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Effects of Ozone on Normal and Potentially Sensitive Human Subjects

Part I: Airway Inflammation and Responsiveness to Ozone in Normal and Asthmatic Subjects

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Part II: Airway Inflammation and Responsiveness to Ozone in Nonsmokers and Smokers

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Part III: Mediators of Inflammation in Bronchoalveolar Lavage Fluid from Nonsmokers, Smokers, and Asthmatic Subjects Exposed to Ozone: A Collaborative Study

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

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HEI Statement

Synopsis of Research Report Number 78

Effects of Ozone on Pulmonary Function and Airway Inflammation in Normal and Potentially Sensitive Human Subjects

BACKGROUND

Ozone is a common air pollutant and a major component of smog. The current National Ambient Air Quality Standard (which is currently being revised) is 0.12 parts per million (ppm), a level not to be exceeded for more than one hour, once per year. This standard is based largely on evidence that, in sensitive individuals, short-term exposure to ozone causes symptoms such as cough and shortness of breath, and reversible changes in some tests of lung function.

A procedure called spirometry is commonly used to measure lung function, particularly the measurement of forced expiratory volume in one second (FEV_1), which is the volume of air a subject can forcibly exhale in the first second following maximal inhalation. Exposure to ozone causes a transient decrease in FEV_1 in many people. This response is highly reproducible within an individual; however, there are marked differences among individuals in their sensitivities to ozone. Ozone also can cause the airways to become hyperresponsive to inhaled substances (such as the drug methacholine) that constrict the large airways or bronchi; this response is reflected in the airways becoming increasingly resistant to air flow. Finally, inhaling ozone can produce airway inflammation and cell injury, as evidenced by the appearance of cellular and biochemical markers of these reactions in airway fluids.

This report describes the results of two independent studies that were designed to (1) evaluate the range of ozone-induced responses in the general population, (2) study the effects of short-term exposure to ozone in populations with underlying airway inflammation (smokers and people with asthma), and (3) compare the responses in sensitive populations with those in normal subjects. A common goal of both studies was to characterize ozone-induced responses in populations thought to be most sensitive to ozone so that appropriate standards can be set to protect human health.

STUDY DESIGN

The studies described in this report were conducted by two investigator groups: Dr. John Balmes and colleagues of the University of California, San Francisco, and Dr. Mark Frampton and associates of the University of Rochester. Drs. Balmes and Frampton used similar ozone exposure regimens but different study populations: normal and asthmatic men and women exposed to 0.2 ppm ozone or clean air for four hours in the Balmes study, and male and female nonsmokers and smokers exposed to 0.22 ppm ozone or clean air for four hours in the Frampton study. In both studies subjects performed moderate exercise during the exposures. The investigators made a number of pulmonary function measurements and used a procedure called bronchoscopy to collect fluids and tissue samples from the subjects' airways. They analyzed these samples for indicators of inflammation and lung damage.

Balmes separated subjects into two categories on the basis of how much their FEV_1 response decreased after exposure to ozone: those least sensitive (smallest decrease) or most sensitive (greatest decrease) to ozone. The investigators addressed three issues: (1) Is an individual's reactivity to inhaled methacholine predictive of how his or her lung function would change after exposure to ozone? (2) What is the relation between ozone-induced airway inflammation (measured 18 hours after exposure) and changes in lung function (measured during and immediately after exposure)? and (3) Do the changes in lung function and markers of inflammation in response to ozone exposure differ between normal people and people with asthma?

This Statement, prepared by the Health Effects Institute and approved by its Board of Directors, is a summary of two research projects sponsored by HEI from 1991 to 1995. Dr. John R. Balmes and colleagues of the University of California, San Francisco, CA, conducted the first study, *Airway Inflammation and Responsiveness to Ozone in Normal and Asthmatic Subjects*; and Dr. Mark W. Frampton and associates from the University of Rochester School of Medicine and Dentistry, Rochester, NY, conducted the second study, *Airway Inflammation and Responsiveness to Ozone in Nonsmokers and Smokers*. The investigators jointly examined *Mediators of Inflammation in Bronchoalveolar Lavage Fluid from Nonsmokers, Smokers, and Asthmatic Subjects Exposed to Ozone: A Collaborative Study*. The Research Report contains three Investigators' Reports and a Commentary on the studies prepared by the Institute's Health Review Committee.

Dr. Frampton's overall objectives were similar to those of the Balmes study, except that Dr. Frampton studied smokers as a potentially susceptible population. Because he was able to identify only a few smokers as sensitive to ozone (on the basis of their FEV_1 responses), all smokers were grouped together for further study. Dr. Frampton measured pulmonary function and markers of inflammation immediately after air or ozone exposure and 18 hours later, thus allowing examination of the time course of these two responses in the same subject. The two investigator groups collaborated to compare the levels of three markers of airway inflammation among the normal, asthmatic, and smoker groups.

RESULTS AND IMPLICATIONS

These studies produced both confirmatory and new information about the responses of normal and potentially susceptible people to environmentally relevant concentrations of ozone. Both investigators confirmed findings from other laboratories. Namely, that exposure to ozone at levels that occur in ambient settings results in reversible changes in FEV_1. Also, many people develop an inflammatory response in their airways (as determined by the appearance of markers of inflammation and cell injury in their lung fluids) after being exposed to ozone. Furthermore, they found no correlation among ozone-induced respiratory symptoms, changes in pulmonary function (as measured by FEV_1), and markers of airway inflammation.

Both Balmes and Frampton found that a subject's airway responsiveness to methacholine did not correlate with the reduction in FEV_1 observed after ozone exposure. Moreover, normal subjects who were characterized as being most sensitive or least sensitive to ozone (by measurements of FEV_1) did not differ in their ozone-induced inflammatory responses. This implies that even if pulmonary function does not change, other potentially harmful effects of ozone, such as airway inflammation, may occur. Balmes found that breathing ozone caused similar changes in FEV_1 in normal and asthmatic subjects. However, 18 hours after exposure to 0.2 ppm ozone for four hours, the levels of some markers of inflammation in lung fluids from asthmatic subjects were higher than the levels observed in normal subjects. Although this suggests that when people with asthma are exposed to ozone, they may develop more intense respiratory tract inflammation than healthy people, further studies are needed at multiple time points after exposure.

Dr. Frampton and colleagues found that although smokers have a greater degree of underlying airway inflammation than nonsmokers, they actually had smaller ozone-induced decrements in FEV_1 than nonsmokers. The magnitude of the inflammatory response to ozone was similar in smokers and nonsmokers. It was also the same in subjects characterized as most sensitive and least sensitive to ozone on the basis of their FEV_1 responses. The investigators found that some compounds (called cytokines) that are known to be important mediators of inflammation were present immediately after exposure to ozone. However, the overall inflammatory response was greater 18 hours after ozone exposure (when lung function had returned toward normal levels) than immediately after exposure.

An unexpected finding of the collaborative study was that the effects of the bronchoscopic procedures used to obtain the airway fluids and biopsy specimens may persist for many weeks in some subjects. This has critical implications for studies involving repeated invasive procedures.

The results of the studies reported here suggest that measuring symptoms and pulmonary function (using standard measurements of air flow) may not be sufficient to evaluate the potential risks associated with ozone exposure. Many individuals experience airway inflammation after being exposed to ozone, and this response is not reflected in the FEV_1 response. The significance of ozone-induced inflammation in terms of subsequent airway disease has not been determined. More attention needs to be directed toward assessing the effects of ozone on small airway function and toward developing noninvasive measurements of airway inflammation.

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Effects of Ozone on Normal and Potentially Sensitive Human Subjects

Part I: Airway Inflammation and Responsiveness to Ozone in Normal and Asthmatic Subjects

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ABSTRACT

We report here the results of a multiphase project to assess the significance of airway responsiveness and airway injury in ozone (O_3)* sensitivity. In Phase I, we measured the preexposure methacholine responsiveness of 66 normal subjects and then exposed these subjects to 0.2 ppm O_3 for 4 hours with moderate exercise. Preexposure methacholine responsiveness was weakly correlated with O_3-induced increases in specific airway resistance (sRaw) but not O_3-induced declines in forced expiratory volume in one second (FEV_1) or forced vital capacity (FVC). In addition, O_3-induced lower respiratory symptoms were not well correlated with O_3-induced changes in lung function. In Phase II, we exposed 23 normal subjects to O_3, following an identical protocol to that of Phase I, and then performed bronchoscopy with proximal airway lavage (PAL), bronchoalveolar lavage (BAL), and bronchial biopsy at 18 hours after exposure. Ozone-induced increases in percentage of neutrophils and total protein concentration were observed in both bronchial fraction and BAL fluids; increased percentage of neutrophils also was observed in PAL fluid. These increases were correlated with O_3-induced increases in sRaw, but not with O_3-induced declines in FEV_1 or FVC. Ozone also appeared to increase expression of intercellular adhesion molecule-1, an important mediator of neutrophil recruitment, in bronchial mucosa. In Phase III, we exposed a group of 19 asthmatic subjects to O_3, following a protocol identical to that of Phase II. We then compared the lower respiratory symptom and lung function responses of the asthmatic subjects to those of the 81 normal subjects who participated in Phase I, Phase II, or both. The changes in the PAL and BAL fluids of the asthmatic subjects were compared with those of the normal subjects who participated in Phase II. Although both the asthmatic and nonasthmatic subjects showed significant O_3-induced changes in lower respiratory symptoms, FEV_1, FVC, and sRaw, no significant differences were found between the groups. For sRaw, however, a nonsignificant trend toward a greater O_3-induced increase was noted for the asthmatic subjects. In contrast, the O_3-induced increases in percentage of neutrophils and total protein concentration in BAL fluid were significantly greater for the asthmatic subjects than for the nonasthmatic subjects. These data suggest that although the lower respiratory symptom and lung function responses to O_3 are not markedly greater in asthmatic subjects than in healthy subjects, the inflammatory response of the asthmatic lung may be more intense.

