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Effects of Nitric Acid Vapor and Ozone on the Response to Inhaled Allergen in Allergic Asthmatic Subjects

CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY
AIR RESOURCES BOARD
Research Division

EFFECTS OF NITRIC ACID VAPOR AND OZONE ON THE RESPONSE TO INHALED ALLERGEN IN ALLERGIC ASTHMATIC SUBJECTS

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ABSTRACT

Although epidemiologic data suggest that people with asthma are at increased risk of exacerbations due to elevated ozone levels, controlled human exposure studies have not consistantly shown asthmatic subjects to be more sensitive to ozone. A possible explanation for this discrepancy may be that ozone increases the response to the natural triggers of an asthma attack. Other factors, such as the presence of acidic pollutants, may also influence the respiratory effects of ozone. This study investigates whether exposure to ozone or the combination of ozone and nitric acid vapor enhances the response to inhaled allergen in allergic asthmatic subjects. In phase I of our study, 14 asthmatic subjects were exposed to 0.2 ppm ozone vs. filtered air for 1 h while exercising with a minute ventilation of 25 L/min/m^2 body surface area. Phase II consisted of 10 subjects exposed to 0.2 ppm ozone combined with 150 $\mu g/m^3$ nitric acid vapor vs. 0.2 ppm ozone for one hour under similar conditions. The ozone concentration used is similar to high ambient levels known to occur in some southern Californian urban centers, and although much higher than ambient conditions, a nitric acid vapor level of 150 $\mu g/m^3$ was chosen to give definitive results as to whether its presence could effect the response to ozone. Following the 1 h exposure, subjects were challenged with doubling doses of dust mite D. Farinae allergen by inhalation until a drop in FEV1 of >15% occurred (PC_{15}). At 6 h post-allergen challenge, bronchoscopy with bronchoalveolar lavage (BAL), left mainstem proximal airway lavage, and endobronchial biopsy and brushings were done. The same subjects were restudied with the second exposure condition after ≥ 4 weeks. Although individual PC_{15} data for phase I revealed a trend toward an increase in sensitivity to inhaled allergen after ozone exposure compared to air, the results for the group as a whole did not reach statistical significance ($p=0.42$, sign-rank). Bronchoscopy during the late-phase response did show a near significant increase in neutrophils in proximal airway lavage fluid after ozone exposure ($p=0.06$, sign-rank), but no increase in markers of inflammation in BAL fluid. In phase II, no significant difference was seen in the response to allergen between the ozone/nitric acid vapor vs. ozone alone exposures ($p=0.11$, sign-rank). A significant increase in eosinophils ($p=0.02$, sign-rank) was seen in the bronchial fraction fluid after the combination exposure. In conclusion, the final results of this study can not confirm the hypothesis that exposure to ozone or the combination of ozone and nitric acid vapor significantly enhances the response to inhaled allergen in allergic asthmatic subjects.

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DISCLAIMER

The statements and conclusions in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their sources, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products.

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SUMMARY

Results from this study do not confirm the hypothesis that exposure to ozone or the combination of ozone and nitric acid vapor significantly enhances the response to inhaled allergen in allergic asthmatic subjects.

CONCLUSIONS AND SIGNIFICANCE

Although the results of this study revealed a non-significant trend toward an enhancing effect of ozone on the early bronchoconstrictor response to allergen, as well as some evidence of an enhancement of proximal airway inflammation during the late-phase response, there was no evidence of a dramatic effect of ozone likely to cause asthma exacerbations. Our data complement recent findings of a study completed by the U.S. Environmental Protection Agency which also found no evidence that ozone pre-exposure enhances the early bronchoconstrictor response to allergen. Our work is the first to look at the effect of ozone on the late-phase inflammatory response to allergen. The results of these two studies are contradictory to those of two earlier studies which did find an enhancing effect of ozone exposure on the early bronchoconstrictor response to allergen. All of these studies are substantially limited by the small number of subjects studied. Therefore, based on a summary analysis of the known literature, no conclusive comments can be made regarding the effects of ozone on the allergen response in allergic asthmatics.

This investigation is the only study of which we are aware that has looked at the effect of combined exposure to ozone and an acidic pollutant on the subsequent response to allergen. Although there was some evidence for an enhanced late phase inflammatory effect, no significant difference between the effects of ozone alone versus ozone plus nitric acid vapor was seen on the early bronchocondtrictor response to ozone. Thus, the results of this phase of our study also do not support the hypothesis that exposure to ozone or the combination of ozone and nitric acid vapor significantly enhances the response to inhaled allergen in allergic asthmatic subjects.

RECOMMENDATIONS

Our results, taken together with the results of other reported studies, suggest that the issue of whether ozone or ozone/nitric acid vapor increases the response to allergen in allergic asthmatic subjects is not resolved. Therefore, further research incorporating a greater number of subjects with more severe asthma, or the use of higher exposure doses of ozone, may help to clarify the role of ozone exposure in the response to allergen in asthma.

INTRODUCTION

The Clean Air Act mandates that the National Ambient Air Quality Standard for ozone, a major component of urban smog, provides adequate protection for even those subgroups of the general population who may be more susceptible to its detrimental effects. Although epidemiologic data suggest that people with asthma are at increased risk of exacerbations due to elevated ozone levels (1, 2), controlled human exposure studies have not consistently shown asthmatics to be more sensitive to ozone (3, 4). A possible explanation for this discrepancy may be that ozone increases the response to the natural triggers of an asthma attack. Other factors, such as the presence of acidic pollutants, may also influence the respiratory effects of ozone. The purpose of this study was to investigate whether ozone or the combination of ozone and nitric acid vapor enhances the effects of inhaled allergen in allergic asthmatic subjects.

Although conflicting data exist (5 - 7), some animal toxicologic studies have suggested that ozone may exacerbate asthma by acting to increase sensitivity to inhaled allergen (8 - 12). A study by Molfino et al. (13) found similar results in allergic asthmatic human subjects in which exposure to 0.12 ppm ozone x 1 h increased the sensitivity to inhaled allergen as measured by spirometry during the early response to allergen. Data from our own laboratory have suggested that spirometric parameters of lung function do not necessarily reflect the inflammatory effects of ozone in the respiratory tract (14), and it is also known that the degree of airway inflammation is an important predictor of the severity of asthma (15). Therefore, phase I of our study investigates whether ozone enhances the effects of inhaled allergen on airway inflammation during the late-phase response, as well as on the immediate bronchoconstriction of the early response to allergen in allergic asthmatic subjects.

A second factor which may influence the respiratory effects of ozone is the presence of co-pollutants. Nitric acid, a common co-pollutant in west coast urban smog, has not been shown to impair pulmonary function (16) or produce acute respiratory tract injury (17) in studies of normal, healthy subjects. However, epidemiologic studies have associated an increased risk of asthmatic exacerbations to the presence of acidic pollution (18, 19), and animal toxicologic data suggest that co-exposure to acidic pollutants and ozone can increase ozone-induced lung injury

(20 - 24). Phase II of our study investigates whether the combination of ozone with nitric acid vapor increases the effects of ozone on the response to inhaled allergen in allergic asthmatic subjects.

Pollutant exposures were done using a high ambient concentration of ozone (0.2 ppm) which is known to occur often in southern California urban smog. A concentration of nitric acid vapor (150 $\mu g/m^3$) that is several-fold higher than known ambient conditions was used in order to give definitive results as to whether its presence could effect the response to ozone.

