (HDMA + O₃). R_{aw} , airway resistance; I_{aw} , airway inertance; R_{ti} , tissue resistance; I_{ti} , tissue inertance; C_{ti} , tissue compliance; GFS, goodness-of-fit statistic. Data are mean ± SEM.

* A significant difference from FA ($P < 0.05$).

† Significant difference from HDMA ($P < 0.05$).

†† A significant difference from O₃ ($P < 0.05$).

parameter estimates. Baseline I_{aw} , R_{ti} , I_{ti} and C_{ti} were not affected by any treatment. Baseline R_{aw} was significantly elevated after 6 cycles of exposure in the HDMA + O₃ group compared to the filtered air and HDMA groups (Fig. 3A). Baseline R_{aw} was significantly elevated after 10 cycles of exposure in the HDMA + O₃ group compared to the filtered air, HDMA, and O₃ groups (Fig. 3B).

There were no significant differences for EC150 R_{aw} between any group following 6 cycles of exposure (Fig. 4A). In contrast, the EC150 R_{aw} for the HDMA + O₃ group following 10 cycles of exposure was significantly reduced compared to the filtered air, HDMA, and O₃ groups (Fig. 4B), indicating an increased in airway responsiveness to histamine aerosol in this group. In addition, the histamine EC150 R_{aw} at cycle 10 was significantly less than the histamine EC150 R_{aw} at cycle 6 for the HDMA + O₃ group (Fig. 4A versus Fig. 4B), indicating the development of airway hyperresponsiveness in this group somewhere between cycles 6 and 10.

Bronchoalveolar cell differential

Mean values for bronchoalveolar cell differentials for each group are given in Table 2. There were no significant differences between the study groups in the percentage of lymphocytes, neutrophils, and macrophages recovered in bronchoalveolar lavage fluid. The percentage of bronchoalveolar lavage eosinophils in the HDMA + O₃ group was significantly elevated compared to those in the filtered air and HDMA groups (Fig. 5A).

Accumulation of eosinophils in airways

Allergen sensitization and challenge (HDMA group) and allergen sensitization and challenge in combination with episodic ozone inhalation (HDMA + O₃ group) resulted in

a significantly greater eosinophil accumulation in the conducting airways (Fig. 5B) compared to the filtered air and O₃ groups. There was no significant difference in the volume of conducting airway eosinophil load between the HDMA group and HDMA + O₃ group (Fig. 5B).

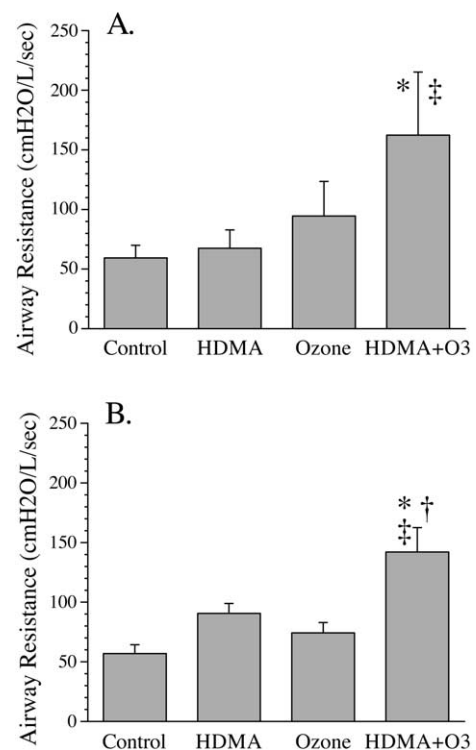


Fig. 3. Baseline airway resistance for cycle 6 (A) and cycle 10 (B) of exposure protocol for filtered air control, house dust mite allergen sensitized and challenged (HDMA), ozone exposed, and HDMA in combination with ozone exposure (HDMA + O₃) infant rhesus monkeys. Data are reported as mean ± standard error of the mean. (*) A significant difference from filtered air control. (†) A significant difference from ozone exposed. (‡) A significant difference from HDMA.

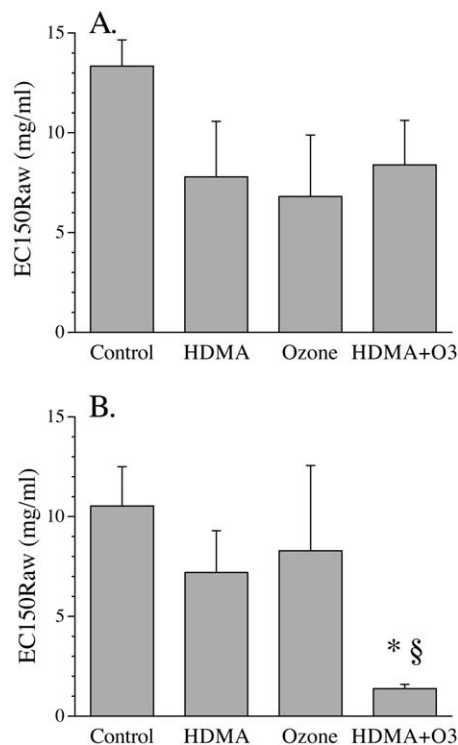


Fig. 4. The effective concentration of histamine required to produce a 150% increase in airway resistance (EC150_{Raw}) for cycle 6 (A) and cycle 10 (B) of exposure protocol for filtered air control, house dust mite allergen sensitized and challenged (HDMA), ozone exposed, and HDMA in combination with ozone exposure (HDMA + O₃) infant rhesus monkeys. Data are reported as mean $\pm$ standard error of the mean. (*) A significant difference from filtered air control. (§) A significant difference from cycle six HDMA + O₃.