INTRODUCTION

Ozone is a major component of urban air pollution to which millions of people are periodically exposed in many areas of North America. In contrast to certain other air pollutants, a relatively extensive scientific data base has been gathered on the health effects of O_3. A number of controlled human exposure studies suggest that the National Ambient Air Quality Standard (NAAQS) for O_3 may not provide a margin of safety to protect the most sensitive members of the population from adverse health effects (McDonnell et al. 1983, 1985a, 1993; Gong et al. 1986; Horstman et al. 1990; Devlin et al. 1991). Implicit in this statement is that the O_3-sensitive segment of society has been identified. But, in fact, this is not the case. Although the pulmonary function responses to O_3 have been characterized in small groups of subjects, the determinants of these responses have been elusive. Furthermore, although sensitivity to O_3 is most often defined in terms of decreases in FEV_1, it is not clear that this is the best measure of the adverse effects of exposure to O_3. Failure to pinpoint the

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

This Investigators' Report is Part I of Health Effects Institute Research Report Number 78, which also includes *Part II: Airway Inflammation and Responsiveness to Ozone in Smokers and Nonsmokers*, by Mark Frampton and associates; *Part III: Mediators of Inflammation in Bronchoalveolar Lavage Fluid from Nonsmokers, Smokers, and Asthmatic Subjects Exposed to Ozone: A Collaborative Study*, by Mark Frampton, John R. Balmes, and colleagues; a Commentary by the HEI Health Review Committee, and an HEI Statement about the research project. Correspondence concerning this Investigators' Report may be addressed to Dr. John R. Balmes, Lung Biology Center, Box 0854, University of California, San Francisco, CA 94143-0854.

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factors that determine O_3 sensitivity hampers regulatory efforts aimed at protecting public health. With the current U.S. Environmental Protection Agency's (EPA) emphasis on the FEV_1 response as the primary adverse health effect of O_3 exposure, large numbers of O_3-sensitive individuals may be exposed to levels capable of inducing oxidant injury to the respiratory tract without experiencing warning symptoms. Knowing the predictors of O_3 sensitivity would allow regulatory agencies to establish and implement measures to minimize the exposure of those individuals at greatest risk during high-O_3 periods. Therefore, elucidating the determinants of O_3 sensitivity would have far-reaching health benefits.

The results of many controlled human exposure studies conducted over the past several decades have documented that short-term inhalation of O_3 causes dose-dependent decrements in FVC and relatively smaller increases in sRaw, in addition to decrements in FEV_1 as described above (McDonnell et al. 1983; Hazucha 1987; Horstman et al. 1990). Many studies also have demonstrated marked interindividual, but minimal intraindividual, differences in response to O_3 (Glinder et al. 1983; Avol et al. 1984; Kulle et al. 1985; McDonnell et al. 1985b). This observation suggests that the response to O_3 may be determined predominantly by characteristics inherent in the individual. Demographic differences such as age, gender, race, weight, and height have not been useful for predicting O_3 sensitivity (McDonnell 1989). Until recently, individuals with airway hyperresponsiveness, whether asthmatic or not, were not considered to be at increased risk of adverse effects of O_3 (Linn et al. 1978; Koenig et al. 1987; McDonnell et al. 1987). However, in a previous study, we found that normal subjects selected for O_3 sensitivity as defined by a 10% or greater fall in FEV_1 after a 4-hour exposure to 0.2 ppm O_3 (exercising 50 min/hour, which produced a volume of expired gas [$\dot{V}_E$], or ventilatory rate, of approximately 40 L/min) had a significantly lower preexposure PC_{100} (the provocative concentration of methacholine that causes a 100% increase in sRaw) than did the O_3-insensitive subjects (2.95 mg/mL vs. 18.67 mg/mL, $p < 0.005$) (Aris et al. 1991). The Phase I study reported here was performed prospectively to test the hypothesis that nonspecific airway responsiveness can predict O_3 sensitivity in normal adults.

Several human studies have demonstrated that O_3 exposures capable of causing changes in lung function can also cause cellular and biochemical evidence of injury or inflammation in PAL and BAL fluids (Seltzer et al. 1986; Koren et al. 1989; Aris et al. 1993a,b). As was noted for lung function responses, considerable interindividual variability is apparent in the magnitude of the changes in the lavage fluid contents in response to O_3. What was unknown when we began the project reported here was whether the magnitude of change in FEV_1, FVC, or sRaw could predict the degree of respiratory tract inflammation. Recently, Weinmann and coworkers (1995b) demonstrated in a small number of subjects that decrements in forced expiratory flow (from 25% to 75% of the vital capacity [FEF_{25-75}] measured isovolumetrically) persisted longer than other O_3-induced changes in lung function, and that these decrements also correlated with one marker of O_3-induced inflammation, fibrinogen concentration in BAL fluid.

The mechanism by which O_3 induces decrements in FEV_1 and FVC appears to be neurally mediated involuntary inhibition of inspiratory effort (Hazucha et al. 1989) involving stimulation of airway C-fibers (Coleridge et al. 1993). The mechanism by which O_3 causes mild increases in sRaw is less clear, but is presumably due to airway narrowing (Weinmann et al. 1995a). If airway narrowing does occur, it is likely due to mucosal edema, but whether this is a result of direct O_3-induced injury or neurogenic inflammation is also unclear. Ozone-induced changes in PAL and BAL fluids may result from direct cytotoxicity to the airway epithelium, but secondary injury due to the inflammatory response may also play a role (Castleman et al. 1980; Aris et al. 1993b). Although O_3-induced spirometric decrements and sRaw increases are more easily measured, the degree of airway inflammation may have more important long-term consequences. We designed the Phase II study reported here to test the hypothesis that acute O_3-induced lung function changes are correlated with PAL and BAL fluid indicators of respiratory tract inflammation measured 18 hours after exposure.

Although normal people may experience adverse respiratory effects from exposure to O_3, it has been suggested that people with asthma may be an especially susceptible subgroup (Boushey 1989; Balmes 1993; Koenig 1995; Koren and Bromberg 1995). This is reasonable given that two characteristic features of asthma, airway inflammation and increased nonspecific airway responsiveness, are acute responses induced by O_3 inhalation. Several controlled exposure studies of asthmatic and atopic subjects, however, have failed to show enhanced spirometric responses to short-term O_3 inhalation (Linn et al. 1978; Koenig et al. 1987; McDonnell et al. 1987). This result is not as surprising as it might seem when one considers that the primary mechanism for O_3-induced decrements in FEV_1 is decreased inspiratory capacity rather than airway obstruction (Hazucha et al. 1989). One study involving exposure to a relatively high dose of O_3, however, did show enhanced lung function responses in asthmatic subjects compared with normal subjects (Kreit et al. 1989). In contrast to the results of controlled exposure studies of asthmatic subjects,

several epidemiologic studies have provided evidence that high ambient O_3 concentrations are associated with an increased rate of asthma attacks (Whittemore and Korn 1980; Holguin et al. 1985). Other studies have documented increased hospital admissions or emergency department visits for respiratory disease, including asthma, after days of high ambient O_3 levels (Bates and Sizto 1983; White et al. 1994). The mechanism of increased asthma morbidity associated with O_3 pollution is more likely related to airway inflammation than lung function responses. We designed the Phase III study reported here to test the hypothesis that O_3 exposure induces a greater inflammatory response in asthmatic than in nonasthmatic persons.

Previous work from our laboratory has demonstrated that O_3 exposure can cause neutrophil infiltration of the bronchial mucosa in human subjects (Aris et al. 1993b). Therefore, we obtained bronchial biopsies during bronchoscopies of Phase II and Phase III subjects for leukocyte morphometry. We also obtained biopsies from Phase II subjects for ultrastructural study of O_3-induced airway epithelial injury by electron microscopy. Because of our interest in the mechanism of neutrophil recruitment to the lung after O_3 exposure, we hypothesized that O_3 would induce increased expression of certain cell adhesion molecules known to be involved in leukocyte recruitment in other models: intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM), and E-selectin (also known as endothelial leukocyte adhesion molecule, or ELAM). We also hypothesized that O_3 would induce increased expression of certain integrins, a family of adhesion molecules that often recognize specific components of the extracellular matrix as ligands, but which also can interact with cell surface proteins to mediate both heterotypic and homotypic cell-cell interactions. The integrin subunits of interest in this study are those that can be expressed in the airway epithelium: $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$, $\alpha 9$, $\beta 3$, $\beta 5$, and $\beta 6$. We used immunohistochemistry to assess the roles of these adhesion molecules in O_3-induced airway injury and inflammation.

SPECIFIC AIMS

1. To determine if airway responsiveness to methacholine is associated with sensitivity to O_3 (as measured by decrement in FEV_1 following exposure).
2. To determine the effects of prolonged exposure to ambient levels of O_3 on proximal airway and distal lung injury and inflammatory responses in "sensitive" and "insensitive" subjects (as assessed by evidence of inflammatory cells recruited to the airways, increased epithelial permeability, and initiation of injury repair).
3. To determine if the degree of O_3-induced proximal airway or distal lung injury or inflammation can be predicted by lung function responses to exposure in normal subjects and in subjects with asthma.