METHODS

Subjects

A total of 44 atopic asthmatic subjects, ages 18-35, were recruited into the study. Fourteen subjects completed phase I (air vs. ozone exposure) and ten completed phase II (ozone vs. ozone/nitric acid vapor exposure). Nine of the subjects participated in both phases. Those subjects not completing the study either did not meet inclusion criteria or chose not to finish. Recruitment was done through letter solicitation to allergic asthmatic patients followed at the University of California, San Francisco, Allergy/Immunology Clinic and through general advertisement. All subjects had a documented physician diagnosis of asthma or met the NIH-sponsored National Asthma Education Program guidelines (15) for the diagnosis of asthma. Subjects were confirmed atopic by allergen skin-prick testing, had a positive sensitivity to dust mite Dermatophygoides farinae (DF), were non-smokers (<1 pack-year, no smoking within the last 6 months), and had no medical contraindications to bronchoscopy. All subjects received financial compensation for their participation. Further subject characteristics are listed in table 1. Predicted values for the spirometric parameters are those of Knudson and coworkers (25).

Experimental Protocol

Initial screening. A brief telephone interview with potential subjects was used to confirm a history of asthma and exclude smokers. Because of the complexity of the

study and to facilitate recruitment, each potential subject was invited for an initial visit to allow an onsite explanation of the study protocol and equipment. Subjects were informed of the risks of the study protocol and those enrolled signed consent forms approved by the Committee on Human Research of the University of California, San Francisco. Allergen skin-prick testing to verify atopic status and sensitivity to the allergen dust mite DF was done on enrolled subjects.

Baseline testing. Baseline pulmonary function tests, which included forced expired volume in 1 sec (FEV_1), forced vital capacity (FVC), specific airway resistance (SRaw), and methacholine challenge, were done on a subsequent visit. A 15-minute exercise test on a cycle ergometer (No. 18070, Gould Godart, Bilthoven, The Netherlands) or treadmill (Model M9.1, Precor Co., Bothell, WA) was used to determine the appropriate workload necessary to achieve a target ventilatory rate of 25 $L/min/m^2$ body surface area. To screen out asthmatic subjects with significant exercise-induced symptoms, subjects who had a greater than 15% decrease in FEV_1 from baseline after exercise were dropped from the study.

No subject took inhaled beta-adrenergic agonists within 6 h; nonsteroidal anti-inflammatory agents within 24 h; sodium cromolyn, theophylline, or antihistamines within 48 h; or inhaled, oral, or injectable steroids within 8 wk of any testing. No caffeinated beverages or chocolate were allowed for 4 h before or anytime during testing. Subjects reported no symptoms of respiratory infection or asthma exacerbations for at least one month prior to any tests. FEV_1 at the start of each new study day was required to be within 10% of baseline in order to proceed.

Study exposures. At least three days after methacholine challenge, subjects were exposed in a random, double-blind fashion to 0.20 ppm ozone versus filtered air for phase I, or 0.20 ppm ozone versus 0.20 ppm ozone combined with 150 $\mu g/m^3$ nitric acid vapor for phase II, in a monitored exposure chamber. The exposures were 1 h in length during which each subject exercised on a cycle ergometer and/or treadmill at the pre-determined workload. The tidal volume and respiratory rate were measured with a pneumotachograph (No. 3, A. Fleisch, Lausanne, Switzerland) four times during each 1-h exercise period to calculate ventilatory rate and allow adjustment of the workload as needed to maintain the target value. SRaw, FEV_1, and FVC were measured immediately before and after

each exposure. A symptom questionnaire consisting of a 5-point rating scale for each of 13 symptoms (chest pain on inspiration, chest tightness, shortness of breath, cough, sputum production, throat irritation, wheezing, nasal irritation, eye irritation, back pain, headache, nausea, and anxiety) was self-administered immediately before and after each exposure.

One-half hour after the end of each exposure, subjects underwent allergen challenge. Allergen was administered in doubling doses until a 15% decrease in FEV_1 from the post-diluent baseline was measured (PC_{15}). FEV_1 was measured each hour for 5 h from the time of the last allergen dose. At 6 h after the final dose of allergen, fiberoptic bronchoscopy was performed. Vital signs and peak flow measurements were monitored overnight at the General Clinical Research Center of San Francisco General Hospital.

Subjects returned after at least four weeks for the second exposure condition, allergen challenge, and bronchoscopy using the same protocol.

Pulmonary Function Measurements

Spirometry was performed on a dry, rolling seal spirometer (S400, Spirotech Division, Anderson Instruments, Inc., Atlanta, GA). During each visit, the daily baseline FEV_1 and FVC were calculated from the three best of six FVC maneuvers (two sets of three maneuvers, 5 min between sets) to minimize the effect of first-time spirometry variability. Thereafter, mean values for FEV_1 and FVC were calculated from three acceptable FVC maneuvers (26) obtained approximately 30 sec apart. SRaw was determined as the product of airway resistance and thoracic gas volume, both calculated from the average of 5 measurements taken 30 sec apart in a constant-volume body plethysmograph (Warren E. Collins, Braintree, MA).

Methacholine responsiveness was tested by measuring the subject's FEV_1 before and after five 6 sec inhalations of phosphate-buffered saline combined with doubling concentrations of methacholine (0.16, 0.31, 0.63, 1.25, 2.5, 5 , 10, 20, 40, 80, 160, 320 mg/ml) delivered by a nebulizer (No. 646; Devilbiss Co., Somerset, PA) with a dose-metering device (Rosenthal dosimeter, Laboratory for Applied

Immunology Inc., Fairfax, VA) calibrated to deliver 0.01 ml/breath. The concentration of methacholine (PC_{20}) that produced a 20% decrease in FEV_1 from the post-saline baseline was calculated by log-linear interpolation.

Exposure Chamber and Atmospheric Monitoring

All exposures took place in a 2.5 m x 2.5 m x 2.4 m steel and glass chamber (Model W00327-3R, Nor-Lake, Inc., Hudson, WI) filled with filtered air at 20° C and 50% relative humidity to which ozone or ozone/nitric acid vapor were added. The custom-built chamber was designed to maintain temperature and relative humidity within 1.0°C and 2% of the chosen set points, respectively (DSC 8500, Johnson Controls, Poteau, OK). The exposure chamber, and air filtration, humidification and conditioning systems have been previously described in detail (17). Relative humidity and temperature, were recorded every 30-sec., displayed in real-time (LabVIEW 2, National Instruments, Austin, TX) and stored by a microcomputer (Model IIsi, Apple Computer Inc., Cupertino, CA), for the duration of the exposure. Humidity and temperature means were similar for the air (20.5 ± 0.6°C and 49.9 ± 1.7%), ozone (20.3 ± 0.5°C and 50.2 ± 0.9%), and ozone/nitric acid vapor exposures (20.3 ± 0.4°C and 49.7 ± 0.6%).

Ozone was produced with a corona-discharge ozone generator (Model T 408, Polymetrics, Inc., San Jose, CA) and analyzed with an ultraviolet light photometer (Model 1008 PC, Dasibi, Glendale, CA). The ozone concentration, which was measured every 3 min, displayed in real-time and stored by microcomputer, averaged 0.19 ppm, 0.00 ppm, and 0.20 ppm for the ozone, air, and ozone/nitric acid vapor exposures, respectively. The ozone analyzer was calibrated biannually by the California Air Resources Board with a standard ozone generator/analyzer instrument (Model 1009 IC, Dasibi) and precision-checked in the laboratory on a monthly basis.