Allergen sensitization and challenge (HDMA group) resulted in a significantly greater eosinophil accumulation in the terminal/respiratory bronchioles (Fig. 5C) compared to the filtered air, O₃, and HDMA + O₃ groups.

Distribution of mucous cells

The mean mass of mucous cells within the fifth generation intrapulmonary airway and terminal bronchioles of the left cranial lobe for each group is shown in Fig. 6 with examples of the images that were used for analysis shown in Fig. 7. HDMA sensitization and inhalation alone and in

combination with ozone inhalation resulted in a significant increase in the mass of mucous cells within the fifth intrapulmonary airway (Fig. 6A). There was no difference in the mean mass of mucous cells within the fifth intrapulmonary airway between filtered air inhalation and ozone inhalation and then HDMA sensitization and inhalation and HDMA sensitization and inhalation in combination with ozone inhalation (Fig. 6A). HDMA sensitization and inhalation in combination with ozone inhalation resulted in a significant increase in the mass of mucous cells within the terminal bronchioles (Fig. 6B).

Tracheal basement membrane thickness

The BMZ was variable in thickness in all of the monkeys studied. In monkeys maintained in filtered air the mean BMZ thickness was $4.5 \pm 0.1 \mu\text{m}$. The range of individual measurements was 1.5–8.3 μm . The mean BMZ thickness of monkeys exposed to HDMA alone was significantly increased compared to filtered air ($6.6 \pm 0.6 \mu\text{m}$). The range of individual measurements was 1.0–14.5 μm . The mean BMZ thickness of monkeys exposed to O₃ alone was similar to monkeys in FA and averaged $5.5 \pm 0.9 \mu\text{m}$. The range of individual measurements was 1.0–9.5 μm . In monkeys exposed to HDMA and O₃ the mean BMZ thickness was much more variable in thickness with many areas very thin and other regions much thicker. However, the average width was significantly greater than compared to controls ($6.2 \pm 0.6 \mu\text{m}$). The range of individual measurements was 1.5–12.5 μm .

Airway diameters of proximal and midlevel airways

Ozone inhalation and allergen sensitization and challenge, alone, and/or in combination did not significantly influence the diameter of the first or seventh generation intrapulmonary airway of left cranial lobe. The mean diameter of the first generation intrapulmonary airway were 2.01 ± 0.18 , 2.38 ± 0.52 , 1.70 ± 0.31 , and $1.65 \pm 0.11 \text{ mm}$ for the filtered air, HDMA, O₃ and HDMA plus O₃ groups, respectively. The mean diameter of the seventh generation intrapulmonary airway were 0.85 ± 0.06 , 0.93 ± 0.10 , 0.83 ± 0.13 , and $0.90 \pm 0.06 \text{ mm}$ for the filtered air, HDMA, O₃, and HDMA plus O₃ groups, respectively.

Table 2

Cell differentials in bronchoalveolar lavage fluid obtained from infant rhesus monkeys previously sensitized to HDMA/and or episodically exposed to ozone at time of necropsy

Group	Macrophages	Lymphocytes	Neutrophils	Eosinophils
Control	85.15 $\pm$ 1.86	12.8 $\pm$ 1.85	0.67 $\pm$ 0.27	1.45 $\pm$ 0.47
HDMA	78.9 $\pm$ 7.07	19.13 $\pm$ 7.04	1.05 $\pm$ 0.28	0.85 $\pm$ 0.19
Ozone	81.02 $\pm$ 2.53	13.13 $\pm$ 2.00	0.72 $\pm$ 0.26	4.88 $\pm$ 1.19
HDMA + O ₃	76.93 $\pm$ 2.43	14.40 $\pm$ 1.88	0.60 $\pm$ 0.32	7.87 $\pm$ 2.84*

Note. Data are reported as mean $\pm$ standard error of the mean.

* Significantly different from control and HDMA alone, $P \leq 0.05$.

