Effect of Ozone Exposure on Airway Responses to Inhaled Allergen in Asthmatic Subjects*

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Background: Controlled human exposure studies have produced conflicting results regarding the effect of ozone on the early bronchoconstrictor response to inhaled allergen in specifically sensitized asthmatic subjects. Spirometric parameters do not necessarily reflect the airway inflammatory effects of inhaled ozone or allergen.

Objective: This study was designed to investigate whether exposure to ozone enhances the late airway inflammatory response, as well as the early bronchoconstrictor response, to inhaled house dust mite allergen in sensitized asthmatic subjects.

Design: Randomized, counter-balanced, cross-over study.

Setting: Human exposure laboratory.

Methods: Fourteen subjects were exposed to 0.2 ppm O₃ or filtered air, on separate days, for 1 h during exercise. After each exposure, the subjects were challenged with doubling doses of Dermatophagoides farinae (DF) allergen (provocative concentration of DF causing a 15% decrease in FEV₁ [PC₁₅]). At 6 h after allergen challenge, bronchoscopy with BAL, proximal airway lavage (PAL), and endobronchial biopsy were performed. The second exposure/allergen challenge/bronchoscopy sequence was performed at least 4 weeks after the first sequence.

Results: No significant difference in cellular or biochemical markers of the late inflammatory response after allergen was found between the ozone and air exposures (although a trend toward increased neutrophils was noted after ozone exposure in the PAL fluid, p ≤ 0.06). For the group as a whole, no significant difference in PC₁₅ was demonstrated after ozone exposure compared to air exposure. However, subjects with the greatest ozone-induced decrements in FEV₁ tended to have lower PC₁₅ values after ozone exposure.

Conclusion: Exposure to a relatively low-level concentration of ozone does not enhance the late inflammatory or early bronchoconstrictor response to inhaled antigen in most allergic asthmatic subjects. Our results do suggest, however, that a subgroup of asthmatics may acquire increased sensitivity to aeroallergens after exposure to ozone.

Key words: airway inflammation; allergen challenge; asthma; BAL; ozone

Abbreviations: AU = allergen units; DF = Dermatophagoides farinae; ECP = eosinophil cationic protein; GM-CSF = granulocyte-macrophage colony-stimulating factor; IL = interleukin; MPO = neutrophil myeloperoxidase; PAL = proximal airway lavage; PC₁₅ = concentration of Dermatophagoides farinae causing a 15% decrease in FEV₁ from the post-saline solution baseline; PC₂₀ = concentration of methacholine causing a 20% decrease in FEV₁ from the post-saline solution baseline; Sraw = specific airway resistance

Epidemiologic data suggest that people with asthma are at increased risk for exacerbation when exposed to high levels of ambient ozone. Contrary to expectations, controlled human exposure studies have not consistently shown people with asthma to be more sensitive to ozone in terms of physiologic response. In prior work with asthmatic and nonasthmatic subjects, we demonstrated that FEV₁ and FVC measurements do not correlate with ozone-induced cellular and biochemical indexes of lung injury and inflammation, and that asthmatic subjects have greater ozone-induced inflammatory...
responses than normal subjects. Asthma is a disease characterized by airway inflammation, particularly during the late-phase response to allergen, and the degree of airway inflammation is an important predictor of asthma severity. Thus, one possible explanation for the epidemiologic findings is that ozone exposure may enhance the inflammatory response to triggers of asthma, such as allergen, reflected in prior controlled human studies measuring physiologic parameters alone. Several studies in humans have evaluated the effect of ozone on the early bronchoconstrictor response to inhaled allergen in asthmatic subjects, but the results have been conflicting, and again may have missed potential changes in airway inflammation during the late-phase response. This study was designed to investigate the potential for ozone to enhance markers of airway inflammation during the late-phase response to inhaled allergen, as well as the early bronchoconstrictor response, in specifically sensitized asthmatic subjects.

