

occupational and environmental lung disease

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

Lisa L. Chen, MD; Ira B. Tager, MD, MPH; David B. Peden, MD; Dorothy L. Christian, BA; Ronald E. Ferrando, MA; Barbara S. Welch, BS; and John R. Balmes, MD, FCCP

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. (CHEST 2004; 125:2328–2335)

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

Abbreviations: AU = allergen units; DF = *Dermatophyoides farinae*; ECP = eosinophil cationic protein; GM-CSF = granulocyte-macrophage colony-stimulating factor; IL = interleukin; MPO = neutrophil myeloperoxidase; PAL = proximal airway lavage; PC₁₅ = concentration of *Dermatophyoides 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.¹ Contrary to expectations, controlled human exposure studies^{2–5} have not consistently shown people with asthma to be more sensitive to ozone in terms of

physiologic response. In prior work^{6,7} 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

Table 1—Subject Characteristics

Age, yr	Sex	FEV ₁ , L (% predicted)	Sraw, L × cm H ₂ O/L/s	PC ₂₀ , mg/mL	Medications*
29	Male	3.36 (83)	4.7	0.49	B, IS
33	Male	3.70 (86)	4.8	0.72	B, T, IS
32	Male	3.53 (82)	2.7	1.95	B
29	Male	4.17 (104)	4.3	7.62	B
36	Male	4.34 (114)	2.3	3.94	B, IS
28	Male	3.99 (85)	6.2	3.24	B
31	Female	3.26 (99)	4.3	0.18	B
32	Male	2.83 (72)	7.8	0.24	B
25	Male	3.33 (85)	8.7	0.43	None
35	Male	3.31 (78)	1.6	0.35	B
23	Male	3.60 (88)	7.8	0.60	B
27	Male	4.73 (105)	2.7	0.90	B
18	Male	3.69 (90)	3.8	0.97	B
22	Male	3.54 (88)	4.1	1.40	B

*B = inhaled bronchodilator; T = theophylline; IS = inhaled steroid.

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.⁸ Thus, one possible explanation for the epidemiologic findings is that ozone exposure may enhance the inflammatory response to triggers of asthma, such as allergen, not reflected in prior controlled human studies measuring physiologic parameters alone. Several studies^{9–13} 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.

*From the Lung Biology Center, Center for Occupational and Environmental Health (Drs. Chen and Balmes, Ms. Christian, Mr. Ferrando, and Ms. Welch), Cardiovascular Research Institute, and Medical Service, San Francisco General Hospital, University of California, San Francisco, CA; Division of Public Health Biology and Epidemiology (Dr. Tager), School of Public Health, University of California, Berkeley, CA; and Center for Environmental Medicine and Lung Biology (Dr. Peden), Department of Pediatrics, University of North Carolina, Chapel Hill, NC.

Supported by California Air Resources Board Contract #A133–150 and the National Institutes of Health (R01 ES08970 and M01RR00083–41).

Manuscript received August 1, 2003; revision accepted November 21, 2003.

Reproduction of this article is prohibited without written permission from the American College of Chest Physicians (e-mail: permissions@chestnet.org).

Correspondence to: John R. Balmes, MD, FCCP, University of California, San Francisco, Box 0843, San Francisco, CA 94143-0843; e-mail: jrbalmes@itsa.ucsf.edu

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 guidelines¹⁴ 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.¹⁵

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 $> 2 \times 2$ -mm skin-wheal response.¹⁶

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; Bithoven, 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; 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, FEV₁, 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 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 maneuvers¹⁷ 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 (Laboratory for Applied Immunology; Fairfax, VA) calibrated to deliver 0.01 mL per breath. The concentration of methacholine causing a 20% decrease in FEV₁ from the post-saline solution baseline (PC₂₀) was calculated by log-linear interpolation.

Environmental Chamber and Atmospheric Monitoring

All exposures took place in a 2.5 × 2.5 × 2.4-m steel and glass chamber (Model W00327-3R; Nor-Lake; Hudson, WI) filled with filtered air at 20°C and 50% relative humidity to which

ozone was 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 chamber and air filtration, humidification, and conditioning systems have been previously described.¹⁵ Relative humidity and temperature were recorded every 30 s, displayed in real-time (LabVIEW 2; National Instruments; Austin, TX), and stored by a microcomputer (Model IIsi; Apple Computer; Cupertino, CA) for the duration of the exposure. Temperature and humidity means were similar for the air (20.5 ± 0.6°C and 49.9 ± 1.7%) and ozone exposures (20.3 ± 0.5°C and 50.2 ± 0.9%), respectively (± SD).