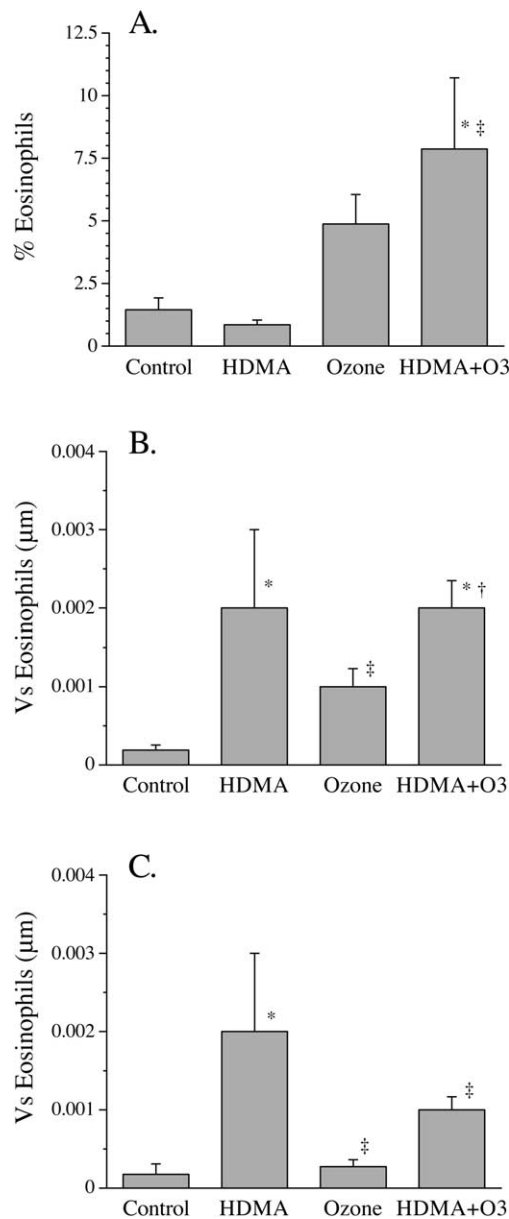


Fig. 5. Percentage of eosinophils in bronchoalveolar lavage (A), eosinophil content of the proximal airway (B), and terminal bronchioles (C) of the left cranial lobe for filtered air control, house dust mite allergen sensitized and challenged (HDMA), ozone exposed, and HDMA in combination with ozone exposure (HDMA + O₃) infant rhesus monkeys. Data are reported as mean $\pm$ standard error of the mean. (*) A significant difference from filtered air control. (†) A significant difference from ozone exposed. (‡) A significant difference from HDMA.

Airway, parenchyma, and blood vessel volume

The total volume of the right middle lobe as a ratio of body weight and the fraction of total volume for parenchyma, conducting airway and blood vessels are given in Table 3. The total volume per body weight and volume fraction of parenchyma of the right middle lobe was significantly greater in the HDMA + O₃ group compared to the filtered air group. In contrast, the volume fraction of con-

ducting airway and blood vessel were significantly less in the HDMA + O₃ group compared to the filtered air group. In addition the volume fraction of conducting airway was significantly less in the HDMA + O₃ group compared to the HDMA and O₃ groups.

Discussion

The findings from this study are consistent with the notion that repeated cycles of ozone inhalation amplifies immune responses to allergen in sensitized infants. Specifically, we observed that the combination of repeated cyclic inhalation of 0.5 ppm ozone with HDMA sensitization and inhalation results in alterations in 9 of 10 immune, structural, and functional end-points examined, with six of these end-points demonstrating greater than additive effects. These observations indicate that the combination of cyclic ozone and HDMA inhalation in HDMA sensitized infant monkeys act synergistically to produce an allergic-reactive airway phenotype. This reactive phenotype is characterized by elevated HDMA-induced histamine release, a significant eosinophilia of midlevel conducting airways that was associated with a transmigration of eosinophils into the airway

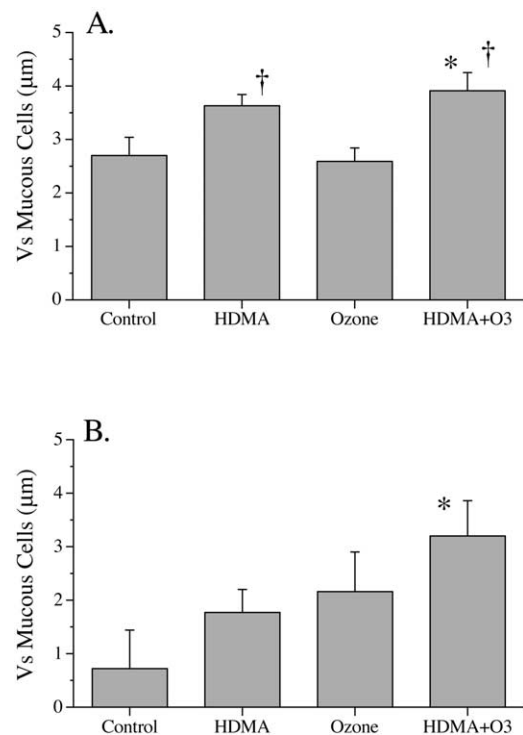


Fig. 6. Mucous cell content of the fifth intrapulmonary airway (A) and terminal bronchioles (B) of the left cranial lobe for filtered air control, house dust mite allergen sensitized and challenged (HDMA), ozone exposed, and HDMA in combination with ozone exposure (HDMA + O₃) infant rhesus monkeys. Data are reported as mean $\pm$ standard error of the mean. (*) A significant difference from filtered air control. (†) A significant difference from ozone exposed. (‡) A significant difference from HDMA.