PHASE I. RELATIONSHIP OF NONSPECIFIC AIRWAY RESPONSIVENESS TO OZONE SENSITIVITY

METHODS AND STUDY DESIGN

Subjects

Based on our previous data (Aris et al. 1991), we estimated that 66 subjects would be required to test a difference in methacholine responsiveness (PC_{100} of 8 mg/mL) between the upper and lower tertiles of FEV_1 responses to O_3 with a beta error = 0.15 and an alpha error = 0.05. We recruited normal, athletic adults, ages 18 through 50, by advertising at two large college campuses in San Francisco. Telephone interviews were conducted to collect data on athletic activity, smoking status, and history of respiratory or medical problems. After explaining our protocol, we invited 88 nonsmoking (defined as having smoked fewer than 50 cigarettes in the last year and none in the last 6 weeks) adults without active medical problems to participate in the study. Six individuals refused to participate, largely because of time constraints. The other 82 subjects had no history of recent (i.e., within the last 6 weeks) respiratory infection and came to the laboratory for Day #1 of the protocol. Each subject completed a medical history questionnaire. Eight subjects dropped out of or were excused from the study before completing the first exposure because of either a language barrier (1), time constraints (1), chronic antihistamine use (1), a preexisting knee injury (1), an inability to complete the methacholine test (1), failure to return (2), or an inability to perform sustained exercise (1). Eight additional subjects dropped out after completing one exposure because of either an injury sustained outside of the protocol (2), time constraints (3), failure to return (2), or a recent hysterectomy (1). The remaining 66 subjects completed the entire protocol. Twenty-two had a history of light smoking (defined as less than 10 pack-years of total consumption), but none had smoked more than 50 cigarettes in the past year. Subject characteristics are listed in Table 1. Predicted values for the spirometric parameters are those of Knudson and coworkers (1983). No subject took prescription medications, inhaled beta-adrenergic agonists, antihistamines, nonsteroidal antiinflammatory agents, or vitamins C and E within 1 week of testing. No subject

consumed tea, coffee, or hot chocolate within 4 hours of any part of the experiment. All subjects received financial compensation for their participation.

Experimental Protocol

Day 1: Methacholine Airway Responsiveness and Allergy Skin Testing We chose methacholine responsiveness as the primary independent variable in Phase I because it is considered to be the best measure of nonspecific airway responsiveness and is a commonly used clinical test. Each subject completed a medical history questionnaire, was informed of the risks of the experimental protocol, and signed consent forms approved by the Committee on Human Research of the University of California, San Francisco. Each subject then underwent methacholine responsiveness testing as previously described (Aris et al. 1991) with minor modifications. We measured sRaw before and after inhalation of five deep breaths of phosphate-buffered saline or doubling concentrations of methacholine (0.63, 1.25, 2.5, 5, 10, 20, 40, 80 mg/mL) delivered by a nebulizer (No. 646; Devilbiss Co., Somerset, PA) with a dose-metering device calibrated to deliver 0.01 mL/breath. Serially increasing concentrations were administered until there was an increase in sRaw greater than 100% from the post-saline baseline value (PC_{100}), or until the 80 mg/mL dose had been given. The PC_{100} was calculated by log-linear interpolation. A 100% increase in sRaw was chosen as the target endpoint for methacholine responsiveness testing because it is more sensitive than PC_{20} (i.e., the concentration of methacholine that produces a 20% decrease in FEV_1). A PC_{100} was obtained for all 66 subjects (Figure 1). The PC_{100} values were log-transformed before further analysis.

All subjects underwent allergy skin-prick testing on the anterior forearm by intraepidermal injection (Morrow-Brown needles, Aller//guard, Topeka, KS) of 11 allergens (mixed grasses, mixed trees, mixed weeds, cat- and dog-hair epithelium, house dust-HS, house dust-BB, *Alternaria*, *Hormodendrum*, *Dermatophagoides pteronyssinus*, and *Dermatophagoides farinae*) and both negative (saline) and positive (5 mg/mL histamine) control substances, according to the method of Pepys, with minor modifications (Pepys 1975; Brown et al. 1981). All allergen preparations except for the histamine and house dust-BB (Berkeley Biologicals, Berkeley, CA) were obtained from Hollister Steir (Miles Inc., Elkhart, IN). The length and width of both the erythema and induration at each test site were measured with a metric ruler 15 minutes after injection. If a histamine response of less than 5 mm was observed, the skin tests were repeated at a later date. A subject was considered to have a positive skin test if the mean of the length and width of the induration for any antigen was at least 2 mm larger than that for histamine.

Days 2 and 3: Ozone and Air Exposure Protocols On two subsequent days, each separated from the other by 3 weeks, the subjects were exposed in random, double-blind fashion to 0.2 ppm O_3 or to filtered air. Each subject had his or her baseline sRaw, FEV_1, and FVC measured 5 minutes before undergoing each exposure. These lung function tests were selected as the primary dependent variables for Phase I because abundant data from previous controlled human exposure studies, including some from our laboratory, indicate that O_3-induced changes in these tests are reproducible within subjects. The exposure protocol was 4 hours in duration with 50-minute exercise periods alternating with 10-minute measurement and rest periods. Hourly measurements of lung function were made during exposure also because considerable evidence from previous controlled human studies indicates that O_3-induced changes can be detected with this measurement interval. The exposures

Table 1. Phase I. Subject Characteristics[a]

	Mean	SD
Age (years)	27.0	4.5
FEV_1 (L)	3.96	0.75
FEV_1 (% predicted)	97.3	10.4
FVC (L)	4.72	0.92
FVC (% predicted)	98.8	9.8
sRaw (L × cm H_2O/L/sec)	3.2	1.3
PC_{100} (mg/mL)	8.5	9.6
Gender (M/F)	42/24	–
Number of positive skin tests	20	–

[a] $n = 66$.

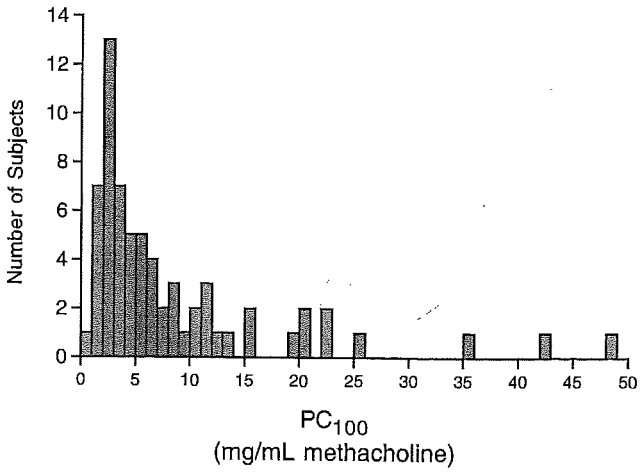

Figure 1. Phase I. Distribution of methacholine responsiveness in 66 subjects measured as PC_{100}.

took place in a chamber filled with filtered air at 20°C and 50% relative humidity, to which O_3 either was or was not added. Each subject exercised on a treadmill (Model M9.1, Precor Co., Bothell, WA) or a cycle ergometer (No. 18070, Gould Godart, Bilthoven, The Netherlands), or both; the equipment was individually adjusted to produce a targeted $\dot{V}_E$, as measured with a pneumotachograph (No. 3, A. Fleisch, Lausanne, Switzerland), of 25 L/min/m^2 body surface area. The subjects chose to either cycle, jog, or alternate between the two. The tidal volume and respiratory rate were measured, and the ventilatory rate was calculated at the 10-minute, 25-minute, and 40-minute points of each 50-minute exercise period, and the workload was adjusted to maintain the targeted ventilatory rate. The mean ventilatory rate for the subjects undergoing exposure to air (44.1 L/min) or O_3 (43.5 L/min) were similar. During each 10-minute measurement and rest period, each subject immediately exited the chamber and sRaw, FEV_1, and FVC were measured, in that order. Each subject was allowed to rest, drink water or juice, and eat *ad libitum* during the remainder of the measurement and rest period. Symptom questionnaires consisting of a 5-point rating scale (0 = none and 4 = severe) for each of 12 symptoms (lower respiratory: chest discomfort or tightness, chest pain on deep inspiration, shortness of breath, cough, phlegm or sputum production, and wheeze; upper respiratory: throat or nasal irritation; nonrespiratory: anxiety, eye irritation, headache, and nausea) were self-administered immediately before and immediately after each exposure.

Pulmonary Function Measurements

Specific airway resistance was determined as the product of airway resistance and thoracic gas volume, both having been measured in a constant-volume body plethysmograph (Warren E. Collins, Braintree, MA). Subjects did not breathe deeply prior to sRaw measurements, and these measurements were made at functional residual capacity. Specific airway resistance was calculated as the mean of five measurements, each taken 30 seconds apart. For 11 subjects, sRaw measurements were not obtained after one of the two exposures due to technical problems in operating the body plethysmograph. Spirometry was performed on a dry, rolling-seal spirometer (S400, Spirotech Division, Anderson Instruments, Inc., Atlanta, GA). Mean values for FVC and FEV_1 were calculated from three acceptable FVC maneuvers (American Thoracic Society 1987), each obtained approximately 30 seconds apart. On the first visit to the laboratory, the mean values for FEV_1 and FVC were calculated from six (two sets of three, 5 minutes apart) FVC maneuvers to minimize the effect of first-time spirometry variability in establishing a baseline.

The Exposure Chamber and Atmospheric Monitoring

All exposures took place in a 2.5-m × 2.5-m × 2.4-m steel and glass chamber (Model W00327-3R, Nor-Lake, Inc., Hudson, WI), which was custom-built and designed to maintain chamber temperature and relative humidity within 2.0°C or 4% of their respective set points (DSC 8500, Johnson Controls, Poteau, OK). The exposure chamber, and air filtration, humidification, and conditioning systems have been previously described in detail (Aris et al. 1990, 1991). Temperature and relative humidity, which were recorded every 30 seconds for the duration of the exposure and averaged, were similar for the air (20.3°C ± 1.0°C and 50.9% ± 3.2%) and O_3 exposures (20.1°C ± 0.6°C and 49.2% ± 3.8%).