Ozone was produced with a corona-discharge generator (Model T 408; Polymetrics; San Jose, CA) and analyzed with an ultraviolet light photometer (Model 1008 PC; Dasibi; Glendale, CA). The ozone concentration, which was measured every 30 s, displayed in real-time, and stored by microcomputer, averaged 0.2 ppm and 0.0 ppm for the ozone and air 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.

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 determined using a formula described by Cockcroft et al.¹⁹ 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 predictive value of Cockcroft et al.¹⁹ 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 calculated 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,¹⁵ with minor alterations. Briefly, nebulized 0.5% albuterol solution was inhaled, 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 spiked, 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; 200g $\times$ 5 min) and stained in Diff-Quik (American Scientific Products; McGaw Park, IL) as previously described.¹⁷ Lavage fluids were immediately centrifuged at 200g for 15 min, and the supernatants were separated and recentrifuged at 3,000g 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.¹⁸ Interleukin (IL)-5, IL-6, and IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and transforming growth factor (TGF)- β_1 and TGF- β_2 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 PC_{15} 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 $p \leq 0.05$ 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 PC_{15} decreased after ozone and whether their FEV_1 or Sraw decreased after ozone. The odds ratio of a decrease in PC_{15} after ozone for those subjects with a decrease in FEV_1 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 PC_{15} 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 PC_{15} due to ozone exposure would be at least one doubling dose lower than the PC_{15} measured after air exposure.

RESULTS

There was no difference in paired comparisons of baseline FEV_1 between ozone and air 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 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 PC_{15} 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 PC_{15} value after ozone exposure (Fig 1). Although a trend was noted, the difference in PC_{15} 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 (FEV_1 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 PC_{15} after ozone (Fig 2). The odds ratio for a decrease in PC_{15} after ozone in subjects with a decrease in FEV_1 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-

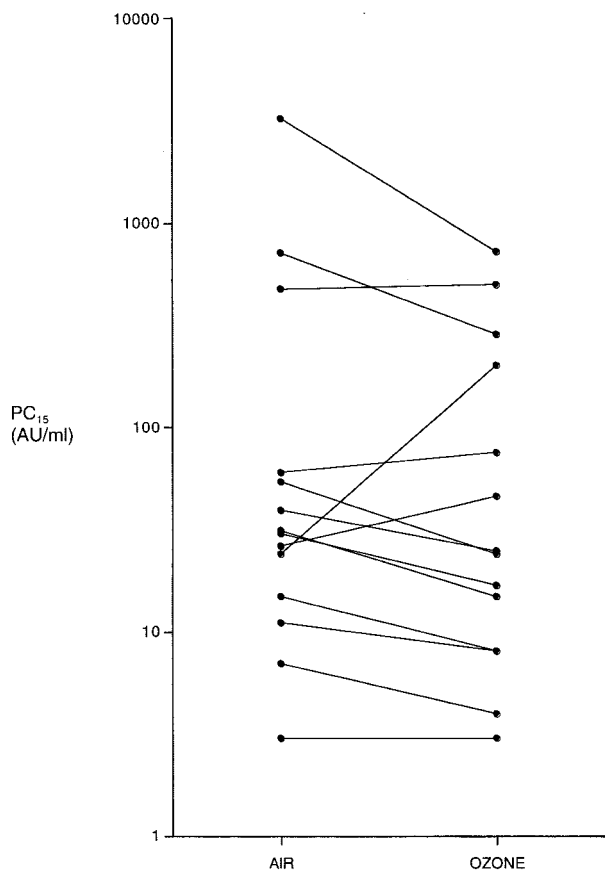


FIGURE 1. Comparison of individual allergen challenge PC₁₅ results (n = 14) after exposure to filtered air or 0.2 ppm ozone for 1 h with exercise.

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- β_1 , TGF- β_2 , 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.²⁰ Ozone exposure also causes an airway neutrophilia in both normal and asthmatic subjects,^{6,7,21,22} especially pronounced in lavage fluid from the proximal airways.^{7,21} 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.^{6,7,22} 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.²³ The inflammatory response to ozone has been demonstrated to include increases in IL-6, IL-8, and GM-CSF levels in BAL fluid,^{21,24} and asthmatic subjects have been shown to have enhanced cytokine responses to ozone.^{7,22} 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.²⁵ 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.