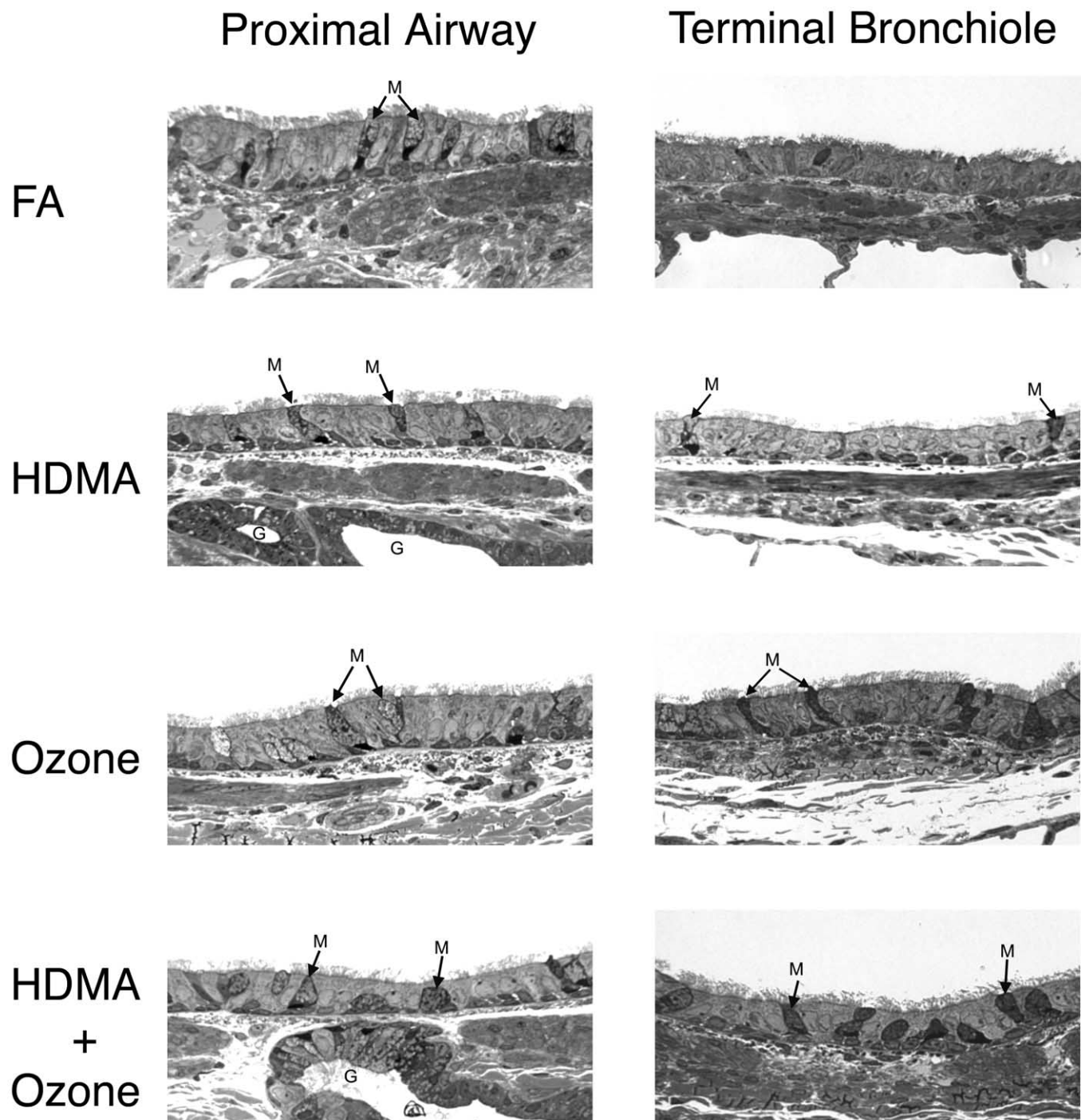


Fig. 7. Example of toluidene blue stained airway sections sample at the fifth intrapulmonary airway (Proximal Airway) and the terminal bronchiole of the left cranial lobe for filtered air control (FA), house dust mite allergen sensitized and challenged (HDMA), ozone exposed, and HDMA in combination with ozone exposure (HDMA + Ozone) infant rhesus monkeys. M, an identified mucous-containing cell.

lumen, and an altered structural development of the conducting airways that was associated with an elevation of airway resistance and nonspecific reactivity. In contrast, HDMA sensitization followed with repeated cyclic inhalation of HDMA allergen resulted in a significant eosinophilia of midlevel conducting airways and terminal/respiratory bronchioles, yet did not result in an increase in HDMA-

induced histamine release, BAL eosinophils, or alterations in airway structure, resistance, and/or reactivity. In addition, the repeated cyclic inhalation of 0.5 ppm ozone alone did not result in significant alterations in any of our measures of lung structure and/or function.