Table 1—Subject Characteristics

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Sex</th>
<th>FEV1, L (% predicted)</th>
<th>Sraw, L × cm H2O/L/s</th>
<th>PC20, mg/mL</th>
<th>Medications*</th>
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<td>29</td>
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<td>3.36 (83)</td>
<td>4.7</td>
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*B = inhaled bronchodilator; T = theophylline; IS = inhaled steroid.

Materials and Methods

Subjects

A total of 44 allergic asthmatic subjects (aged 18 to 36 years) were recruited for the study through letter solicitation to patients followed up at the University of California, San Francisco Allergy/Immunology Clinic and general advertisement. Of those recruited, 14 subjects completed the study. Those subjects who did not complete the study either did not meet inclusion criteria or chose not to finish. All subjects had a documented physician diagnosis of asthma or met the National Asthma Education Program guidelines for the diagnosis of asthma. Subjects were confirmed atopic by allergen skin-prick testing, were nonsmokers (<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.

No subjects received inhaled β-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 weeks of any testing. No caffeinated beverages or chocolate were allowed for 4 h before or any time during testing. Subjects reported no symptoms of respiratory infection or asthma exacerbation for at least 1 month prior to enrollment.

Experimental Protocol

Initial Screening: A brief telephone interview with potential subjects was used to confirm a history of asthma and exclude smokers. At the first visit to the laboratory, subjects were informed of the risks of the study protocol, and those enrolled signed consent forms approved by the University of California, San Francisco Committee on Human Research. Epicutaneous skin-prick testing with a battery of nine local aeroallergens, including Dermatophagoides farinae (DF), and controls of saline solution/50% glycerol and histamine, was performed to verify atopic status and sensitivity to DF. Sensitivity was defined as a >2 × 2-mm skin-wheal response.

Baseline Testing: Baseline pulmonary function tests, which included FEV1, FVC, specific airway resistance (Sraw), and metha-
choline challenge, were performed on a subsequent visit. A 15-min exercise test on a cycle ergometer (Model 18070; Gould Godart; Bilthoven, the Netherlands) or treadmill (Model M9.1; Precor; Bothell, WA) was used to determine the appropriate workload necessary to achieve a target ventilatory rate of 25 L/min/m² body surface area. Subjects who had a > 15% decrease in FEV₁ from baseline after exercise were dropped from the study. FEV₁, at the start of each new study day was required to be within 10% of the baseline value from enrollment to proceed.

Study Protocol: At least 3 days after methacholine challenge, subjects were exposed in a random, double-blind fashion to either 0.2 ppm O₃ or filtered air in an environmental chamber. The exposures were 1 h in length, during which each subject exercised on a cycle ergometer and/or treadmill at the predetermined workload. The tidal volume and respiratory rate were measured with a pneumotachograph (No. 3; A. Fleisch; Lau- sanne, 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, FEV₁, and FVC were measured immediately before and after each exposure. A symp- tom 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 under- went DF allergen challenge. Spirometry was performed each hour for 5 h from the time of the last allergen dose. At 6 h after the final dose of antigen, fiberoptic bronchoscopy was performed. Vital signs and peak flow measurements were monitored overnight at the General Clinical Research Center at San Francisco General Hospital. Subjects returned after at least 4 weeks for the second exposure condition, DF allergen challenge, and bronchoscopy following an identical protocol.

Pulmonary Function Measurements

Spirometry was performed on a dry, rolling-seal spirometer (S400; Spirotech Division, Anderson Instruments; Atlanta, GA). During each visit, the daily baseline FEV₁ 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 FEV₁ and FVC were calculated from three acceptable FVC maneuvers obtained approximately 30 s apart. Sraw was determined as the product of airway resistance and thoracic gas volume, both calculated from the average of five measurements obtained 30 s apart in a constant-volume body plethysmograph (Warren E. Collins; Braintree, MA).