Nitric acid gas was generated by flash vaporization of a 3% HNO_3 solution using an infusion pump (Harvard Apparatus Co., Millis, MA) to instill the solution into a 1000 ml round-bottom flask maintained at 200° C by a heating mantle. Nitric acid gas was transferred to the exposure chamber via the excurrent port of the generation flask through a 1-m length teflon tube by providing a driving pressure

of 4.7 L/min with compressed air at the incurrent port of the flask. The infusion pump was adjusted as needed to achieve the target concentration of 150 $\mu g/m^3$. Nitric acid concentration was monitored at the 15, 30, 45, and 55-min points of each 1-h exposure by drawing chamber air at 13.6 L/min for 4 min through a 47-mm nylon filter (1-μm pore size, Gelman Sciences, Ann Arbor, MI) contained in an open-faced cassette, which was positioned within 1 m of the subject's face. Nitrate, eluted from each filter with 4 ml of buffered extraction fluid, was measured with high performance ion chromatography, using a separator column (IonPac A 54A, P/N 37041, Dionex, Sunnyvale, CA), a 2.5 ml/min flow rate, an eluent of 5.6 mM sodium bicarbonate and 4.8 mM sodium carbonate, and a 0.25 mM sulfuric acid regenerant. The nitric acid vapor concentration averaged 0.162 $\mu g/m^3$ during the ozone/nitric acid vapor exposures.

Skin-prick test and Allergen Challenge

Skin-prick testing was done using nine common west coast aeroallergens (Port Orford cedar, Monterey cypress, olive tree, English plantain, standardized dust mite DF, standardized dust mite DP, standardized cat pelt, perennial ryegrass, mugwort sage; Miles/Hollister-Stier, Westhaven, CT), lyophilized dust mite DF (lot XPB64-D5-15.9, Greer Laboratories Inc., Lenoir, NC), and controls (normal saline/50% glycerin, Miles/Hollister-Stier; and histamine, Allermed, San Diego, CA) applied to the volar forearm surface. Atopy was defined by a > 2 x 2 mm skin wheal response to at least one of the nine allergens. On the opposite forearm, a dilutional skin-prick test was done using log concentrations (0.1 AU to 1000 AU) of the lyophilized dust mite DF.

The starting dose for each allergen challenge was determined using a formula described by Cockcroft et al (27) which predicts the concentration of allergen necessary to cause a 20% decrease in FEV_1 based on the results of the dilutional allergen skin-prick test and methacholine PC_{20} for each subject. The starting dose of allergen used was four doubling doses below the Cockcroft predicted value. Allergen inhalation was done with a nebulizer attached to a dose-metering device calibrated to deliver 0.01 ml/breath as described for the methacholine challenge. A set of five 6 sec inhalations of phosphate-buffered saline combined with doubling concentrations of reconstituted lyophilized dust mite DF were

administered every 10 min until a 15% drop in FEV_1 was reached. If the drop in FEV_1 was between 10 to 15% of baseline, the next allergen dose was held for an additional 10 min and spirometry retested before proceeding to a higher dose. The starting concentration of allergen was identical for each subject on the two different exposure days.

Bronchoscopy, Lavage and Biopsy Procedures

Bronchoscopy was performed in a dedicated suite at San Francisco General Hospital. The procedure of bronchoscopy, and proximal airway and bronchoalveolar lavage were done as described in detail previously (17) with minor alterations. Briefly, intravenous access was established, supplemental oxygen was delivered via nasal canula, and the upper airways were anesthetized with topical lidocaine spray and 4% cocaine-soaked (1 cc) cotton pledgets. Midazolam was given intravenously, and the dose titrated to maintain subject comfort. All subjects received intravenous atropine (0.4 mg) as a standard premedication. The bronchoscope (FB 18x, Pentax Precision Instruments Corp., Orangeburg, NY) was introduced through the mouth and the flow of supplemental oxygen was increased during proximal airway lavage (PAL) to 10 L/min to prevent the transient desaturation that may be seen with left mainstem bronchus occlusion. A custom-designed, 6-French, double-balloon, double-port catheter (Baxter Healthcare Corp., Irvine, CA), with a 1.5-cm inter-balloon distance, was positioned in the left mainstem bronchus by inflating the proximal balloon at the level of the carina and the distal balloon superior to the left upper lobe orifice. PAL was done using 12 ml (8 aliquots of 1.5 ml) of warmed saline. Bronchoalveolar lavage (BAL) was obtained from the right middle lobe with three 50-ml aliquots of warmed saline. The first 15 ml of lavage fluid retrieved was labeled bronchial fraction. All lavage samples were immediately put on ice. Endobronchial brushings were done in the left upper lobe orifice using a 3-mm diameter cytology brush (Millrose Labs, Mentor, OH). Five brush strokes each were taken at two separate mucosal sites. Five endobronchial biopsy specimens were then obtained from the right upper lobe carina using a spiked, cup-forceps (2.4 mm diameter, Pentax Precision Instrument Corporation, Orangeburg, NY). Biopsy and brush specimens were done on opposite upper lobe sites during subsequent bronchoscopies.

Measurement of Cells and Biochemical Constituents of Lavage Fluids

Total cells were counted in unspun aliquots of PAL and BAL fluids using a hemacytometer. Differential cell counts were performed on slides prepared with a cytocentrifuge (Cytospin 2, Shandon Southern Products, Ltd., Astmoor, UK; 200 g x 5 min) and stained in Diff-Quik (American Scientific Products, McGaw Park, IL) as previously described (17). PAL and BAL fluids were immediately centrifuged at 200 g for 15 min, and the supernatant was separated and re-centrifuged at 3000 g for 15 min to remove any cellular debris.

Cellular and biochemical constituents were measured in PAL, bronchial fraction, and BAL specimens. Lactate dehydrogenase (LDH) was measured within 30 min after bronchoscopy with a commercially available kit (#228-10, Sigma Chemical Co., St. Louis, MO) and a spectrophotometer (DU 65, Beckman Instruments Inc., Fullerton, CA). The other biochemical studies were performed on lavage supernatants which had been frozen at -70° C. Total protein was assayed by a modification of the Lowry procedure (28). Lavage concentrations of fibronectin were determined with an antibody-capture immunoassay, as described by Miles and Hales (29) with minor modifications (17). Interleukins 6 and 8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and transforming growth factors β1 and β2 (TGFβ1, TGFβ2) levels were analyzed using commercially available immunoassays (R&D Systems, Minneapolis, MN). Commercially available kits were also used for measuring eosinophil cationic protein and mast cell tryptase (KABI Pharmacia Diagnostics, Piscataway, NJ), and neutrophil myeloperoxidase (Cayman Chemical, Ann Arbor, MI).

Bronchial Biopsy Immunohistochemistry and Histology

Four biopsy specimens were immediately placed in 20% sucrose and stored for 2 h at 4°C. These biopsies were embedded in OCT compound (Miles Inc., Elkhart, IN) and allowed to equilibrate for 5-10 min. The OCT molds were then placed in isopentane (Fisher Scientific, Santa Clara, CA) and snap frozen using liquid

nitrogen. Embedded biopsies were stored in liquid nitrogen until further processing.