The combined effect of ozone exposure and aerosolized antigen has been studied in murine models. In a similar, but

Table 3

Fractional lobe and tissue compartment volumes obtained from the right middle lung lobe of infant rhesus monkeys previously sensitized to HDMA/and or episodically exposed to ozone at time of necropsy

Group	Total lobe volume/body wt (ml/kg)	Volume fraction of parenchyma	Volume fraction of conducting airways	Volume fraction of blood vessels
Control	5.35 ± 0.60	0.970 ± 0.003	0.018 ± 0.0005	0.012 ± 0.003
HDMA	6.09 ± 0.31	0.974 ± 0.001	0.019 ± 0.001	0.007 ± 0.001
Ozone	5.62 ± 0.20	0.972 ± 0.001	0.021 ± 0.002	0.007 ± 0.001
HDMA + O ₃	6.88 ± 0.38*	0.980 ± 0.002*†	0.014 ± 0.001*†‡	0.006 ± 0.002*

Note. Data are reported as mean ± standard error of the mean.

* Significantly different from control.

† Significantly different from ozone alone.

‡ Significantly different from HDMA alone, all $P \leq 0.05$.

not identical, episodic exposure to 0.8 ppm ozone with ovalbumin (OVA) aerosol following each exposure, Swiss Webster mice were found to significantly increase IgE synthesis in the lung when exposed to both agents as compared to controls exposed to OVA and ozone alone (Gershwin et al., 1981). More recently, priming of an allergic response was demonstrated in BALB/c and C57BL/6 mice after intermittent ozone exposure and OVA aerosol. In this model increases in total serum IgE, interleukin-(IL) 4 and IL-5, airway responsiveness, and recruitment of eosinophils and lymphocytes into the airways were documented in dual-exposed animals. These findings were significant because they demonstrate development of a T helper 2-(Th2) type response in both a high IgE and a low IgE responder mouse strain (Neuhaus-Steinmetz et al., 2000). In addition, other air pollutants have been shown in rodent models to enhance allergic sensitization and reactivity. For example, exposure to 5 ppm NO₂ for 3 h prior to immunization significantly enhanced levels of house dust mite specific IgE and pulmonary inflammation in rats (Gilmour, 1995). Studies that combine ozone and allergic asthma in a primate model are few. However, in the 1980s a study was performed in which cynomolgus monkeys were exposed to ozone and a platinum salt by inhalation for 12 weeks. Bronchoprovocation challenge and skin testing were performed. The data showed that ozone and platinum exposure significantly increased specific and nonspecific bronchial hyperreactivity, as well as positive skin testing with platinum compared to either alone (Biagini et al., 1986).

Although the previously cited studies used young adult animals, the focus of the present study was on the immune, structural, and functional responses of neonatal monkeys. To date, only one other study has focused on the effects of air pollutants on allergic responses to aerosolized antigen in infants (Hamada et al., 2000). In this study, neonatal mice were exposed to leachate of residual oil fly ash and aerosolized OVA. The combined effect of the pollutant and the allergen resulted in increased airway hyperresponsiveness and increased eosinophils in bronchoalveolar lavage of neonatal mice. In contrast to adult mice, measurement of OVA-specific IgE was not demonstrated after initial sensitization

of neonates, but was present only in the combined exposure group after secondary exposure to aerosolized OVA. The lack of airway sensitization in the neonatal mice was attributed to a possible lack of dendritic cells, which are critical for airway sensitization. This hypothesis was supported by increased numbers of Ia⁺ cells in airway mucosa of dual exposed neonatal mice compared with those exposed to only OVA (Hamada et al., 2000). Although we do not know if the infant monkeys in the present study exhibit a similar response, their overall IgE response was of a much lesser magnitude when compared to young adult monkeys (approximately 3 years of age) undergoing a similar protocol (unpublished data).

In the present study, the strongest evidence that cyclic ozone inhalation amplifies the immune response in HDMA sensitized and challenged infant monkeys was the observed increase in serum histamine following allergen challenge (Fig. 2B) and the increase in eosinophils present in the BAL (Fig. 5A). Histamine is a biogenic amine released via exocytosis of secretory granules of mast cells and basophils. In allergy, histamine release is the result of cross-linking FcεR1 molecules subsequent to the binding of antigen to antigen-specific IgE that is bound to the receptor domain of the FcεR1 molecule. FcεR1 molecule cross-linking in turn activates phospholipase C to catalyze the breakdown of phosphatidyl inositol biphosphate into inositol triphosphate (IP₃) and diacylglycerol. Inositol triphosphate in turn increases the release of Ca²⁺ from the endoplasmic reticulum that binds to calmodulin and activates myosin light chain kinase to phosphorylates myosin light chain, resulting in exocytosis of histamine containing secretory granules (Abbas et al., 1991). Whether the cyclic inhalation of ozone influences this cascade of events is unknown. The observed trend ($P = 0.082$) toward increased serum HDMA-specific IgE in the HDMA plus ozone group (Fig. 2A) would be consistent with the notion that priming of Th2 responses by ozone maybe partly responsible for the observed elevation in serum histamine levels. Alternatively, the cyclic inhalation of ozone may result in elevated levels of cytokines that are known to cause the release of histamine from mast cells by IgE-independent mechanisms. One likely candidate cy-