Methacholine responsiveness was tested by measuring FEV₁ before and after five 6-s inhalations of phosphate-buffered saline solution combined with doubling concentrations of methacholine (0.16 mg/mL, 0.32 mg/mL, 0.63 mg/mL, 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL, and 10 mg/mL) delivered by a nebulizer (Model 646; Devilbiss; Somerset, PA) with a Rosenthal dosimeter. 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.

Skin-Prick Testing and Inhalation Challenge With DF Allergen

Epicutaneous skin-prick testing with the battery of nine local aeroallergens was done on the volar forearm surface of one forearm. If the subject had sensitivity to DF on this screening test, dilutional skin-prick testing was done on the opposite forearm using log concentrations (0.1 to 1,000 allergen units [AU]) of DF allergen (Greer Laboratories; Lenoir, NC). The starting dose for each inhalation challenge with DF was deter- mined using a formula described by Cockcroft et al.² The formula predicts the concentration of allergen necessary to cause a 20% decrease in FEV₁ based on the results of the dilutional allergen skin-prick test and PC₂₀ for each subject. The starting dose of allergen used was four doubling doses below the predic- tive value of Cockcroft et al. Inhalation challenge with DF allergen was performed as described above for methacholine challenge. The concentration of DF causing a 15% decrease in FEV₁, from the post-saline solution baseline (PC₁₅) was calcu- lated by log-linear interpolation. If the drop in FEV₁ was between 10% and 15% of baseline, spirometry was repeated 10 min later before proceeding to a higher dose. The starting concentration of allergen was identical for each subject on the 2 different exposure days.

Bronchoscopy, Lavage, and Biopsy Procedures

Bronchoscopy was performed in a dedicated room at San Francisco General Hospital as described previously, with minor alterations. Briefly, nebulized 0.5% albuterol solution was in- haled, IV access was established, and the upper airways were anesthetized with topical 4% lidocaine spray and 4% cocaine-soaked cotton pledgets. All subjects received IV atropine (0.4 mg) and supplemental oxygen (2 L/min) via nasal cannula. Midazolam was administered IV to most subjects, and the dose was titrated to maintain subject comfort. The bronchoscope (FB 18x; Pentax Precision Instruments; Orangeburg, NY) was introduced through the mouth, and the lower airways were anesthetized with 1% lidocaine as needed. A custom-designed, 6F, double-balloon, double-port catheter (Baxter Healthcare; Irvine, CA), with a 1.5-cm interballoon 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. Proximal airway lavage (PAL) was performed using eight
1.5-mL aliquots of warmed saline solution. After PAL was completed, BAL was performed in the right middle lobe using three 50-mL aliquots of warmed saline solution. The first 15 mL of lavage fluid retrieved was labeled bronchial fraction. All lavage samples were immediately put on ice. Five endobronchial biopsy specimens were then obtained from the right upper lobe carina using a spoked, cup-forceps (2.4 mm in diameter, Pentax Precision Instruments). Biopsy specimens were obtained from the left upper lobe carina during the second bronchoscopy.

**Measurement of Cells and Biochemical Constituents of Lavage Fluids**

Total cells were counted in unspun aliquots of PAL, bronchial fraction, and BAL fluids using a hemocytometer. Differential cell counts were performed on slides prepared with a cytocentrifuge (Cytospin 2; Shandon Southern Products; Astmoor, UK; 200 g × 5 min) and stained in Diff-Quik (American Scientific Products; McGaw Park, IL) as previously described. Lavage fluids were immediately centrifuged at 200 × g for 15 min, and the supernatants were separated and recentrifuged at 3,000 g for 15 min to remove any cellular debris.