The fifth biopsy was embedded in plastic (glycol methacrylate) for histology. Initially, the biopsy was immediately placed in acetone and allowed to incubate at -20°C for 4 to 24 h. After incubation, the biopsy was placed in methyl benzoate for 15 minutes, followed by a basic resin and benzoyl peroxide infiltrating solution for 3-4 h, and then incubated in hardening solution for 48 h at 4°C (Historesin Embedding kit, Reichert Jung, Germany). Plastic embedded biopsies were stored at -20°C.

Frozen tissues were sectioned at 5 μm and stained using immunohistochemical techniques described in detail previously (30). Histologic evaluation was done on 2 μm sections of the plastic embedded endobronchial biopsies using hemotoxylin-eosin stain. For each section of tissue, the numbers of eosinophils were counted in several non-overlapping high power fields until all the available areas of the submucosa were examined. Results were expressed as number of eosinophils per square mm. To avoid reader bias, both frozen and plastic embedded slides were coded and read in blinded-fashion.

Statistical Analysis

The comparisons of PC_{15} values, baseline and across-exposure spirometry, lavage fluid cellular and biochemical endpoints, and tissue eosinophil counts were done using paired t-tests and Wilcoxon signed-rank tests as appropriate for normally and non-normally distributed results. Group data were presented as the median and range values or the mean and standard deviation of the mean (mean ± SD). Correlations between data were made using both Pearson and Spearman correlation tests. Differences or correlations with a $p \leq 0.05$ were accepted as statistically significant.

RESULTS

Phase I: Air vs. Ozone

Exposure to ozone prior to allergen challenge did result in median PC_{15} values (24 AU/ml, range 3 - 723; AU = allergen units) lower than those measured after air exposure (31 AU/ml, range 3 - 3276) in the 14 asthmatic subjects tested (figure 1). However, these findings were not statistically significantly (p=0.42, sign-rank). The difference between the PC_{15} levels from the air and ozone days for each individual did not correlate with the FEV_1 response to ozone exposure. Nor was a correlation found between the difference in PC_{15} between the two exposure conditions and the level of exercise-induced bronchoconstriction during the 1-h air exposure, or the level of baseline non-specific airway responsiveness as measured by methacholine challenge (PC_{20}).

A spirometric late-phase response, defined as a >15% drop in FEV_1 during hours 3 to 5 after allergen, was seen in five subjects after air exposure compared to two subjects after ozone exposure. Only one subject had a late-phase response after both exposure conditions. A significantly greater drop in mean percent FEV_1 during the late phase occurred after the air exposure (12.4% ± 0.1) as compared to the ozone exposure (6.8% ± 0.1).

The starting baseline FEV_1 was not significantly different between air and ozone exposure days. Although 1 h of exercise during ozone exposure caused a mean decrease in FEV_1 of 10%, this decrease was not significantly different than the 4% decrease in FEV_1 due to exercise alone during the control air exposure. The mean percent changes in SRaw and FVC across the 1-h exposures also were not significantly different between the air and ozone days.

Bronchoscopy during the late-phase response did show a near significant increase in neutrophils in the PAL fluid after ozone exposure (p=0.06, sign-rank) (table 2). Pre-allergen exposure to ozone did not show a significant effect on lavage fluid eosinophil, lymphocyte, or total leukocyte cell counts.

Contrary to our hypothesis, pre-allergen exposure to ozone was associated with a significant decrease in LDH in bronchial fraction fluid (p = 0.03, sign-rank), and a

near significant decrease in BAL fluid ($p = 0.06$, sign-rank), compared to air exposure (table 3). No significant difference between the two exposure conditions was seen in lavage fluid total protein, fibronectin, GM-CSF, interleukins 6 and 8, TGFβ1, TGFβ2, or markers of inflammatory cell degranulation (i.e. eosinophil cationic protein, mast cell tryptase, or neutrophil myeloperoxidase).

Immunohistochemical staining of both endobronchial biopsy and brush specimens revealed no difference in the expression of adhesion molecules α2, α3, α5, α6, α9, β3, β4, β5, β6, ICAM, VCAM, or E-selectin between the air and ozone exposures (figures 2 - 4). No significant difference in the number of submucosal eosinophils were seen on histolgic evaluation of endobronchial biopsy specimens.

Phase II: Ozone vs. Ozone/Nitric acid vapor

No difference was measured ($p=0.11$, sign-rank) between the pre-allergen exposure to the combination of nitric acid vapor and ozone compared to ozone alone in the sensitivity to allergen as measured by allergen challenge PC_{15} (median 18 AU/ml, range 3 - 417, for ozone/nitric acid vapor combination; median 21 AU/ml, range 3 - 499, for ozone alone), in the ten allergic asthmatic subjects exposed to both conditions (figure 5).

None of the ten subjects displayed a physiologic late-phase response to allergen after exposure to the ozone/nitric acid vapor combination, while three of the ten did have a late-phase response after ozone alone. There was no significant difference in the percent drop in FEV_1 during the late phase between the two exposure conditions.

Starting baseline FEV_1 values and the mean percent change in SRaw, FEV_1, and FVC across the exposures were not significantly different between the ozone/nitric acid vapor and ozone alone days.

Lavage fluid results did show a significant increase in eosinophils ($p=0.02$, sign-rank) in the bronchial fraction after the ozone/nitric acid vapor exposure compared to ozone alone (table 2). No significant differences were seen in lavage neutrophil, lymphocyte, or total leukocyte cell counts. Conversely, total protein in PAL fluid trended toward an increase after the ozone alone exposure ($p = 0.07$,

sign-rank). LDH, fibronectin, GM-CSF, interleukins 6 and 8, TGFβ1, TGFβ2, eosinophil cationic protein, mast cell tryptase, and neutrophil myeloperoxidase measurements were not significantly different between the two exposures (table 3).

Endobronchial biopsy and brush specimens for the expression of the same adhesion molecules as described for phase I revealed no difference due to the addition of nitric acid vapor to ozone (figures 2 - 4). No significant difference was found in the number of submucosal eosinophils seen on histolgic evaluation of endobronchial biopsy specimens between the two exposure conditions.

DISCUSSION

The results of Phase I demonstrated no significant effect of ozone pre-exposure on the subsequent response to allergen in allergic subjects with mild asthma. While there was a slight trend toward an ozone effect to enhance the early bronchoconstrictor response to allergen, there was no evidence of an enhancing effect of ozone on the late inflammatory response as measured by cellular or biochemical endpoints in bronchoalveolar lavage fluid. Of some interest, however, there was some evidence of enhanced proximal airway inflammation during the late-phase response after ozone pre-exposure. Given that asthma is a disease characterized by airway rather than alveolar inflammation, this component of our results deserves note. This apparent effect of ozone pre-exposure on allergen-induced proximal airway inflammation may be due to chance since many lavage endpoints were analyzed and the issue of multiple comparisons must be raised.

The results of Phase II showed no significant difference between the effect of pre-exposure to the combination of ozone and nitric acid vapor on the subsequent response to allergen challenge compared to that of ozone alone. There was, however, some evidence of an enhanced inflammatory cell response after the combined exposure compared to ozone alone in the bronchial fraction of BAL, which reflects inflammation in the airways more than the alveoli.