tokine is IL-8, which has been known to be produced by rhesus monkey airway epithelium following ozone-induced injury (Chang et al., 1998) and has been shown to cause the release of histamine from mucosal mast cells (Yakabi et al., 2002). IL-8 mediated release of histamine appears to be the result of binding to CXCR1 and CXCR2 and is associated with a dose-dependent increase in intercellular Ca^{2+} (Inamura et al., 2002). Interestingly, ozone exposure of cultured human nasal mucosa results in greater histamine release from tissue obtained from atopic versus nonatopic subjects (Schierhorn et al., 1999). Similarly nasal tissue from the atopic subjects also demonstrated a marked increase in IL-8 production induced by ozone (Schierhorn et al., 1999). These observations suggest ozone-induced IL-8 secretion by airway epithelium may also be responsible for the synergistic interaction of HDMA and cyclic ozone inhalation on histamine release observed in the current study.

In addition to IL-8, the targeted induction of chemokine expression by airway epithelium may be an important mechanism by which ozone can provide a transepithelial chemotactic signal for eosinophils already present within the airway mucosa of HDMA sensitized and challenged monkeys (Fig. 5B and C). Ozone inhalation and pulmonary eosinophil recruitment have been previously correlated; it has been demonstrated that acute inhalation of ozone alone can promote significant airway eosinophil accumulation in the resus monkey, independent of allergen exposure (Hyde et al., 1992). Further, several rodent models of ozone inhalation have reported enhanced mRNA expression of eotaxin, a potent eosinophil chemokine (Ishi et al., 1998; Michelson et al., 1999). The relevance of our findings in the infant monkey is highlighted by the recent observation that in human subjects with mild allergic asthma, ozone inhalation can potentiate the eosinophilic inflammation induced by allergen challenge (Vagaggini et al., 2002). Whether the observed ozone-induced transmigration of eosinophils is associated with their increased activation and release of granular contents is unknown, but the observed increase in airways reactivity would be consistent with this possibility.

Results from this study indicate that allergen sensitization and challenge in combination with repeated cyclic inhalation of ozone alters not only the immune response of the airways but their structural development as well. At birth, the primate respiratory tract undergoes a transition from a relatively dormant developing organ to a functional gas exchange system that is in a stage of rapid growth. This stage of rapid growth (alveolar stage of lung development) lasts up to 8–10 years of age in the human and approximately 3 years in the rhesus monkey and is characterized by the extension of the alveolar ducts and the formation of alveoli with further thinning of type 1 epithelium. During this period the epithelium, smooth muscle, and neural components of the conducting airways further differentiate toward an adult phenotype. Growing evidence supports the notion that successful morphogenesis of the airway postna-

tally is dependent on a complex interaction among differentiating airway epithelium, smooth muscle, nerves and ganglia, and the mesenchymal extracellular matrix (McGowan, 1992). This interaction is characterized by the release of numerous growth factors from the cellular components that in turn determine the composition of the extracellular matrix that in turn influences cellular differentiation. The observation in the present study of a marked reduction in the ratio of the volume of airways with diameters greater than 400 μm to volume of lung parenchyma in HDMA sensitized monkeys that inhaled HDMA and ozone suggests that the resulting pattern of repeated injury and inflammation associated with this regimen of exposure alters the profile of growth factors at critical airway sites important for normal airway morphogenesis. The importance of this altered airway morphogenesis for airway function is supported by the significant negative correlation (-0.795 , $P = 0.0011$) between baseline airway resistance and the ratio of conducting airway volume to volume of lung parenchyma. This relationship is important since it occurs without a significant alteration in more proximal conducting airway (generations 3 and 7) diameters and suggests a marked increase in the resistance of more distal airways. This increased resistance of distal airways may also be partially the result of increased mucous secretion within these airways. This possibility is supported by the observed increase in the volume of mucous cells within the terminal bronchioles of HDMA-sensitized monkeys that inhaled HDMA and ozone (Fig. 6B).

In conclusion, the results of these studies to date serve to illustrate the need to better understand the interaction of ozone and inhaled allergen in infants and how these two challenges to the respiratory tract alter normal lung development. Of particular importance is determining the critical time points and airway sites for postnatal airway morphogenesis and how allergen in combination with ozone inhalation alters the profile of growth factors at these sites. Also of critical importance is defining the mechanisms that are responsible for the observed synergism of cyclic ozone inhalation and allergen sensitization and challenge on immune and functional responses of the developing lung.