Assays of biochemical constituents of lavage fluids were performed on aliquots of supernatants that had been frozen at −70°C. Total protein was assayed by a modification of the Lowry procedure. Lavage concentrations of fibronectin were determined with an antibody-capture immunoassay, as described previously. Interleukin (IL)-5, IL-6, and IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and transforming growth factor (TGF)–β1 and TGF–β2 levels were analyzed using commercially available immunoassays (R&D Systems; Minneapolis, MN). Commercially available kits were also used for measuring eosinophil cationic protein (ECP), tryptase (KABI Pharmacia Diagnostics; Piscataway, NJ), and neutrophil myeloperoxidase (MPO) [Cayman Chemical; Ann Arbor, MI].

**Bronchial Biopsy Histology**

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

Histologic evaluation was done on 2 μm sections of the plastic-embedded biopsy specimens using hematoxylin-eosin stain. For each section of tissue, the numbers of eosinophils were counted in several nonoverlapping high-power fields until all the available areas of the submucosa were examined. Results were expressed as number of eosinophils per square millimeter. To avoid reader bias, tissue slides were coded and read in blinded fashion.

**Statistical Analysis**

The comparisons of PC15 values, pulmonary function measurements, lavage fluid cellular and biochemical parameters, and tissue eosinophil counts were done using Wilcoxon signed-rank tests. Correlations between variables were evaluated with Spearman coefficients. Differences or correlations at p ≤ 0.05 were accepted as statistically significant. To determine whether subjects with increased sensitivity to DF allergen after ozone were a definable subgroup, subjects were dichotomized into groups based on whether their PC15 decreased after ozone and whether their FEV1 or Sraw decreased after ozone. The odds ratio of a decrease in PC15 after ozone for those subjects with a decrease in FEV1 or Sraw after ozone and their 95% confidence limits were calculated. One-sided power calculations were carried out to determine the magnitude of decrease in PC15 response after ozone (relative to filtered air) that could be detected with the number of subjects studied. Estimates of the variances of paired differences were those obtained in the present study. In all cases, the largest variance estimates were used to provide a conservative assessment of power. We assumed that a reasonable change in PC15 due to ozone exposure would be at least one doubling dose lower than the PC15 measured after air exposure.

**RESULTS**

There was no difference in paired comparisons of baseline FEV1 between ozone and air exposure days. Although 1 h of exercise during ozone exposure caused a mean decrease in FEV1 of 10%, this decrease was not significantly different than the 4% decrease in FEV1 due to exercise alone during the air exposure. The mean percent changes in Sraw and FVC across the 1-h exposures also were not significantly different between the ozone and air days. There were significantly higher scores for lower respiratory symptoms (chest pain on inspiration, chest tightness, shortness of breath, cough, and sputum production) after ozone exposure as compared to after air exposure.

The group median PC15 value for the allergen challenge was lower after ozone exposure (24 AU/mL; range, 3 to 723 AU/mL) compared to air exposure (31 AU/mL; range, 3 to 3,276 AU/mL). Individually, 9 of the 14 asthmatic subjects tested had a lower PC15 value after ozone exposure (Fig 1). Although a trend was noted, the difference in PC15 between the two exposure conditions for the group was not statistically significant.

Further analyses plotting the distributions of subject responses were done to potentially define a subgroup responsible for the trend toward an ozone-induced increase in allergen sensitivity. These plots suggested a possible correlation between ozone sensitivity (FEV1 response across the 1-h ozone exposure) predicting increased allergen sensitivity after ozone exposure, as seen in seven of the nine individuals with the lower PC15 after ozone (Fig 2). The odds ratio for a decrease in PC15 after ozone in subjects with a decrease in FEV1 after ozone was 1.66 (95% confidence interval, 0.45 to 12.3). The odds ratio for an increase in Sraw after ozone was 5.25 (95% confidence interval, 0.49 to 56.8).