Ozone was produced from oxygen (O_2) supply gas with a corona-discharge O_3 generator (Model T 408, Polymetrics, Inc., San Jose, CA) and analyzed with an ultraviolet light photometer (Model 1008 PC, Dasibi, Glendale, CA). The O_3 concentration, which was measured every 3 minutes, displayed in real-time (LabVIEW 2, National Instruments, Austin, TX), and stored by a microcomputer (Model IIsi, Apple Computer Inc., Cupertino, CA), averaged 0.19 ± 0.01 ppm and 0.00 ± 0.01 ppm for the O_3 and air exposures, respectively. The O_3 analyzer was calibrated biannually by the California Air Resources Board with a standard O_3 generator and analyzer instrument (Model 1009 IC, Dasibi), and was checked for precision in the laboratory on a monthly basis.

STATISTICAL METHODS AND DATA ANALYSIS

For each exposure, a set of differences for each measure of pulmonary function was obtained by subtracting the baseline value from each of the four hourly measurements made during the exposure. These differences were then used in a repeated multivariate analysis of variance (MANOVA) to test the null hypothesis that no differences would be found between the exposures (SAS 1988). To determine if the responses to O_3 were greater than the responses to air, the differences of the values for the O_3-air differences were computed [e.g., (4-hour FEV_1 – baseline FEV_1)O_3 – (4-hour FEV_1 – baseline FEV_1)air] and these differences-of-the-differences were subjected to a MANOVA. Separate analyses were carried out for FEV_1, FVC, and sRaw. A second set of repeated MANOVA was carried out that included log PC_{100} as a main effect and as an interaction with time.

Linear regression analyses were carried out to further evaluate the effect of PC_{100} on the response to O_3, and to specifically test the principal hypothesis that baseline nonspecific airway hyperresponsiveness would predict the FEV_1 response to O_3. The dependent variable for each analysis was the maximum O_3-minus-air difference for

each measure of pulmonary function (FEV_1, FVC, and sRaw). We carried out a second set of regression analyses using the air-corrected area under the curve (AUC) for each measure of pulmonary function (FEV_1 AUC, FVCAUC, and sRawAUC) as the dependent variable and PC_{100} as the independent variable. The AUC was calculated by the trapezoidal method and the AUC for air was subtracted from the AUC for O_3 (Figure 2).

Based on the power calculation described above, we compared the subjects most sensitive to O_3 with those least sensitive by dividing the group into tertiles based on the air-corrected FEV_1AUC responses, and made comparisons between the upper and lower tertiles (22 subjects were included in each tertile with the following ranges: upper: −19.6% to −57.1% change; middle: −13.3% to −19.4% change; lower: +1.6% to −11.1% change). Differences between these two groups with regard to history of respiratory symptoms or tobacco use, baseline lung function, antigen skin test positivity, and symptom responses after O_3 were assessed with the Wilcoxon rank sum test. We also divided the group into responders and nonresponders by air-corrected, O_3-induced maximum decreases in FEV_1 of more than 10%. The results (data not presented) were similar to the tertile analysis.

Categorical variables were analyzed with the chi-squared test. To determine if there were associations between physiologic and symptomatic responses to O_3, linear regressions of FEV_1AUC on each of the 12 symptom scores and on total respiratory symptom scores were performed.

RESULTS OF PHASE I

Pulmonary Function Responses

With exposure to O_3 and regardless of time after baseline, the mean ± SD maximum change (and mean ± SD percentage of change) in FEV_1 was −0.82 ± 0.63 L (−18.6% ± 13.7%),

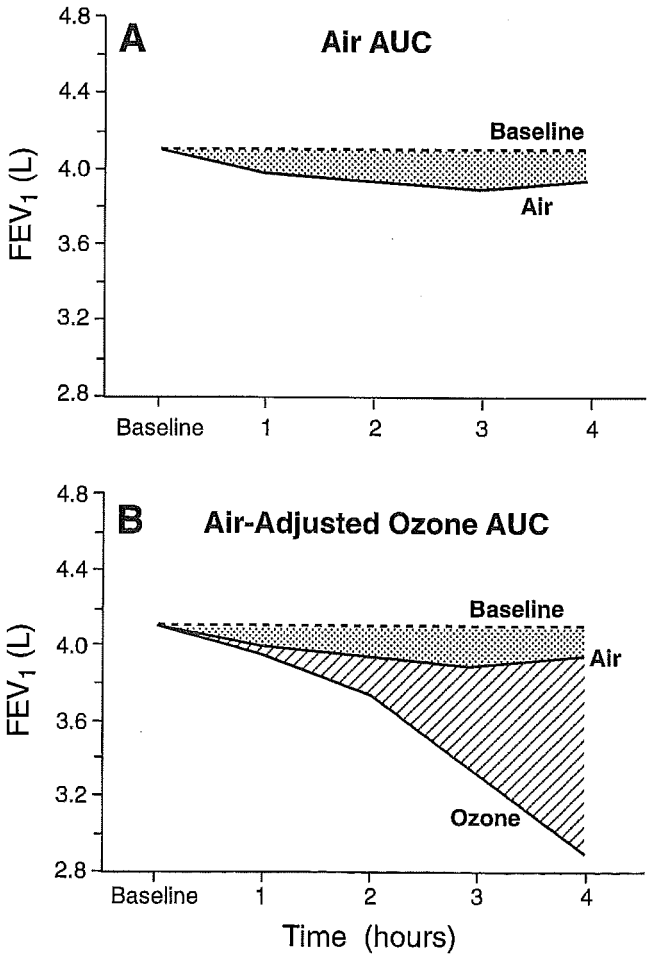

Figure 2. Phase I. Sample air FEV_1AUC determination (A) and sample air-adjusted O_3 FEV_1AUC determination (B).

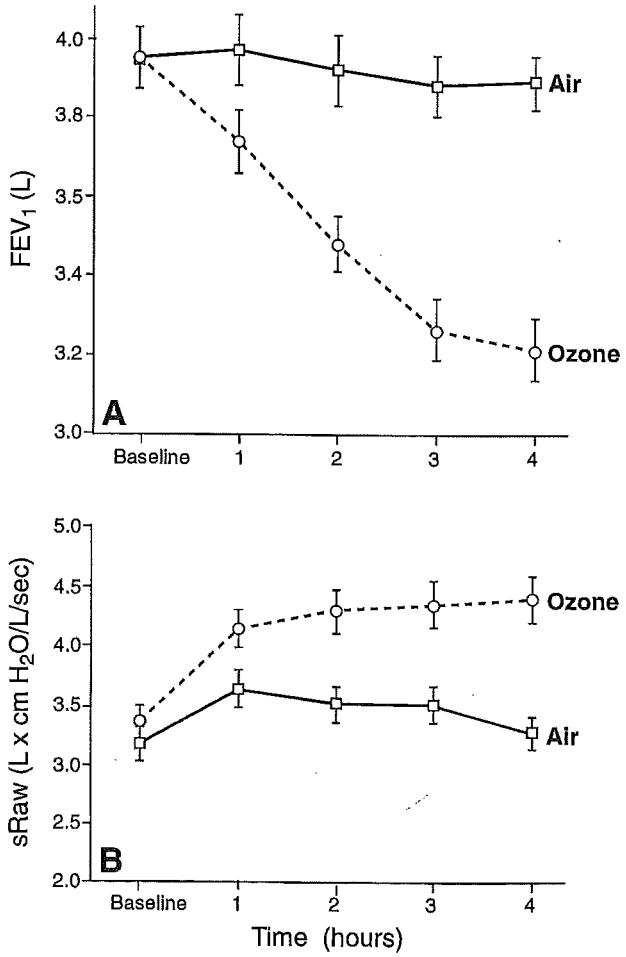

Figure 3. Phase I. Mean ± SE values for (A) FEV_1 (n = 66) and (B) sRaw (n = 55) at baseline and at hourly intervals for normal subjects who completed 4-hour exposures to both O_3 (0.2 ppm) and air.

in FVC was −0.69 ± 0.48 L (−14.6% ± 10.2%), and in sRaw was +1.5 ± 1.1 L × cm H_2O/L/sec (+35.4% ± 34.2%). With exposure to air, the change in FEV_1 was −0.14 ± 0.22 L (−3.5% ± 5.5%), in FVC was −0.16 ± 0.20 L (−3.3% ± 4.2%), and in sRaw was +0.74 ± 0.63 L × cm H_2O/L/sec (+17.6% ± 12.6%). Using the baseline-minus-postexposure difference for each measure of lung function rather than the maximal change during the exposure produced similar results. The mean baseline and hourly FEV_1 and sRaw values for the 66 subjects who completed both the air and O_3 exposures are illustrated in Figure 3. The group mean decreases in FEV_1 and FVC, and the group mean increases in sRaw were significantly larger at every time-point during exposure to O_3 when compared with air exposure (MANOVA, $p < 0.05$). The MANOVA also demonstrated significantly greater changes in FEV_1 ($p < 0.01$), FVC ($p < 0.01$), and sRaw ($p = 0.03$) with the exposure to O_3 compared with exposure to air.

The PC_{100} was not associated with air-corrected, O_3-induced maximum changes in FEV_1 or FEV_1AUC ($p > 0.5$ for both), but was weakly associated with O_3-induced maximal changes in sRaw ($F_{1,54} = 2.85$, $p = 0.09$). A similar association was observed between PC_{100} and sRawAUC ($r = -0.14$, $p = 0.08$). Figure 4 shows the FEV_1AUC and the sRawAUC plotted against log PC_{100}. A weak association ($r = -0.28$, $p = 0.02$) was noted between the air-corrected, O_3-induced changes in FEV_1AUC and sRawAUC, which is shown in Figure 5.