There are several limitations to these studies that should be considered when interpreting the results. First, as is virtually always the case with controlled human exposure studies involving complicated protocols and multiple

bronchoscopies, the sample size for both Phase I and Phase II is relatively small. The trend toward an enhanced early bronchoconstrictor response with ozone in Phase I might have become significant with a larger sample size. Second, the effective ozone dose in both studies was relatively low. The concentration (0.2 ppm) and the duration (1 h) of exposure were such that most subjects did not have > 10% across-exposure decrements in FEV_1 when adjusted for their response to exercise in filtered air alone. A higher effective dose of ozone would have caused more airway injury and perhaps would have had greater influence on the subsequent response to allergen challenge. It should be noted, however, that the effective dose in our studies was greater than that administered in the Molfino et al. study (13) in which an enhancing effect of ozone on the early bronchoconstrictor response to allergen was observed. Third, since all of our subjects inhaled allergen following chamber exposures, a small effect of ozone or ozone/nitric acid vapor pre-exposure on the subsequent late-phase inflammatory response to allergen could have gone undetected in the background of allergen-induced inflammation. Finally, our protocol required subjects to be able to exercise for 1-h periods without suffering severe exercise-induced bronchoconstriction (i.e., a ≤ 15% decrease in FEV_1). Thus, we may have selected relatively mild asthmatic subjects. Perhaps subjects with more severe asthma should be studied in a protocol similar to ours, although it would be challenging to conduct such a study.

Even with the above-noted limitations, our results do not provide support for the hypothesis that ambient concentrations of ozone cause exacerbations of asthma by enhancing the response to inhaled allergen. There are also major limitations to the two previous studies that provide data in support of this hypothesis. The study by Molfino et al. (13) had an exceptionally small sample size (n=7) and a potential problem with the non-random order of exposure to air and ozone. Six of the seven subjects had a lower PC_{15} with allergen challenge after ozone pre-exposure as compared to that after air pre-exposure. Unfortunately all six of these subjects had air exposure followed by allergen challenge prior to ozone exposure followed by allergen challenge; in three of these subjects the interval between allergen exposures was ≤ 3 weeks. There is a well-described priming effect of a previous allergen challenge on the response to a subsequent challenge. Thus, the apparent enhancing effect of ozone on the early response in the Molfino et al. study may be due to the priming of the previous allergen challenge in that protocol. The second study to describe an enhanced response to allergen after ozone exposure was that

of Jorres et al. (the results of this study have only been reported in preliminary form) (31). A much higher effective dose of ozone was administered (0.25 ppm for 3 h by mouthpiece with intermittent exercise) than in our study and the allergen challenge was given at 3 h rather than immediately after the ozone exposure.

Recently, the U.S. Environmental Protection Agency attempted to replicate the Molfino et al. protocol with the exception that the order of exposures was random. These investigators reported no effect of ozone to enhance the early bronchoconstrictor response to inhaled allergen (32). They did not perform bronchoscopy to assess the effect of ozone pre-exposure on the late-phase inflammatory response.

Taken together, the four studies which have investigated the effect of ozone on the early bronchoconstrictor response to allergen yield totally contradictory results. The results of our Phase I study are the only data regarding the effect of ozone on the late inflammatory response to allergen. Although there was some evidence of an increased allergen-induced inflammatory cell response in the proximal airways after ozone pre-exposure, most of our late-phase results do not show an ozone effect. Our Phase II study is the only study of which we are aware that has looked at the effect of a combined exposure to ozone and an acidic pollutant on the subsequent response to allergen. Because we administered a concentration of nitric acid vapor (150 $\mu g/m^{3)}$ that is higher than that found even in southern California smog, the lack of a significant difference between the effect of the combined exposure as compared to ozone alone can be considered somewhat reassuring.

In conclusion, the results of the studies reported here do not confirm the hypothesis that exposure to ozone or the combination of ozone and nitric acid vapor significantly enhances the response to inhaled allergen in allergic asthmatic subjects. Further research will be required to determine why asthmatic individuals appear to be at increased risk of developing exacerbations of this disease with high ambient ozone levels.

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Chen LL, Scannell CH, Tager I, Christian DL, Kelly T, Ferrando R, Balmes JR. Nitric acid vapor does not enhance the effects of ozone in allergic asthmatic subjects exposed to inhaled antigen. Am J Respir and Crit Care Med 1995; 151 (4 part 2): A502

Table 1: SUBJECT CHARACTERISTICS

ID #	Age	Sex	FEV_1 * (L) (%predicted)	SR_{aw} * (LxcmH20/L/s)	Methacholine PC_{20} (mg/ml)
Phase I: Air vs. Ozone		**n = 14**			
A003	29	M	3.36 (83%)	4.7	0.49
A004	33	M	3.70 (86%)	4.8	0.72
A006	32	M	3.53 (82%)	2.7	1.95
A007	29	M	4.17 (104%)	4.3	7.62
A009	36	M	4.34 (114%)	2.3	3.94
A014	28	M	3.99 (85%)	6.2	3.24
A017	31	F	3.26 (99%)	4.3	0.18
A018	32	M	2.83 (72%)	7.8	0.24
A031	25	M	3.33 (85%)	8.7	0.43
A032	35	M	3.31 (78%)	1.6	0.35
A035	23	M	3.60 (88%)	7.8	0.60
A038	27	M	4.73 (105%)	2.7	0.90
A039	18	M	3.69 (90%)	3.8	0.97
A040	22	M	3.54 (88%)	4.1	1.40
Phase II: Ozone vs. Ozone/Nitric acid vapor		**n = 10**			
A003	29	M	3.36 (83%)	4.7	0.49
A006	32	M	3.53 (82%)	2.7	1.95
A009	36	M	4.34 (114%)	2.3	3.94
A014	28	M	3.99 (85%)	6.2	3.24
A017	31	F	3.26 (99%)	4.3	0.18
A018	32	M	2.83 (72%)	7.8	0.24
A032	35	M	3.31 (78%)	1.6	0.35
A034	23	F	3.69 (116%)	3.6	0.80
A038	27	M	4.73 (105%)	2.7	0.90
A039	18	M	3.69 (90%)	4.7	0.97

* Mean values

TABLE 2: LAVAGE CELLULAR ENDPOINTS *

	BAL		Bronchial Fraction		PAL	
PHASE I	**Air**	**Ozone**	**Air**	**Ozone**	**Air**	**Ozone**
Total Leukocytes †	21.8 (10.5)	19.6 (10.0)	21.1 (7.7)	17.8 (5.5)	2.5 (2.2)	5.0 (8.3)
Neutrophils (%)	3.5 (3.2)	4.8 (4.0)	8.9 (7.6)	8.4 (5.3)	21.1 (15.5)	29.3 (19.2)
Eosinophils (%)	4.5 (5.6)	2.1 (3.0)	9.5 (10.5)	2.9 (3.5)	16.5 (17.3)	13.2 (13.2)
Lymphocytes (%)	9.2 (12.4)	5.5 (3.2)	4.6 (2.7)	5.6 (3.2)	4.5 (2.0)	5.4 (4.5)
PHASE II	**Ozone**	**Ozone/Nitric Acid Vapor**	**Ozone**	**Ozone/Nitric Acid Vapor**	**Ozone**	**Ozone/Nitric Acid Vapor**
Total Leukocytes †	21.2 (9.3)	18.7 (7.2)	18.0 (5.4)	17.1 (7.9)	2.7 (2.5)	1.9 (1.2)
Neutrophils (%)	3.9 (1.7)	6.7 (6.6)	6.5 (4.6)	10.1 (6.8)	24.7 (20.4)	26.1 (20.4)
Eosinophils (%)	2.1 (3.5)	2.9 (5.0)	1.5 (1.3)	4.2 (3.3)	14.7 (11.7)	17.0 (15.6)
Lymphocytes (%)	5.8 (3.7)	5.9 (3.7)	6.1 (3.1)	6.0 (2.3)	2.6 (1.2)	2.2 (0.6)