Analysis of lavage fluids showed evidence toward an increase in neutrophils in the PAL fluid after ozone exposure that did not reach statistical significance (p = 0.06) [Table 2]. No correlation was evi-
dent between an increased neutrophil response in lavage fluid and increased allergen sensitivity after ozone exposure. No effect of ozone exposure was observed on lavage fluid eosinophil, lymphocyte, or total leukocyte cell counts. There was also no difference between the two exposure conditions in lavage fluid total protein, fibronectin, GM-CSF, IL-5, IL-6, IL-8, TGF-β1, TGF-β2, or markers of inflammatory cell degranulation (ECP, tryptase, or MPO) [Fig 3]. Morphometry demonstrated no difference in the number of submucosal eosinophils, lymphocytes, or neutrophils between the two exposure conditions.

**Discussion**

This study is the first to address the effect of ozone on the late inflammatory response to allergen in allergic asthmatic subjects using bronchoscopy to obtain samples of airway lining fluid and bronchial tissue. Our results do not demonstrate a significant effect of ozone exposure on the late-phase inflammatory response. These results do, however, show a trend toward greater neutrophilia in the proximal airways after the ozone-allergen exposure as compared to the air-allergen exposure. The airway inflammation associated with the late-phase response of asthma is characterized by both neutrophilia and eosinophilia in BAL fluid. Ozone exposure also causes an airway neutrophilia in both normal and asthmatic subjects, especially pronounced in lavage fluid from the proximal airways. Thus, the trend toward an increased proximal airway neutrophilia that we observed is consistent with what would be expected due to an ozone-induced enhancement of the inflammatory response to inhaled allergen.

Evaluating indexes of airway inflammation has been shown to be necessary to fully assess the potential for ozone-induced effects that may not be evident by spirometry alone. To evaluate the airway inflammatory response in depth, several other parameters important to both the airway response to ozone and asthmatic response to allergen were measured. IL-5 and GM-CSF are known mediators of eosinophil recruitment, maturation, and activation. IL-5 is also a product of the T-helper type 2 subset of lymphocytes that serve as important regulatory cells in the asthmatic response to allergen. The inflammatory response to ozone has been demonstrated to include increases in IL-6, IL-8, and GM-CSF levels in BAL fluid, and asthmatic subjects have been shown to have enhanced cytokine responses to ozone. Despite their putative roles in airway inflammation in asthma or induced by ozone, no differences in the levels of these cytokines were demonstrated after allergen between the two exposures in this study.

Ozone has been shown to increase ECP levels in nasal lavage fluid. In this study, however, we found no evidence of increased ECP (as a marker of eosinophil degranulation), MPO (neutrophil degranulation), or tryptase (mast-cell degranulation) after allergen due to ozone exposure. Additional lavage assays did not show increases in markers of vascular permeability or tissue injury (total protein, fibronectin, and TGF-β) due to ozone exposure. Bronchial tissue biopsies also revealed no increase in inflammatory cell influx after allergen between the two exposures.

We suspect that the relatively low effective dose of ozone used in this study (for safety reasons and to model ambient exposures) may have limited our findings. A modest effect on the late-phase inflammatory response from our low dose of ozone could have gone undetected in the background of a larger inflammatory response to allergen. A higher effective dose of ozone in our study would have caused more airway injury and potentially a greater effect on the subsequent late inflammatory response to allergen.
This study was also limited by the exclusion, for reasons of safety, of persons with moderate-to-severe asthma. Those with more severe disease, and a higher degree of preexisting airway inflammation, would presumably be at greatest risk for consequences due to additional ozone-induced airway inflammation. Another limitation of our protocol is that we did not use a fixed dose of allergen for the inhalational challenge to DF; following ozone exposure, the subjects were on average more responsive to the bronchoconstrictor effects of the inhaled allergen so that they received on average less allergen, which may have limited our ability to observe an effect of ozone on the airway inflammatory response. A study using induced sputum to sample airway lining fluid did find an increased percentage of eosinophils, but not of neutrophils, in sensitized asthmatics exposed to ozone 24 h after specific allergen challenge. The results of this study are not directly comparable to ours for several reasons. First, the dose of ozone used was almost three times what we used. Second, the order and timing of allergen