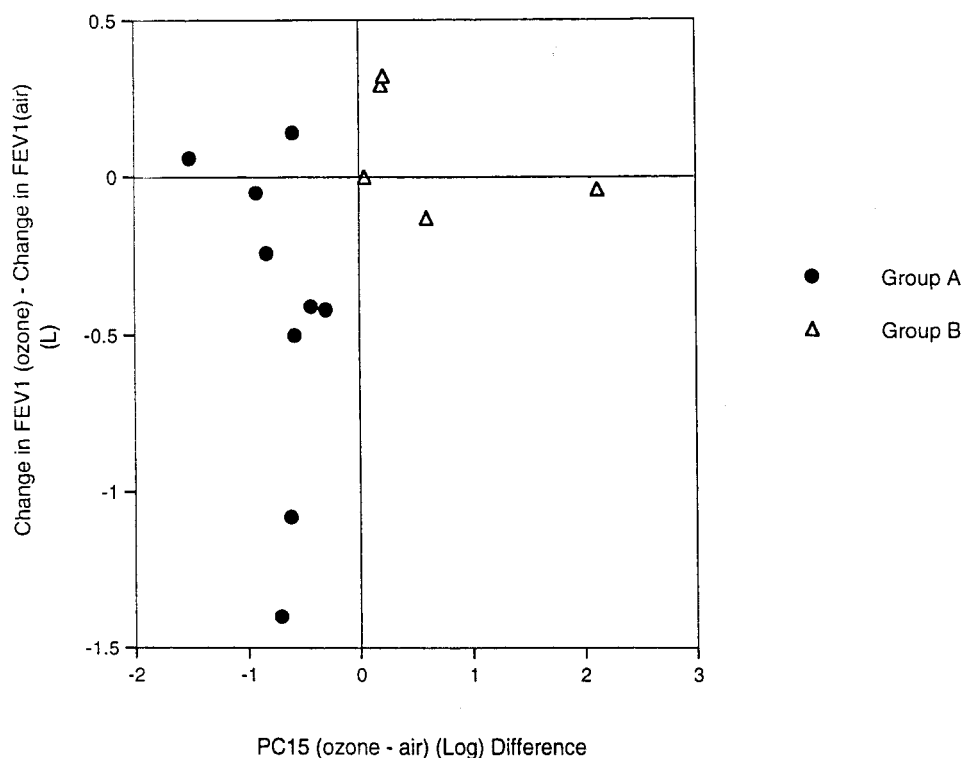


FIGURE 2. Ozone response (change in FEV₁ after 1 h of exercise with ozone exposure minus the change after 1 h of exercise in filtered air) plotted against the change in allergen sensitivity after ozone exposure (PC₁₅ after ozone minus that after filtered air). Group A represents those subjects who were more sensitive to allergen after ozone exposure, *ie*, had a lower PC₁₅ after ozone compared to after air. Group B are those subjects who were less sensitive to allergen after ozone. All of the subjects who had the greatest decrease in FEV₁ after ozone exposure were also found to be more sensitive to allergen after ozone. Note that these subjects are overrepresented in the lower left-hand quadrant (odds ratio, 1.66; 95% confidence limit, 0.45 to 12.3).

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 study²⁶ 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*

Variables	BAL		Bronchial Fraction		PAL	
	Air	Ozone	Air	Ozone	Air	Ozone
Total leukocytes, × 10 ⁴ /mL	23.3 (9.0–43.5)	16.8 (6.8–42.5)	21.0 (7.3–30.8)	17.5 (10.3–28.5)	1.4 (0.5–7.3)	1.9 (0.5–33.0)
Neutrophils, %	3.3 (0.0–12.0)	4.0 (0.0–16.5)	6.3 (0.5–27.3)	6.8 (1.0–18.3)	15.6 (3.8–50.3)	27.8 (0.8–52.8)†
Eosinophils, %	1.5 (0.3–19.0)	1.3 (0.0–11.3)	5.0 (0.0–31.8)	2.0 (0.0–13.5)	10.4 (1.5–54.5)	9.0 (0.3–39.3)
Lymphocytes, %	5.5 (1.0–48.5)	4.8 (1.8–11.8)	3.8 (1.8–11.3)	4.8 (1.8–12.0)	4.1 (1.5–7.8)	4.1 (1.0–17.5)

*Data are presented as median (range).

†Sign-rank *p* = 0.06.