* Mean values (standard deviation)

† ($x10^4$ cells/ml)

TABLE 3: LAVAGE TOTAL PROTEIN AND LDH *

	BAL		Bronchial Fraction		PAL	
PHASE I	**Air**	**Ozone**	**Air**	**Ozone**	**Air**	**Ozone**
Total Protein	0.12 (0.06)	0.12 (0.04)	0.16 (0.07)	0.15 (0.06)	0.08 (0.07)	0.16 (0.18)
LDH	10.74 (4.67)	6.91 (2.23)	18.69 (7.62)	12.78 (5.62)	10.80 (5.37)	14.36 (10.27)
PHASE II	**Ozone**	**Ozone/Nitric Acid Vapor**	**Ozone**	**Ozone/Nitric Acid Vapor**	**Ozone**	**Ozone/Nitric Acid Vapor**
Total Protein	0.14 (0.07)	0.13 (0.09)	0.16 (0.07)	0.20 (0.06)	0.15 (0.15)	0.06 (0.03)
LDH	8.99 (5.38)	12.26 (5.70)	14.27 (5.29)	19.34 (10.36)	12.49 (7.19)	8.89 (6.01)

* Mean values (standard deviation)

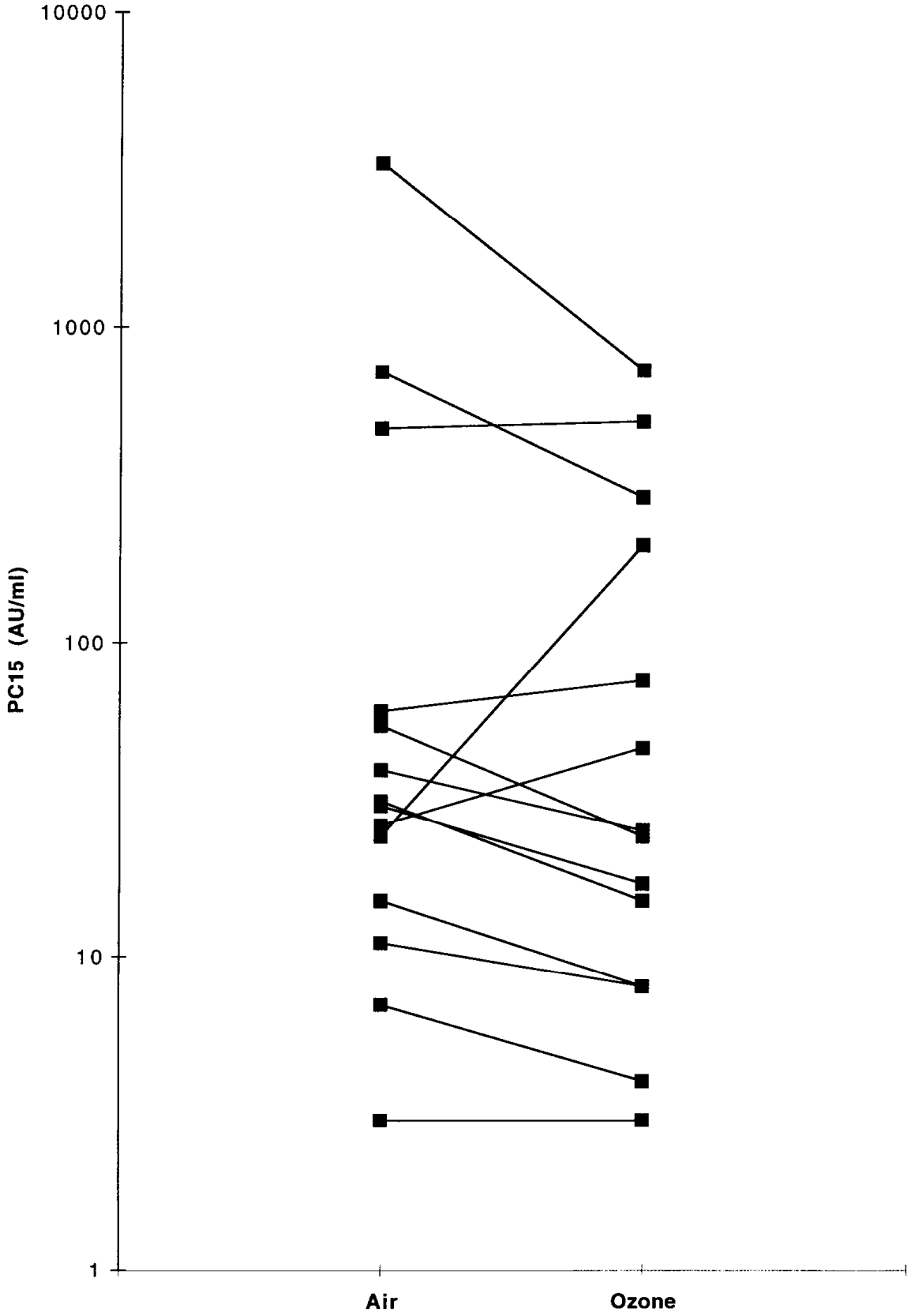


Figure 1. Individual PC15 values for phase I air vs. ozone

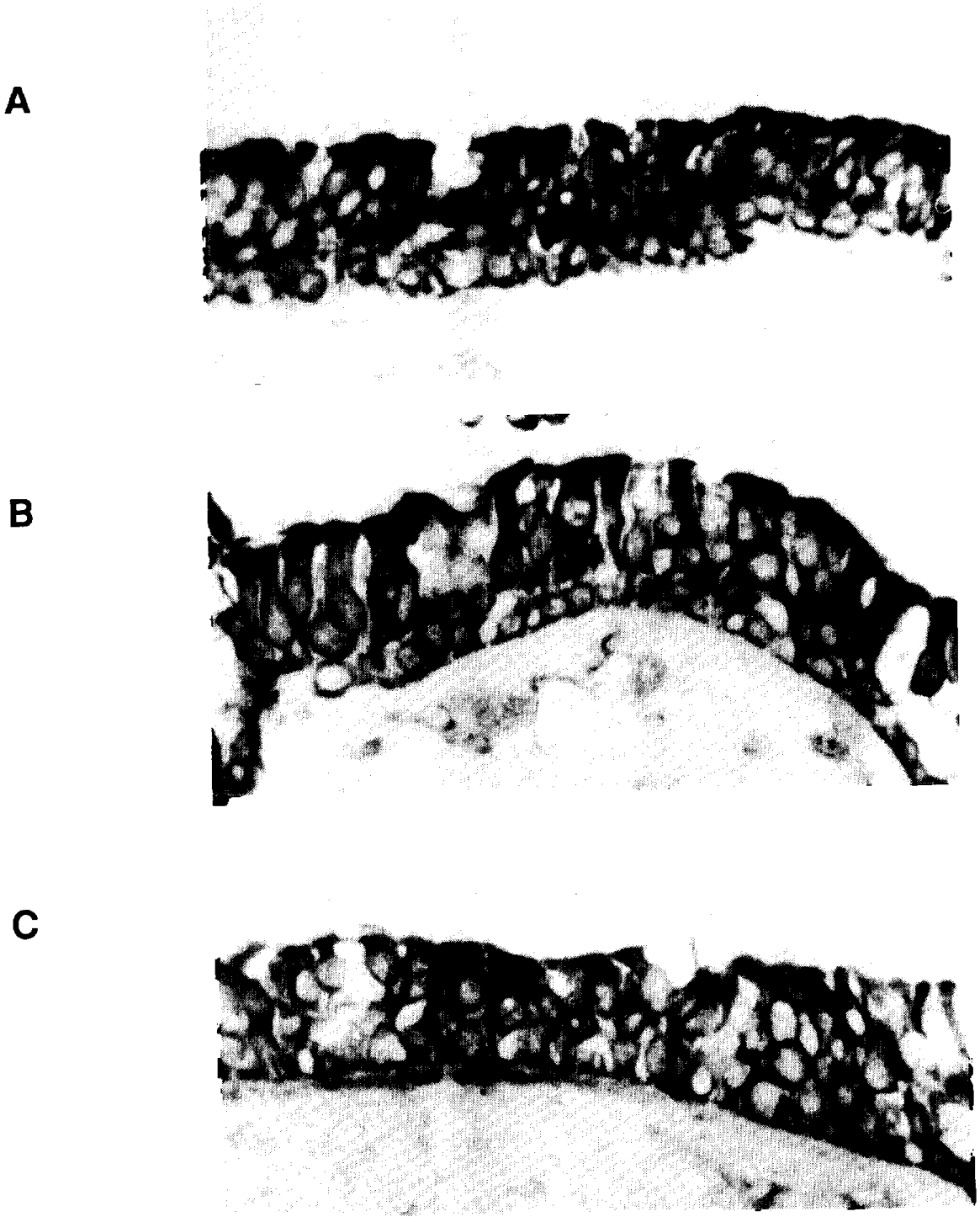


Figure 2. Endobronchial biopsy sections obtained after each exposure condition (A = air, B = ozone, C = ozone/nitric acid vapor) stained with antibodies directed against α9 in asthmatic subjects after allergen challenge.

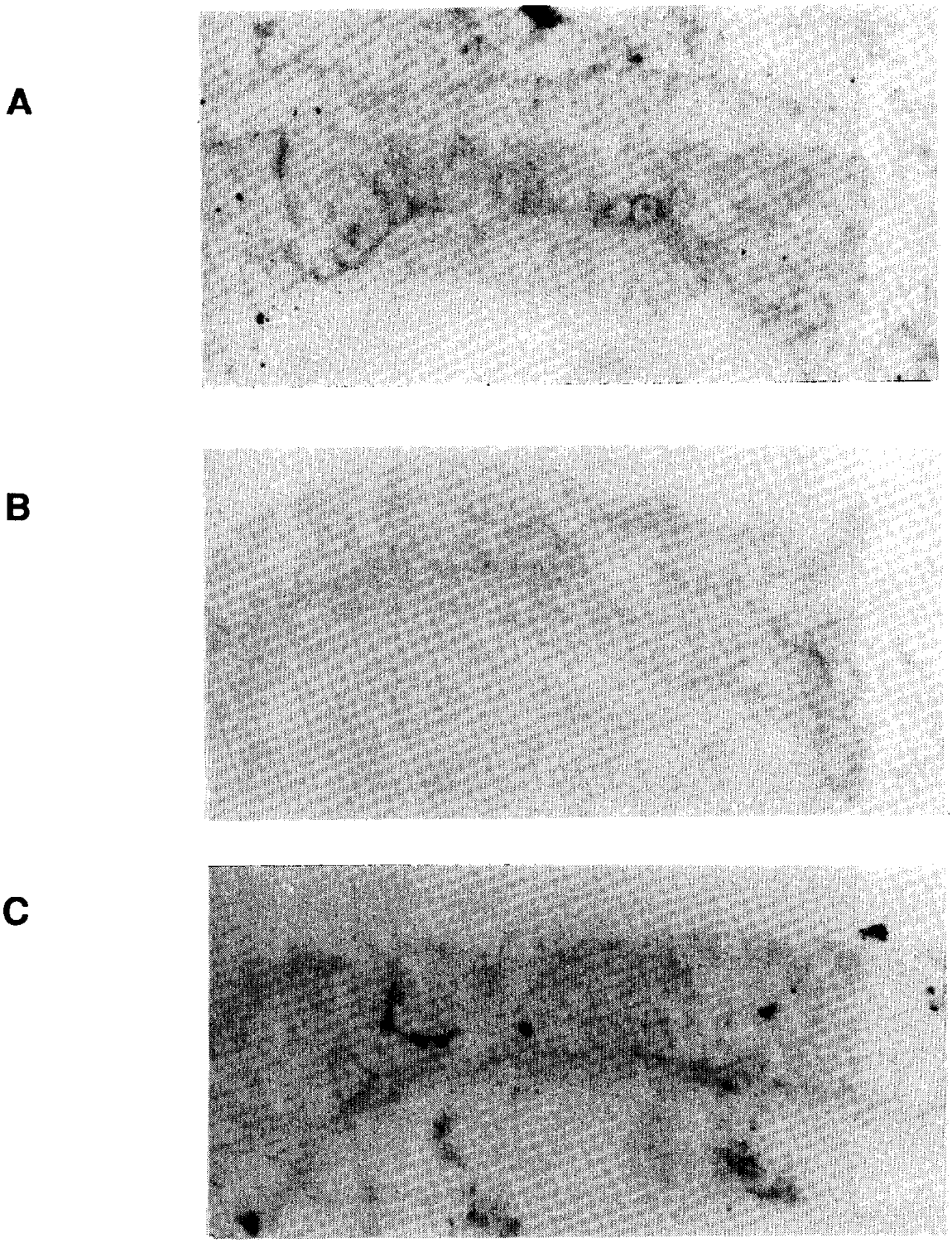
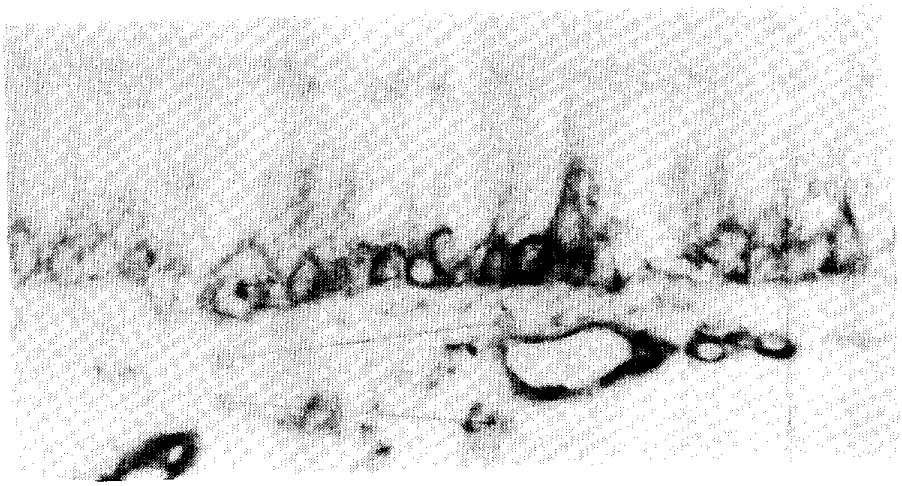


Figure 3. Endobronchial biopsy sections obtained after each exposure condition (A = air, B = ozone, C = ozone/nitric acid vapor) stained with antibodies directed against β6 in asthmatic subjects after allergen challenge.