Table 2—Comparison of Air vs Ozone Lavage Inflammatory Cell Results 6 h After Allergen Challenge

<table>
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<tr>
<th>Variables</th>
<th>BAL</th>
<th>Bronchial Fraction</th>
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</thead>
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<tr>
<td></td>
<td>Air</td>
<td>Ozone</td>
<td>Air</td>
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<tr>
<td>Total leukocytes, × 10³/mL</td>
<td>23.3 (9.0–43.5)</td>
<td>16.8 (6.8–42.5)</td>
<td>21.0 (7.3–30.8)</td>
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<td>Neutrophils, %</td>
<td>3.3 (0.0–12.0)</td>
<td>4.0 (0.0–16.5)</td>
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<tr>
<td>Eosinophils, %</td>
<td>1.5 (0.3–19.0)</td>
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<td>5.0 (0.0–31.8)</td>
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<tr>
<td>Lymphocytes, %</td>
<td>5.5 (1.0–48.5)</td>
<td>4.8 (1.8–11.9)</td>
<td>3.8 (1.5–11.3)</td>
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</table>

*Data are presented as median (range).
†Sign-rank p = 0.06.
and ozone exposures was different (allergen 24 h before ozone vs ozone immediately before allergen). The optimal timing for study of ozone-inhaled allergen interactions needs to be determined.

Our results also did not demonstrate a significant effect due to ozone exposure on the early bronchoconstrictor response to allergen in our group of sensitized asthmatic subjects. This study had a power of 0.80 to detect a decrease of at least one doubling-dose in the PC_{15} due to ozone exposure as compared to that measured after air exposure. Although 9 of the 14 subjects had a lower PC_{15} after ozone, only 4 of these 9 subjects had a decrease in PC_{15} of at least one doubling dose. Comparing published studies, there does appear to be a dose effect of ozone on the early bronchoconstrictor response to allergen. Using a much higher effective dose of ozone (0.25 ppm for 3 h by mouthpiece with intermittent exercise), Jorres and coworkers demonstrated an enhanced early bronchoconstrictor response to allergen due to ozone in subjects with mild asthma. Kehrl and coworkers also demonstrated a modest enhancement in the early bronchoconstrictor response to allergen after 7.6 h of mild exercise with exposure to 0.16 ppm ozone. Using protocols with low-dose ozone exposures (0.12 ppm ozone for 1 h) similar to ours, two other groups of investigators could not demonstrate this enhanced effect.

The most intriguing issue raised by this study is that of a possible association between sensitivity to ozone as measured by acute lung function responses and increased sensitivity to allergen after ozone exposure. Lung function and airway inflammatory responses to ozone vary widely among normal and asthmatic individuals, and there is considerable evidence that there are genetic determinants of this intersubject variability. The lack of statistical significance for the apparent association between FEV_{1} response to ozone and increased sensitivity to allergen after ozone exposure could be due to the small number of subjects we studied in our relatively complicated protocol and warrants further study. Prescreening to identify an ozone-sensitive (by FEV_{1} response) subgroup of asthmatic subjects, or a much larger sample size, is needed to confirm the potential association between ozone sensitivity and increased allergen responsiveness.

In conclusion, although our results do not support the hypothesis that exposure to an ambient level of ozone enhances the overall response to inhaled allergen in sensitized asthmatic subjects, they do suggest that a subgroup is at greater risk for increased sensitivity to aeroallergen after ozone exposure. Further studies are required to better characterize this subgroup and determine the mechanism responsible for the increased sensitivity. This investigation is the first to study the effect of ozone exposure on allergen-induced airway inflammation via lavage and tissue sampling during the late-phase response. At the relatively low level of ozone expo-
sure used in this study, no significant enhancement of the late inflammatory response to allergen was observed in BAL, although there was a trend toward increased proximal airway neutrophilia.

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