Acknowledgments

The authors thank Laurie Brignolo, D.V.M., Sarah Davis, and Bruce Rodello of the California National Primate Research Center for their clinical and organizing expertise. The authors also thank Jessica Arturus, Lei Putney, Jodie Usachenko, Nancy Tyler, Collette Brown, Carmen Ip, and Amanda Omlar for their technical assistance in data collection.

Supported in part by NIEHS P01 ES00628 and NCRR RR00169.

References

- Abbas, A.K., Lichtman, A.H., Pober, J.S., 1991. Cellular and Molecular Immunology. W. B. Saunders Company, Philadelphia.
- Akinbami, L.J., Schoendorf, K.C., 2002. Trends in childhood asthma: prevalence, health care utilization, and mortality. *Pediatrics* 110, 315–322.
- Barr, B.C., Hyde, D.M., Plopper, C.G., Dungworth, D.L., 1990. A comparison of terminal airway remodeling in chronic daily versus episodic ozone exposure. *Tox. Appl. Pharm.* 106, 384–407.
- Biagini, R.E., Moorman, W.J., Lewis, T.R., Bernstein, I.L., 1986. Ozone enhancement of platinum asthma in a primate model. *Am. Rev. Respir. Dis.* 134, 719–725.
- Bjorksten, B., 1999. The environmental influence on childhood asthma. *Allergy* 54, 17–23.
- Calderon-Garciduenas, L., Mora-Tiscareno, A., Chung, C.J., Valencia, G., Fordham, L.A., Garcia, R., Osnaya, N., Romero, L., Acuna, H., Villarreal-Calderon, A., Devlin, R.B., Koren, H.S., 2000. Exposure to air pollution is associated with lung hyperinflation in healthy children and adolescents in Southwest Mexico City: a pilot study. *Inhal. Toxicol.* 12, 537–561.
- Chang, M.M., Wu, R., Plopper, C.G., Hyde, D.M., 1998. IL-8 is one of the major chemokines produced by monkey airway epithelium after ozone-induced injury. *Am. J. Physiol.* 275, L524–L532.
- Frischer, T., Studnicka, M., Gartner, C., Tauber, E., Horak, F., Veiter, A., Spengler, J., Kuhr, J., Urbanek, R., 1999. Lung function growth and ambient ozone: a three-year population study in school children. *Am. J. Respir. Crit. Care Med.* 160, 390–396.
- Galizia, A., Kinney, P.L., 1999. Long-term residence in areas of high ozone: associations with respiratory health in a nationwide sample of nonsmoking young adults. *Environ. Health Perspect.* 107, 675–679.
- Gauderman, W.J., McConnell, R., Gilliland, F., London, S., Thomas, D., Avol, E., Vora, H., Berhane, K., Rappaport, E.B., Lurmann, F., Margolis, H.G., Peters, J., 2000. Association between air pollution and lung function growth in southern California children. *Am. J. Respir. Crit. Care Med.* 162, 1383–1390.
- Gershwin, L.J., Osebold, J.W., Zee, Y.C., 1981. Immunoglobulin E-containing cells in mouse lung following allergen inhalation and ozone exposure. *Int. Arch. Allergy Appl. Immunol.* 65, 266–277.
- Gilmour, M.I., 1995. Interaction of air pollutants and pulmonary allergic responses in experimental animals. *Toxicol. Lett.* 105, 335–342.
- Hamada, K., Goldsmith, C.A., Goldman, A., Kobzik, L., 2000. Resistance of very young mice to inhaled allergen sensitization is overcome by coexposure to an air-pollutant aerosol. *Am. J. Respir. Crit. Care Med.* 161, 1285–1293.
- Howard, C., Reed, M., 1998. *Unbiased Stereology*. Springer-Verlag, New York.
- Hyde, D.M., Hubbard, W.C., Wong, V., Wu, R., Pinkerton, K., Plopper, C.G., 1992. Ozone-induced acute tracheobronchial epithelial injury: relationship to granulocyte emigration in the lung. *Am. J. Respir. Cell Mol. Biol.* 6, 481–497.
- Inamura, H., Kurosawa, M., Okano, A., Kayaba, H., Majima, M., 2002. Expression of the interleukin-8 receptors CXCR1 and CXCR2 on cord-blood-derived cultured human mast cells. *Int. Arch. Allergy. Immunol.* 128, 142–150.
- Ishi, Y., Shirato, M., Nomura, A., Sakamoto, T., Uchida, Y., Ohtsuka, M., Sagai, M., Hasegawa, S., 1998. Cloning of rat eotaxin: ozone inhalation increases mRNA and protein expression in lungs of brown Norway rats. *Am. J. Physiol.* 274, L171–L176.