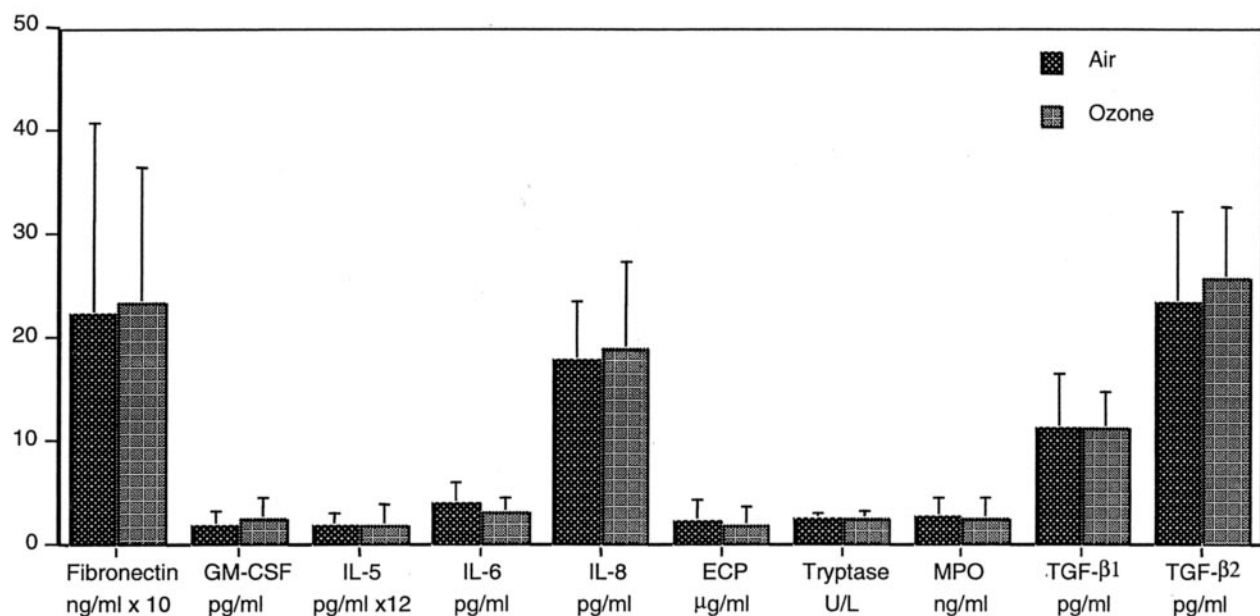


FIGURE 3. Comparison of air vs ozone measurements of various markers of airway inflammation in BAL fluid (mean $\pm$ SD). The mean (SD) for each marker is equal to the product of the ordinate value and the units displayed below each marker. There was no statistically significant difference for any marker between the two exposures.

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,^{10–13} 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 coworkers¹⁰ demonstrated an enhanced early bronchoconstrictor response to allergen due to ozone in subjects with mild asthma. Kehrl and coworkers¹¹ 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.^{12,13}

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,^{7,27} and there is considerable evidence that there are genetic determinants of this intersubject variability.^{28,29} 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.

ACKNOWLEDGMENT: The investigators thank Robert Montanti for assistance with bronchoscopy; Ivo Wortman for the ECP, mast-cell tryptase, MPO, and IL-6 and IL-8 analyses; and Jane Liu from the laboratory of Drs. Homer Boushey and John Fahy at the Cardiovascular Research Institute, University of California, San Francisco, for the TGF- β_1 and TGF- β_2 analyses.