A



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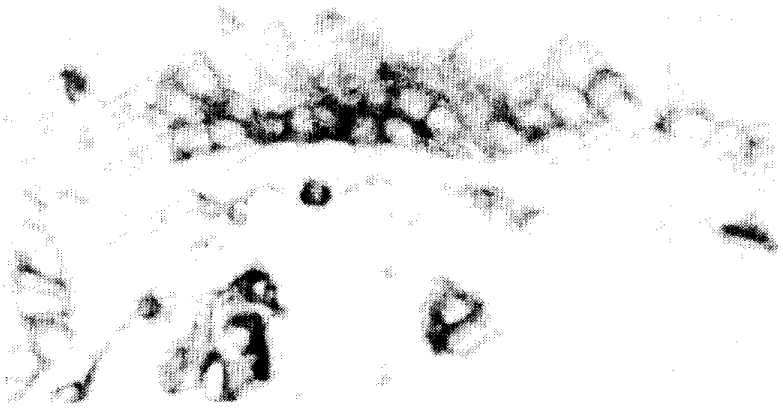

C

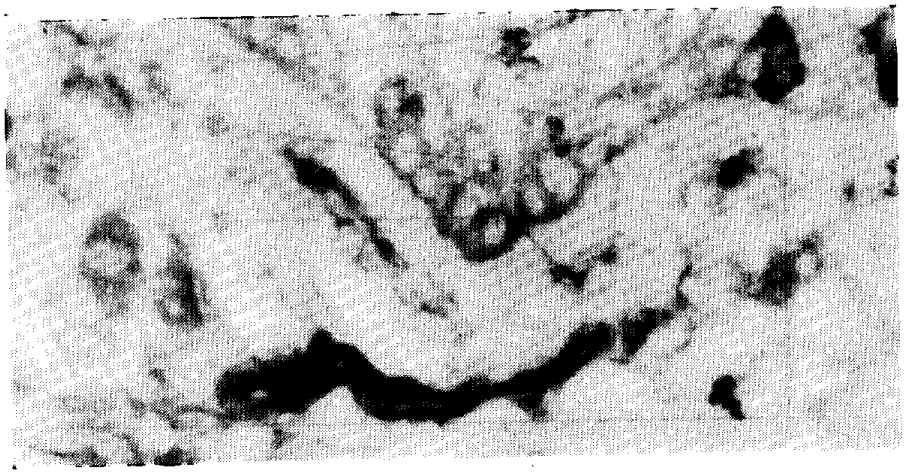

Figure 4. Endobronchial biopsy sections obtained after each exposure condition (A = air, B = ozone, C = ozone/nitric acid vapor) stained with antibodies directed against ICAM in asthmatic subjects after allergen challenge.

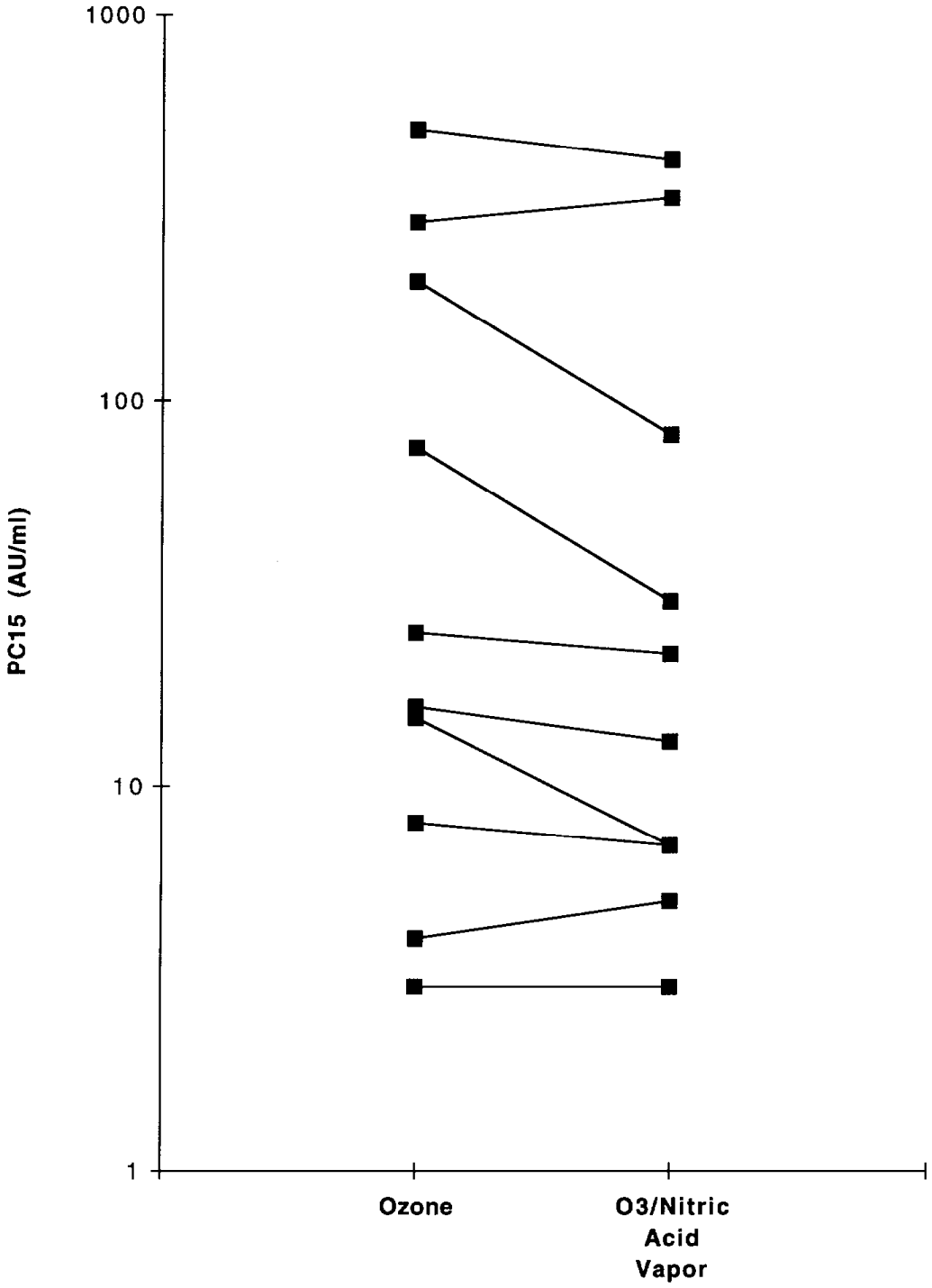

Figure 5. Individual PC15 values for phase II ozone vs. ozone/nitric acid vapor