Pulmonary function responses for the "least-sensitive" (lowest tertile of air-corrected FEV_1AUC distribution) and "most-sensitive" (highest tertile of air-corrected FEV_1AUC distribution) subjects are displayed in Table 2. By definition, the most-sensitive subjects had a larger mean FEV_1AUC decrease after O_3 than the least-sensitive subjects. There was no difference in the O_3-induced sRawAUC between the two groups.

To specifically address whether females were more sensitive to O_3 than males using the FEV_1 criterion, we compared the air-corrected, O_3-induced mean change in FEV_1 for females ($n = 24$) and males ($n = 42$). For the group as a whole, females had mean ± SD maximal percentage of

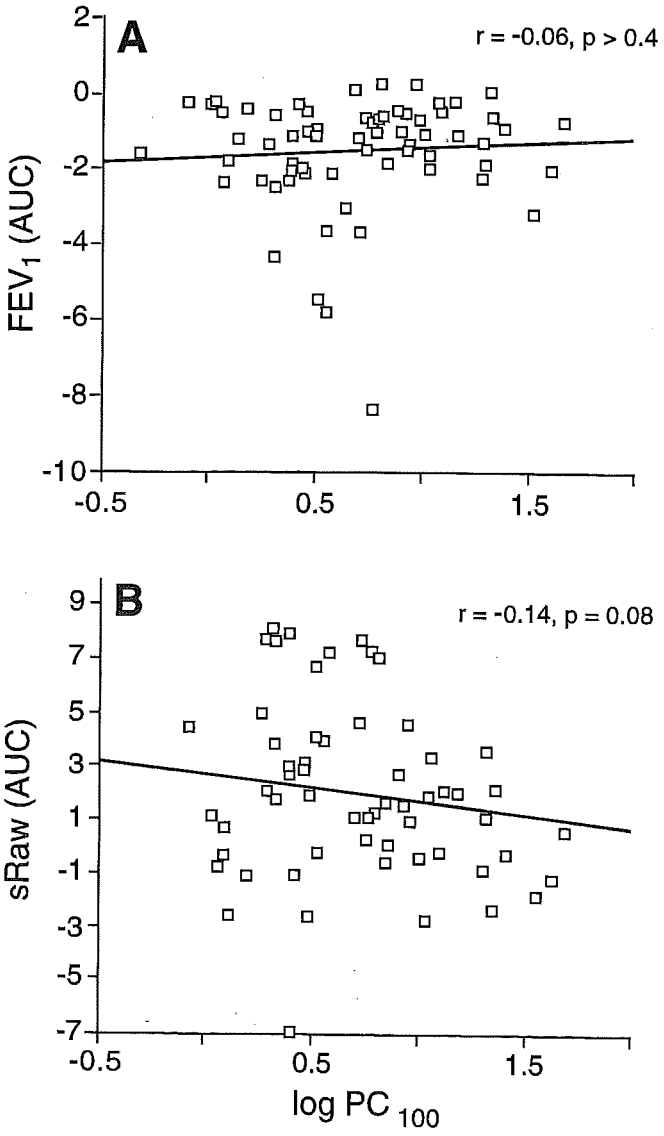

Figure 4. Phase I. Scatter plots of (A) the air-corrected change in FEV_1AUC ($n = 66$) and (B) the air-corrected change in sRawAUC ($n = 55$) across O_3 exposure versus baseline log PC_{100} for normal subjects who completed 4-hour exposures to both O_3 (0.2 ppm) and air.

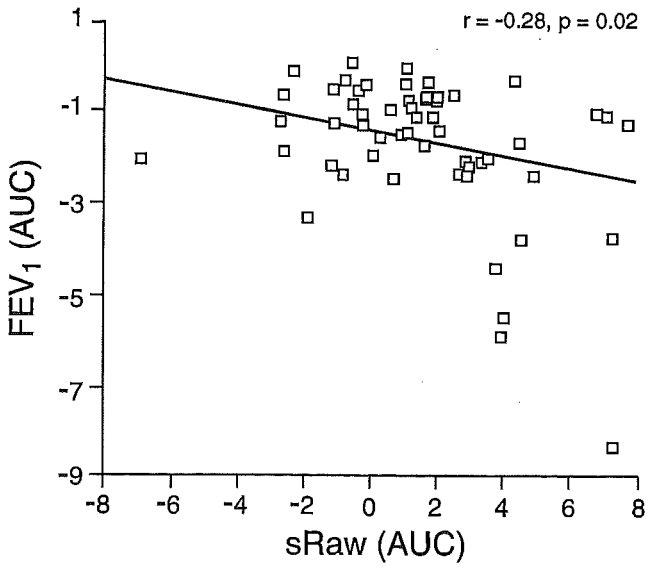

Figure 5. Phase I. Scatter plot of the air-corrected change in FEV_1AUC for O_3 versus the air-corrected change in sRawAUC for O_3 for 55 normal subjects who completed 4-hour exposures to both O_3 (0.2 ppm) and air, and who had both FEV_1 and sRaw measurements during both exposures.

changes in FEV_1 (−15.9% ± 10.2%) and sRaw (+37.7% ± 27.4%) that were comparable to those measured in males (−18.6% ± 13.8% and +34.2% ± 37.6%, respectively).

Symptoms

As expected, exposure to O_3 caused significantly more symptoms (mean ± SD change in symptom score = 8.0 ± 2.6) than exposure to air (mean ± SD change in symptom score = 0.5 ± 0.3) for the group as a whole. The air-adjusted FEV_1AUC for O_3 demonstrated weak to moderate associations with the lower respiratory symptoms of cough (r = −0.37, p = 0.002), wheeze (r = −0.29, p = 0.019), chest pain on deep inspiration (r = −0.31, p = 0.01), and shortness of breath (r = −0.37, p = 0.002), but not with chest discomfort or tightness (r = −0.18, p = 0.14), or sputum production (r = +0.06, p = 0.64). The air-adjusted FEV_1AUC for O_3 was associated with total lower respiratory symptoms (r = −0.36, p = 0.003). The air-adjusted FEV_1AUC was not associated with the upper respiratory symptoms of nasal irritation and throat irritation, nor with the nonrespiratory symptoms of nausea and eye irritation ($p > 0.1$ for all calculations). The air-adjusted sRawAUC for O_3 was not associated with any symptom except wheeze (r = 0.31, $p < 0.01$).

Selected symptom responses for the least- and most-sensitive groups are also displayed in Table 2. As a group, the most-sensitive subjects experienced significantly more coughing and chest discomfort after O_3 than the least-sensitive subjects, but both groups had similar levels of wheezing.

Table 2. Phase I. Physiologic Changes in Lung Function and Symptom Responses After Exposure to 0.2 ppm Ozone

	Least-Sensitive Group	Most-Sensitive Group	
	Lowest Tertile of FEV_1AUC Distribution	Highest Tertile of FEV_1AUC Distribution	p
ΔFEV_1 (L)	− 0.30 ± 0.15	− 1.33 ± 0.58	0.0001[a]
ΔFEV_1AUC	− 0.43 ± 0.28	− 3.15 ± 1.61	0.0001[a]
ΔsRaw(L × cm H_2O/L/sec)	+ 1.02 ± 1.03	+ 1.32 ± 1.05	0.4
ΔsRawAUC	+ 1.1 ± 2.8	+ 2.5 ± 3.4	0.2
Chest discomfort[b]	1.2 ± 1.6	2.1 ± 1.1	0.03[a]
Cough[b]	1.0 ± 1.4	2.2 ± 1.3	0.003[a]
Wheeze[b]	0.3 ± 0.6	0.5 ± 0.8	0.4

[a] Statistically significant at $p < 0.05$.

[b] The symptom questionnaire used a 5-point rating scale, with 0 = none and 4 = severe, for 12 symptoms (see Appendix C).

Table 3. Phase I. Subject Characteristics by Response to Ozone[a]

	Least-Sensitive Group	Most-Sensitive Group	
	Lowest Tertile of FEV_1AUC Distribution	Highest Tertile of FEV_1AUC Distribution	p
Age (years)	28.2 ± 1.3	25.6 ± 0.7	0.08
Gender (M/F)	15/7	18/4	
History of smoking	10 (45%)	4 (18%)	0.05
History of cough	1(5%)	0	NS[b]
History of wheeze	1(5%)	2(9%)	NS
Skin test responders	5 (23%)	6 (27%)	NS
FEV_1 baseline (L)	3.78 ± 0.80	4.27 ± 0.74	0.04
FVC baseline (L)	4.62 ± 1.05	5.06 ± 0.83	0.13
sRaw baseline (L × cm H_2O/L/sec)	3.3 ± 1.7	3.6 ± 1.3	NS
PC_{100} (mg/mL)	7.5 ± 6.5	8.4 ± 11.3	NS

[a] All measurements showing ± values are means ± SD.

[b] NS = not significant.

Characteristics of Least- and Most-Sensitive Groups

Characteristics of the subjects stratified by sensitivity to O_3 are displayed in Table 3. A larger number of the most-sensitive subjects had smoked in the past (45%) compared to the least-sensitive subjects (18%), but the smoking history for these individuals was low-level and remote. A trend toward the most-sensitive subjects being younger than the least-sensitive subjects was identified, as has been reported before (McDonnell et al. 1993). The most-sensitive and least-sensitive subjects had significantly different baseline FEV_1 values on an absolute basis, but when the percentage of predicted FEV_1 values were compared to adjust for the effects of age, height, and gender, this apparent difference disappeared. The two groups did not differ significantly with regard to gender, or the history of cough, sputum production, wheeze, and dyspnea. Thirteen subjects were members of ethnic minority groups (six Asian, four Hispanic, and three Afro-American), but there was no difference in the distribution of these subjects between the most-sensitive and least-sensitive groups. Skin test responses were not different for the two groups. The most-sensitive subjects had a slightly higher mean PC_{100} (i.e., 8.4 ± 11.3 mg/mL) compared with that for the least-sensitive subjects (PC_{100} = 7.5 ± 6.5 mg/mL).