REFERENCES

- Balmes JR. The role of ozone exposure in the epidemiology of asthma. *Environ Health Perspect* 1993; 101(suppl 4):219–224
- Linn WS, Buckley RD, Spier CE. Health effects of ozone exposure in asthmatics. *Am Rev Respir Dis* 1978; 117:835–843
- Koenig JW, Covert DS, Marshall SF, et al. The effects of ozone and nitrogen dioxide on pulmonary function and asthmatic adolescents. *Am Rev Respir Dis* 1987; 136:1152–1157
- Kreit JW, Gross KB, Moore TB, et al. Ozone-induced changes in pulmonary function and bronchial responsiveness in asthmatics. *J Appl Physiol* 1989; 66:217–222
- Horstman DH, Ball BA, Brown J, et al. Comparison of pulmonary responses of asthmatic and nonasthmatic subjects performing light exercise while exposed to a low level of ozone. *Toxicol Ind Health* 1995; 11:369–385
- Balmes JR, Chen LL, Scannell C, et al. Ozone-induced decrements in FEV₁ and FVC do not correlate with measures of inflammation. *Am J Respir Crit Care Med* 1996; 153:904–909
- Scannell C, Chen L, Aris RM, et al. Greater ozone-induced inflammatory responses in subjects with asthma. *Am J Respir Crit Care Med* 1996; 154:24–29
- Busse WW, Calhoun WF, Sedgwick JD. Mechanism of airway inflammation in asthma. *Am Rev Respir Dis* 1993; 147:S20–S24
- Molfino NA, Wright SC, Katz I, et al. Effect of low concentrations of ozone on inhaled allergen responses in asthmatic subjects. *Lancet* 1991; 338:199–203
- Jorres R, Nowak D, Magnussen H. The effects of ozone exposure on allergen responsiveness in subjects with asthma or rhinitis. *Am J Respir Crit Care Med* 1996; 153:56–64
- Kehrl HR, Peden DB, Ball B, et al. Increased specific airway reactivity of persons with mild allergic asthma after 7.6 hours of exposure to 0.16 ppm ozone. *J Allergy Clin Immunol* 1999; 104:1198–1204
- Ball BA, Folinsbee LJ, Peden DB, et al. Response to allergen bronchoprovocation of mild allergic asthmatics following low level ozone exposure. *J Allergy Clin Immunol* 1996; 98:563–572
- Hanania NA, Tarlo SM, Silverman F, et al. Effect of exposure to low levels of ozone on the response to inhaled allergen in allergic asthmatic patients. *Chest* 1998; 114:752–756
- National Asthma Education Program Expert Panel. Guidelines for the diagnosis and management of asthma. Bethesda, MD: National Heart, Lung, and Blood Institute, 1991; NIH Publication No. 91–3042
- Knudson RJ, Lebowitz MD, Holberg CJ, et al. Changes in the maximum expiratory flow-volume curve with growing and aging. *Am Rev Respir Dis* 1983; 127:725–734
- Nelson HS, Szeffler SJ, Jacobs J, et al. The relationships among environmental allergen sensitization, allergen exposure, pulmonary function, and bronchial hyperresponsiveness in the Childhood Asthma Management Program. *J Allergy Clin Immunol* 1999; 104:775–785
- American Thoracic Society. Standardization of spirometry, 1987 update. *Am Rev Respir Dis* 1987; 136:1285–1298
- Aris R, Christian D, Tager I, et al. Effects of nitric acid gas alone or in combination with ozone on healthy volunteers. *Am Rev Respir Dis* 1993; 148:965–973
- Cockcroft DW, Murdock KY, Kirby J, et al. Prediction of airway responsiveness to allergen from skin sensitivity to allergen and airway responsiveness to histamine. *Am Rev Respir Dis* 1987; 135:264–267
- Kay AB. Asthma and inflammation. *J Allergy Clin Immunol* 1991; 87:893–910
- Aris RM, Christian D, Hearne PQ, et al. Ozone-induced airway inflammation in human subjects as determined by airway lavage and biopsy. *Am Rev Respir Dis* 1993; 148:1363–1372
- Basha MA, Gross KB, Gwizdala CJ, et al. Bronchoalveolar lavage neutrophilia in asthmatic and healthy volunteers after controlled exposure to ozone and filtered purified air. *Chest* 1994; 106:1757–1765
- Busse WW, Coffman RL, Gelfand EW, et al. Mechanisms of persistent airway inflammation in asthma: a role for T cells and T-cell products. *Am J Respir Crit Care Med* 1995; 152:388–393
- Devlin RB, McDonnell WF, Mann R, et al. Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. *Am J Respir Cell Mol Biol* 1991; 4:72–81
- Peden DB, Setzer RW, Devlin RB. Ozone exposure has both a priming effect on allergen-induced responses and an intrinsic inflammatory action in the nasal airways of perennially allergic asthmatics. *Am J Respir Crit Care Med* 1995; 151:1336–1345
- Vagaggini B, Taccola M, Cianchetti S, et al. Ozone exposure increases eosinophilic airway response induced by previous allergen challenge. *Am J Respir Crit Care Med* 2002; 166:1073–1077
- Aris RM, Tager I, Christian D, et al. Methacholine responsiveness is not associated with ozone-induced decreases in FEV₁. *Chest* 1995; 107:621–628
- Kleeberger SR, Reddy S, Zhang LY, et al. Genetic susceptibility to ozone-induced lung hyperpermeability: role of toll-like receptor 4. *Am J Respir Cell Mol Biol* 2000; 22:620–627
- Bergamaschi E, De Palma G, Mozzoni P, et al. Polymorphism of quinone-metabolizing enzymes and susceptibility to ozone-induced acute effects. *Am J Respir Crit Care Med* 2001; 163:1426–1431