- Lemanske Jr., R.F., 2002. Issues in understanding pediatric asthma: epidemiology and genetics. *J. Allergy Clin. Immunol.* 109, S521–S524.
- Madwed, J.B., Jackson, A.C., 1997. Determination of airway and tissue resistance after antigen and methacholine in nonhuman primates. *J. Appl. Physiol.* 83, 1690–1696.
- McGowan, S.E., 1992. Extracellular matrix and the regulation of lung development and repair. *FASEB J.* 6, 2895–2904.
- Michelton, P.H., Dailey, L., Devlin, R.B., Peden, D.B., 1999. Ozone effects on the immediate-phase response to allergen in the nasal airways of allergic asthmatic subjects. *Otolaryngol. Head Neck Surg.* 120, 225–232.
- Neter, J., Wasserman, W., Kutner, M., 1985. *Applied Linear Statistical Models*. Richard D. Irwin, Inc., Homewood, IL.
- Neuhaus-Steinmetz, U., Uffhausen, F., Herz, U., Renz, H., 2000. Priming of allergenic immune response by repeated ozone exposure in mice. *Am. J. Respir. Cell Mol. Biol.* 23, 228–233.
- Newacheck, P.W., Halfon, N., 2000. Prevalence, impact, and trends in childhood disability due to asthma. *Arch. Pediatr. Adolesc. Med.* 154, 287–293.
- Peters, J.M., Avol, E., Gauderman, J., Linn, W.S., Navidi, W., London, S.J., Margolis, H., Rappaport, E., Vora, H., Gong, H., Thomas, D.C., 1999. A study of twelve southern California communities with differing levels and types of air pollution. II. Effects on pulmonary function. *Am. J. Respir. Crit. Care Med.* 159, 768–775.
- Schelegle, E.S., Gershwin, L.J., Miller, L.A., Fanucchi, M.V., Van Winkle, L.S., Gerriets, J.P., Walby, W.F., Omlor, A.M., Buckpitt, A.R., Tarkington, B.K., Wong, V.J., Joad, J.P., Pinkerton, K.B., Wu, R., Evans, M.J., Hyde, D.M., Plopper, C.G., 2001. Allergic asthma induced in rhesus monkeys by house dust mite (*Dermatophagoides farinae*). *Am. J. Pathol.* 158, 333–341.
- Schelegle, E.S., Walby, W.F., Alfaro, M.F., Wong, V.J., Putney, L., Stovall, M.Y., Sterner-Koch, A., Hyde, D.M., Plopper, C.G., 2003. Repeated episodes of ozone inhalation attenuates airway injury/repair and release of substance P, but not adaptation. *Tox. Appl. Pharmacol.* 186, 127–142.
- Scherle, W.F., 1970. A simple method of volumetry of organs in quantitative stereology. *Mikroskopie* 26, 57–60.
- Schierhorn, K., Zhang, M., Matthias, C., Kunkel, G., 1999. Influence of ozone and nitrogen dioxide on histamine and interleukin formation in a human nasal mucosa culture system. *Am. J. Respir. Cell. Mol. Biol.* 20, 1013–1019.
- Scott, D., Miller, W., Griffin, C., 1995. Skin immune system and allergic skin, in: Muller and Kirk's Small Animal Dermatology, Saunders, W.B. Philadelphia, p. 509.
- Shore, P.A., Burkhalter, A., Cohn, V.H., 1959. A method for the fluorometric assay of histamine in tissue. *J. Pharmacol. Exp. Ther.* 127, 182–186.
- Smyth, R.L., 2002. Asthma: a major pediatric health issue. *Respir. Res.* 3 (Suppl 1), S3–S7.
- Tyler, W.S., Tyler, N.K., Last, J.A., Gillespie, M.J., Barstow, T.J., 1988. Comparison of daily and seasonal exposures of young monkeys to ozone. *Toxicology* 50, 131–144.
- Vagaggini, B., Taccola, M., Cianchetti, S., Carnevali, S., Bartoli, M.L., Bacci, E., Dente, F.L., Di Franco, A., Giannini, D., Paggiaro, P.L., 2002. Ozone exposure increases eosinophilic airway response induced by previous allergen challenge. *Am. J. Respir. Crit. Care Med.* 166, 1073–1077.
- Weitzman, M., Gortmaker, S.L., Sobol, A.M., Perrin, J.M., 1992. Recent trends in the prevalence and severity of childhood asthma. *J. Am. Med. Assoc.* 268, 2673–2677.
- Wilson, D.W., Plopper, C.G., Dungworth, D.L., 1984. The response of the macaque tracheobronchial epithelium to acute ozone injury. *Am. J. Pathol.* 116, 193.
- Yakabi, K., Arimura, T., Koyanagi, M., Uehigashi, Y., Ro, S., Minagawa, Y., Nakamura, T., 2002. Effects of interleukin-8 and Helicobacter pylori on histamine release from isolated canine gastric mucosal mast cells. *J. Gastroenterol.* 37, 10–16.