PHASE II. EFFECTS OF PROLONGED EXPOSURE TO 0.2 ppm OZONE ON PROXIMAL AIRWAYS AND DISTAL LUNG IN NORMAL SUBJECTS

METHODS AND STUDY DESIGN

Subjects

The aim of our Phase II study was to compare the lavage fluid findings after exposure in subjects relatively sensitive to O_3 to those in subjects relatively insensitive to O_3, using spirometric responses as the measure of sensitivity. An "extreme groups" design (only the upper and lower tertiles) was used to increase statistical power to detect relationships between lung function and lavage fluid measurements (Abrahams and Alf 1978). Twenty-three subjects participated in the study; eight had participated in Phase I and 15 additional subjects were recruited. In Phase I, as reported above, 66 normal subjects underwent exposures on separate days to air or O_3 (0.2 ppm) for 4 hours with intermittent exercise, and were ranked into tertiles of sensitivity to O_3 based on their O_3-induced decrements in FEV_1 (corrected for their response to air). The 23 subjects who participated in Phase II also were exposed on separate days to air or O_3 (0.2 ppm) for 4 hours with intermittent exercise, and were ranked into tertiles of sensitivity to O_3 by their O_3-induced decrements in FEV_1 (corrected for their response to air) using the combined Phase I and Phase II O_3 response distribution (n = 81 healthy subjects). Thirteen subjects were in the least-sensitive tertile and 10 subjects were in the most-sensitive tertile of the O_3-response distribution. One of the subjects who had participated in Phase I had an FEV_1 response in Phase II that dropped him from the most-sensitive group; he was not included in the Phase II data analysis. Two other subjects, one from the least-sensitive group and one from the most-sensitive group, were excluded from the data analysis because of unexplainably high percentages of neutrophils (15%) after air exposure. The two major reasons why subjects who had participated in Phase I did not elect to participate in Phase II were scheduling inconveniences and lack of interest in undergoing bronchoscopy twice.

Characteristics of the 20 subjects included in the Phase II data analysis are listed in Table 4. The only significant difference between the least- and most-sensitive groups is the greater percentage of women in the former (42% vs. 13%). The difference in gender composition accounts for the apparent differences in FEV_1 and FVC. When expressed as a percent of the predicted value for each subject, the mean values for FEV_1 and FVC are similar for the least- and most-sensitive groups. Predicted values for the spirometric parameters are those of Knudson and coworkers (1983). All of the subjects were nonsmoking (< 50 cigarettes in the last year and none in the last 6 weeks) adults without active medical problems. Each subject was informed of the risks of the experimental protocol, and each signed a consent form approved by the Committee on Human Research of the University of California, San Francisco. No subject took prescription medications, inhaled beta-adrenergic agonists, inhaled steroids, antihistamines, nonsteroidal antiinflammatory agents, or vitamins C and E within 1 week of testing. No subject consumed caffeine within 4 hours prior to arriving at the laboratory for each day's protocol. All subjects received financial compensation for their participation.

Pulmonary Function Measurements

Specific airway resistance, FEV_1, and FVC were measured in the same manner as described for Phase I.

Experimental Protocol

The exposure protocol for Phase II was identical to that of Phase I.

The Exposure Chamber and Atmospheric Monitoring

All exposures took place in the same chamber and under the same conditions as described above for Phase I.

Table 4. Phase II. Subject Characteristics

Gender	Age	Height (cm)	FEV_{1a} (L)	FEV_1 (%)	FVC[a] (L)	FVC (%)	sRaw[b] (L × cm H_2O/L/sec)	PC_{100} (mg/mL)
Least-Sensitive Group								
M	35	169	3.88	107	4.57	101	1.4	10.0
M	27	177	5.23	123	6.29	120	3.7	6.6
F	24	165	3.19	101	3.60	94	2.3	2.7
M	22	173	4.24	103	5.25	108	2.8	22.1
F	33	163	2.91	100	4.14	118	5.3	1.6
M	30	164	3.90	111	4.68	108	2.7	ND[c]
M	33	184	4.13	92	5.01	90	2.9	4.1
M	32	177	3.50	85	4.36	86	2.7	10.8
M	38	183	4.54	106	5.67	106	4.2	7.1
F	31	174	3.62	111	4.45	112	2.9	29.3
F	24	165	3.65	116	4.34	114	3.1	3.7
F	24	155	3.52	122	4.05	118	1.6	7.1
Mean	29	171	3.86	106	4.70	106	3.0	9.6
SD	5	8	0.62	11	0.75	11	1.1	8.6
Most-Sensitive Group								
M	30	177	4.74	114	5.71	111	1.9	3.0
M	24	180	4.93	105	5.88	107	2.3	12.5
M	23	178	4.76	108	4.83	93	1.1	15.2
M	26	171	4.13	103	4.55	92	1.5	3.0
M	22	180	4.85	108	5.89	113	2.7	14.7
F	24	163	3.41	110	3.68	99	3.0	6.0
M	34	171	4.41	116	5.59	119	4.2	1.4
M	30	187	4.45	95	5.65	97	2.8	1.3
Mean	27	176	4.46	107	5.22	104	2.4	7.1
SD	4	7	0.50	7	0.79	10	1.0	6.0

[a] Mean of six baseline values on exposure days.

[b] Mean of five baseline values on exposure days.

[c] ND = Not done.

Table 5. Phase II. Exposure Characteristics[a]

	Air	Ozone
O_3 (ppm)[b]	0.000 ± 0.003	0.192 ± 0.003
Temperature (°C)[c]	20.1 ± 0.1	20.1 ± 0.2
Relative humidity (%)[c]	50.4 ± 0.5	50.5 ± 0.4

[a] Values are presented as means ± SD.

[b] Number of observations per exposure = 67.

[c] Number of observations per exposure = 400.

The environmental characteristics of the exposures are listed in Table 5. No statistically significant differences in relative humidity or temperature were found between the O_3 and air exposures. There was also no significant difference in the subjects' mean ventilatory rates between the two exposures.

Bronchoscopy, Lavage, and Biopsy Procedures

Bronchoscopies were performed 18 ± 2 hours after each exposure in a dedicated suite at San Francisco General Hospital. We performed bronchoscopy 18 hours after exposure because previous studies by both our laboratory and other investigators had documented the presence of an O_3-induced inflammatory response in many subjects at this time point (Koren et al. 1989; Aris et al. 1993a,b). We elected to analyze fluid from both the proximal airway and the distal lung because previous studies in humans had focused on the latter, except for one study from our laboratory that did demonstrate the presence of proximal airway inflammation after O_3 exposure (Aris et al. 1993b). Our laboratory's procedures for bronchoscopy, PAL, and BAL have been reported in detail previously (Aris et al. 1993a,b). Briefly, intravenous access was established, supplemental O_2 was delivered, and the upper airways were anesthetized with topical lidocaine. The bronchoscope (FB 18x, Pentax Precision Instruments Corp., Orangeburg, NY) was introduced through the mouth and the flow of supplemental O_2 was increased to 10 L/min to prevent the transient desaturation that may be seen with left mainstem bronchus occlusion during PAL. A custom-designed, 6-French, double-balloon, double-port catheter (Baxter Healthcare Corp., 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 (Figure 6). Proximal airway lavage was performed using 12 mL (eight aliquots of 1.5 mL) of saline warmed to 37°C. The bronchoscope was redirected into the right middle lobe orifice, and BAL was performed with four 60-mL aliquots of saline. The first 10 mL of lavage fluid retrieved was labeled the bronchial fraction. After BAL was completed, the bronchoscope was redirected to an upper lobe orifice and at least five bronchial specimens were obtained with spiked biopsy forceps that are 110 cm in length with a total diameter of 2.4 cm (Pentax Precision Instrument Corporation, Orangeburg, NY). The specimens were taken from the right upper lobe carina during the bronchoscopy after the first exposure, and from the left upper lobe carina during the bronchoscopy after the second exposure. After bronchoscopy, the subject was transported to the Lung Biology Center for a 1- to 2-hour recovery period. Total cells were counted in unspun aliquots of PAL, bronchial fraction, and BAL fluids using a hemacytometer. Differential cell counts were made from slides prepared with a cytocentrifuge (Cytospin 2, Shandon Southern Products, Ltd., Astmoor, UK; 200 × *g* for 5 minutes) and stained in Diff-Quik (American Scientific Products, McGaw Park, IL) as previously described (Aris et al. 1993a). Proximal airway lavage and BAL fluids were immediately centrifuged at 200 × *g* for 15 minutes, and the supernatants were separated and recentrifuged at 1800 × *g* for 15 minutes to remove any cellular debris.

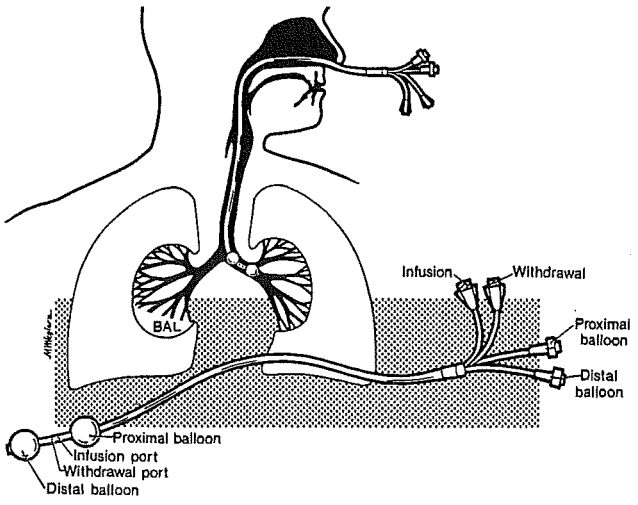

Figure 6. Phase II. Double-balloon airway-sampling catheter and its position in the left main-stem bronchus during isolated PAL. Bronchoalveolar lavage is performed in the right middle lobe.

Measurement of Biochemical Constituents of Lavage Fluids

Lavage fluid biochemical constituents were measured in aliquots of PAL, bronchial fraction, and BAL supernatants that had been frozen at −70°C. We assayed total protein because it correlates with epithelial permeability and used a modification of the Lowry procedure (Markwell et al. 1978). We assayed fibronectin because this protein is involved in tissue injury and repair. Lavage concentrations of fibronectin were determined with an antibody-capture immunoassay, as described by Miles and Hales (Miles and Hales 1968) with minor modifications (Aris et al. 1993a). We measured lavage concentrations of interleukin-8 (IL-8) and granulocyte-macrophage colony-stimulating factor (GM-CSF) concentrations because these cytokines are potent neutrophil chemoattractants. We used commercially available immunoassays (R & D Systems, Minneapolis, MN) to measure cytokines in lavage fluid.

Processing of Biopsy Specimens

The tissue specimens were removed from the biopsy forceps with a pair of needle-nose forceps and placed immediately in fixative. Of the five specimens taken from each

subject, four were placed in 20% sucrose phosphate-buffered saline (PBS) on ice, and the fifth was placed in acetone on ice. The four specimens were frozen as previously described (Weinacker et al. 1995). Briefly, specimens were incubated in 20% sucrose:PBS at 4°C for 1 to 2 hours, embedded in OCT compound (Baxter Scientific Products, McGaw Park, Illinois), quick-frozen in 2-methyl butane, and stored in a liquid nitrogen tank.

The single specimen placed in acetone was incubated an additional 4 hours or more at –20°C and then embedded in glycolmethacrylate (GMA) plastic (Montefort et al. 1994). Briefly, after incubation in acetone fixative, the specimen was immersed in fresh acetone for 15 minutes at room temperature and then in methyl benzoate for an additional 15 minutes. The tisssue was then immersed in GMA monomer:benzoyl peroxide (Historesin Embedding Kit, Reichert-Jung) at room temperature, and the monomer was changed every hour. The tissue was then embedded in GMA resin prepared by mixing GMA monomer–benzoyl peroxide with hardener containing dimethyl sulfoxide, and polymerized overnight at 4°C. The GMA blocks were stored at –20°C until used for immunostaining.

Immunohistochemistry

In order to evaluate the expression of integrins and cell adhesion molecules in airway tissue after O_3 exposure, immunohistochemistry was performed on frozen sections that had been fixed in cold acetone. Frozen specimens were removed from the liquid nitrogen tank and placed into a cryostat 1 hour prior to sectioning in order to allow them to equilibrate to cutting temperature, which was set at –20°C. A fresh staining dish filled with acetone was also placed in the cryostat to use as a fixative. When ready, sections were cut at 5 μm on a Reichert-Jung (Deerfield, IL) Cryocut 1800 cryostat and picked up on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), air-dried for 5 minutes, and fixed in cold acetone in a cryostat. After fixation, sections were air-dried an additional 1 hour to prevent sections from falling off slides.

After air-drying, sections were rehydrated with two 5-minute rinses in PBS and then preblocked in 0.5% casein:0.05% thimerosal:PBS for 15 minutes at room temperature. Sections were carefully blotted for excess preblock buffer, incubated in primary antibody overnight at 4°C. All antibodies were diluted in casein buffer to minimize background effect. The antibodies against α2, α3, and α5 were purified mouse monoclonal antibodies (Gibco BRL Life Technologies, Gaithersburg, MD), and were diluted 1:1000. The mouse monoclonal antibody against α6 was kindly donated by Dr. Caroline Damsky (University of California, San Francisco) in supernate form and was diluted 1:200. The rabbit antibody against α9 was a purified polyclonal antibody that was raised in our research center by Dr. Dean Sheppard (University of California, San Francisco) and was diluted 1:200. The mouse monoclonal antibody against β3 (Upstate Biotechnology Inc., Lake Placid, NY) was diluted 1:100. The mouse β5 antibody was kindly donated by Dr. Martin Hemler (Dana-Farber Cancer Institute, Boston, MA) and was diluted 1:2500. The mouse monoclonal β6 antibody was raised in our research center by Dr. Dean Sheppard and was diluted 1:40. The mouse monoclonal antibody against ICAM-1, kindly donated by Dr. Craig Wegner (Abbott Laboratories, Abbott Park, IL), was diluted 1:40. The mouse monoclonal antibody against VCAM (Genzyme Corp., Cambridge, MA) was diluted 1:1000, and the mouse monoclonal antibody against E-selectin, kindly donated by Dr. Wayne Smith (Baylor College of Medicine, Houston, TX), was diluted 1:100.

Several controls were used to show the specificity of the antibodies. A monoclonal antibody to α2β1 (always expressed in airway epithelium) was used as a positive control, and a monoclonal antibody to α8β1 (only expressed in smooth muscle) was used as an irrelevant antibody control. In addition to these controls, a no-primary antibody control was used to show the specificity of the secondary antibody, and a no-primary–no-secondary antibody control was used to show the specificity of the streptavidin-peroxidase conjugate.

After incubation in primary antibody, sections were rinsed in straight PBS and then incubated in biotinylated secondary antibody for 1 hour at room temperature. The antirabbit and antimouse biotinylated secondary antibodies (Vectastain ABC Elite Peroxidase Kit, Vector Laboratories, Burlingame, CA) were diluted 1:500 in casein buffer. Sections were rinsed and then blocked for endogenous peroxidase activity by incubating sections for 30 minutes in 0.3% hydrogen peroxide:methanol. Sections were rinsed once again and incubated in ABC streptavidin-peroxidase reagent (Vectastain kit) for 1 hour at room temperature. Sections were then rinsed and the chromagen was developed with the DAB Plus Substrate kit (Zymed Laboratories, South San Francisco, CA). The reaction was developed over a period of 10 minutes, when it was stopped by rinsing the sections in double-distilled water. Slides were then dehydrated, cleared in Hemo-De reagent (Fisher Scientific), and fixed permanently in Permount mounting media (Fisher Scientific). Sections were viewed with a Zeiss (Thornwood, NY) Axioskop microscope and immunohistochemistry data were evaluated on a scale of 0 to 4, with 0 indicating no signal present and 4 indicating strongest signal present. The sections were read in a blinded fashion. Photographs were taken with Fuji Reala ASA 100 film. Exposed film was sent out for processing.

Because neutrophils are difficult to view morphologically in plastic-embedded, acetone-fixed tissue without the use of immunohistochemistry, staining with a specific monoclonal antibody against neutrophil elastase (Dako Corporation, Carpinteria, CA) was used. To perform immunohistochemistry, plastic-embedded tissue was removed from −20°C and allowed to come to room temperature. Sections were cut at 3 μm on a Riechert-Jung JB-4 model microtome and floated on deionized water. Sections were picked up on Superfrost Plus slides and air-dried for a minimum of 4 hours. After air-drying, sections were rehydrated in two 5-minute rinses of PBS and then preblocked for 15 minutes at room temperature in 0.5% casein:0.05% thimerosal:PBS. Sections were blotted for excess preblock buffer and incubated in primary antibody for 16 to 20 hours at room temperature. The neutrophil elastase monoclonal antibody was used at a dilution of 1:50 in casein buffer. After primary antibody incubation, sections were rinsed in PBS and then incubated in antimouse biotinylated secondary antibody at a dilution of 1:200 in casein buffer for 2 hours at room temperature. Sections were rinsed and then incubated in 3.0% H_2O_2:methanol for 10 minutes at room temperature to eliminate endogenous peroxidase activity. Sections were rinsed and then incubated in ABC reagent for 2 hours at room temperature. After rinsing, chromagen was developed as described above. Sections were then dehydrated, cleared, mounted, viewed, and photographed as described above.

Neutrophil and Eosinophil Morphometry

To count neutrophils, the region 50 μm below the basement membrane was calculated as the area of interest for counting. A sweep of this region was counted for all the visible basement membrane present, and the total area was calculated in millimeters squared. The total neutrophil count was then divided by the area calculated, resulting in a unit designated as neutrophils/millimeter squared. Counting was done in a blinded fashion.

A standard hematoxylin-eosin stain was sufficient for the counting of eosinophils in airway tissue. Eosinophils were recognizable by their distinct eosinophilic staining granules, which stood out against the light blue stain of the hematoxylin. Therefore, immunohistochemstry was not necessary for this portion of the study. Counting was done in the same manner as for neutrophils.

Electron Microscopy

Tissue samples were fixed (2 hours at room temperature) with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and post-fixed (2 hours) in 1% osmium tetroxide (pH 7.2). The tissue was dehydrated in graded solutions of ethanol and infiltrated with Polybed 812 (Polysciences, Inc., Warrington, PA). Semithin (0.5-μm) sections were cut and stained with toluidine blue for light microscopic examination. From Phase II subjects, 17 bronchial specimens obtained after O_3 exposure and 21 specimens obtained after air exposure were available for ultrastructural study. As assessed by examining toluidine-blue semithin sections, six of the ozone-exposed samples and six of the air-exposed samples contained bronchial mucosa suitable for electron microscopy. Areas containing airway epithelium were selected and thin sections were cut with a diamond knife and mounted on mesh grids. After staining with uranyl acetate and lead citrate, samples were examined and photographed with a JEOL (Tokyo, Japan) 100S electron microscope. The electron photomicrographs were coded and reviewed in a blinded fashion.

STATISTICAL METHODS AND DATA ANALYSIS

The physiologic responses (sRaw, FEV_1, and FVC) during the O_3 exposure were determined in the following ways: (1) the mean percentage of change for each measure was calculated as the preexposure mean value minus the postexposure mean value divided by the postexposure value × 100; and (2) the AUC for each measure was calculated by the trapezoidal method. In both cases, the responses during the O_3 exposure were corrected for those during the air exposure by subtracting the response to air from the response to O_3. The differences in air-corrected responses between the most-sensitive and least-sensitive subjects were compared by two-tailed *t* tests.

Mean values for cellular and biochemical endpoints in PAL, bronchial fraction, and BAL fluids were first compared between the O_3 and air exposures by Wilcoxon signed rank tests to determine if there were differences between the two exposures. Then, cellular and biochemical endpoints were compared between the most- and least-sensitive subjects with two-tailed Wilcoxon rank sum tests to determine if the inflammatory indicators correlated with the magnitude of change in the air-corrected FEV_1. For each lavage fluid endpoint, the value after O_3 exposure was either (1) used by itself or (2) corrected for the air response by subtracting the value after the air exposure. Both of these methods produced similar results. Linear correlation of the magnitude of change in air-corrected values for FEV_1 and sRaw with the magnitude of air-corrected values for several lavage fluid endpoints was also performed using Spearman correlation coefficients.

Symptom scores were categorized as (1) lower respiratory (chest pain, chest tightness, shortness of breath, cough, sputum production, wheezing), (2) upper respiratory (throat irritation, nose irritation), or (3) nonrespiratory (anxiety, headache, eye irritation, nausea). For each exposure, the

symptom scores were determined by subtracting the preexposure symptom score from the postexposure symptom score. The symptom score differences after exposure to O_3 were compared to those after exposure to air using the Wilcoxon signed rank test.

Immunohistochemical staining scores of bronchial specimens for various adhesion molecules as well as counts of neutrophils and eosinophils after exposure to O_3 were compared to those after exposure to air using the Wilcoxon signed rank test for paired specimens and the Wilcoxon rank sum test for unpaired specimens.

In all of these analyses, a p value < 0.05 was considered statistically significant.

RESULTS OF PHASE II

Pulmonary Function Responses

The mean pre- and postexposure FEV_1, FVC, and sRaw data for the least- and most-sensitive groups are shown in Table 6. As expected, statistically significant differences between the groups were noted for the mean percentage of change values in FEV_1 ($p < 0.001$) and FVC ($p < 0.001$). The difference in sRaw response after O_3 exposure was also statistically significant ($p < 0.001$). The mean percentage of change values in FEV_1 during both air and O_3 exposures are also shown graphically in Figure 7.

Symptoms

Lower respiratory symptom scores showed a significant increase after O_3 exposure for all subjects combined (postexposure score – preexposure score [mean ± SD], 6.7 ± 5.1; $p < 0.001$), but no significant difference in this endpoint was noted between the least- and most-sensitive groups.

Table 6. Phase II. Physiologic Changes in Lung Function After Exposure to Air or 0.2 ppm Ozone[a]

		Least-Sensitive Group ($n = 12$)	Most-Sensitive Group ($n = 8$)
FEV_1	Air	+0.3 ± 0.6	−1.7 ± 1.2
	Ozone	−7.0 ± 1.6	−36.0 ± 4.9
FVC	Air	−1.6 ± 0.8	−1.1 ± 1.0
	Ozone	−6.8 ± 1.8	−28.0 ± 4.6
sRaw	Air	+0.6 ± 7.1	+5.8 ± 9.2
	Ozone	+24.2 ± 7.0	+44.1 ± 15.5

[a] All parameters are measured as percentage of change from baseline and values are presented as means ± SE.

Cellular and Biochemical Responses

Proximal airway lavage, bronchial fraction, and BAL data after both air and O_3 exposures for both least- and most-sensitive groups are shown in Table 7. The mean ± SD differential cell counts in BAL fluid after O_3 exposure were 75.3% ± 7.0% macrophages, 12.0% ± 5.6% lymphocytes, 8.9% ± 4.8% neutrophils, and 0.3% ± 0.6% eosinophils. After air exposure, these values were 82.8% ± 5.1% macrophages, 8.5% ± 4.3% lymphocytes, 4.4% ± 2.3% neutrophils, and 0.2% ± 0.4% eosinophils. Most subjects had increases in percentage of neutrophils and total protein concentration in lavage fluids after ozone exposure compared to the values after air exposure (see Figure 8 for individual BAL fluids). Significant increases were found

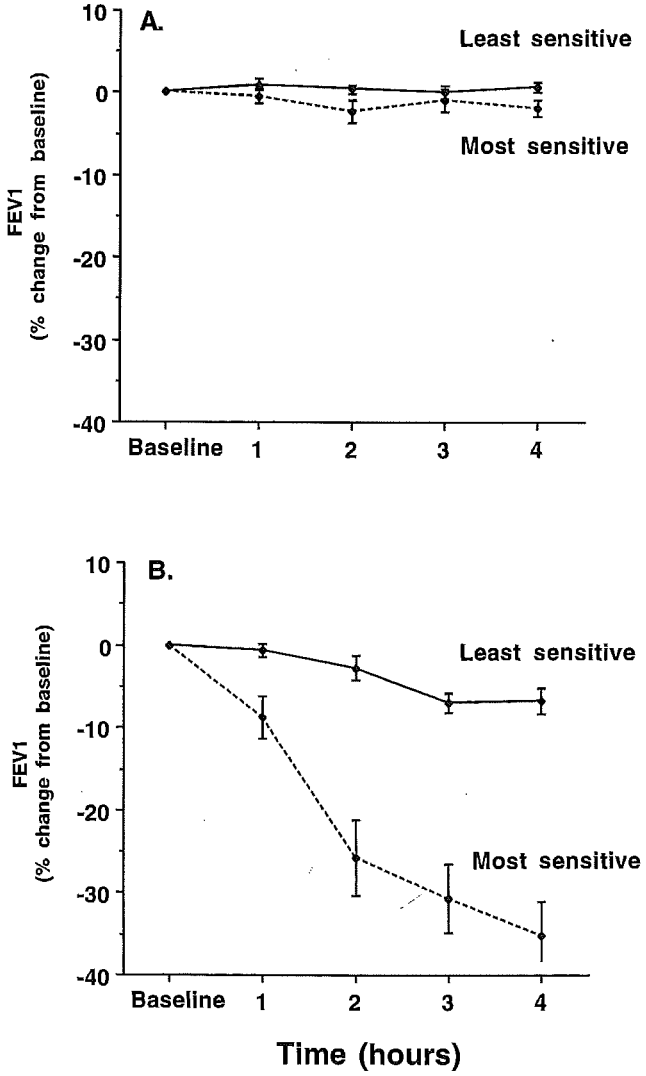

Figure 7. Phase II. Mean ± SE percentage of change in FEV_1 from baseline at hourly intervals during exposure to (A) filtered air or (B) 0.2 ppm O_3 for the least- and most-sensitive subjects.

Table 7. Phase II. Lavage Endpoints[a]

	Least-Sensitive Group (n = 12)		Most-Sensitive Group (n = 8)	
	Air	Ozone	Air	Ozone
Bronchoalveolar Lavage				
Total leukocytes ($\times 10^4$)	11.2 ± 1.3	14.0 ± 1.4	18.6 ± 3.1	22.7 ± 8.0
Neutrophils (%)	5.2 ± 0.7	9.6 ± 1.1	3.1 ± 0.6	7.9 ± 2.3
Total protein (mg/mL)	0.106 ± 0.008 (1.0)	0.155 ± 0.014 (1.0)	0.086 ± 0.011 (1.0)	0.136 ± 0.015 (1.0)
Fibronectin (ng/mL)	495.5 ± 148.5 (4674.5)	851.1 ± 120.3 (5491.0)	605.4 ± 120.4 (7039.5)	888.2 ± 129.0 (6530.9)
IL-8 (pg/mL)	39.6 ± 6.8 (373.6)	60.3 ± 18.8 (389.0)	31.2 ± 4.2 (362.8)	41.9 ± 7.5 (308.1)
GM-CSF (pg/mL)	1.31 ± 0.2 (12.4)	1.99 ± 0.6 (12.8)	1.5 ± 0.7 (17.4)	2.89 ± 0.6 (21.3)
Bronchial Fraction				
Total leukocytes ($\times 10^4$)	9.1 ± 0.9	16.4 ± 2.9	16.3 ± 1.9	20.4 ± 2.5
Neutrophils (%)	7.7 ± 1.9	32.5 ± 5.5	5.4 ± 1.0	29.0 ± 8.2
Total protein (mg/mL)	0.108 ± 0.008 (1.0)	0.158 ± 0.012 (1.0)	0.104 ± 0.012 (1.0)	0.145 ± 0.023 (1.0)
IL-8 (pg/mL)	89.9 ± 11.4 (832.4)	238.4 ± 85.4 (1508.9)	95.3 ± 17.6 (916.3)	187.2 ± 33.7 (1291.0)
Proximal Airway Lavage				
Total leukocytes ($\times 10^4$)	3.7 ± 2.4	4.9 ± 2.1	4.0 ± 1.5	15.2 ± 10.0
Neutrophils (%)	33.0 ± 6.5	61.7 ± 5.3	42.7 ± 10.3	52.6 ± 7.9
Total protein (mg/mL)	0.062 ± 0.019 (1.0)	0.109 ± 0.039 (1.0)	0.096 ± 0.026 (1.0)	0.157 ± 0.060 (1.0)
IL-8 (pg/mL)	104.6 ± 21.5 (1687.1)	210.6 ± 43.3 (1932.1)	222.5 ± 87.7 (2317.7)	241.8 ± 72.5 (1540.1)

[a] Values are presented as means ± SE, with the ratio to total protein concentration given in parentheses where applicable.

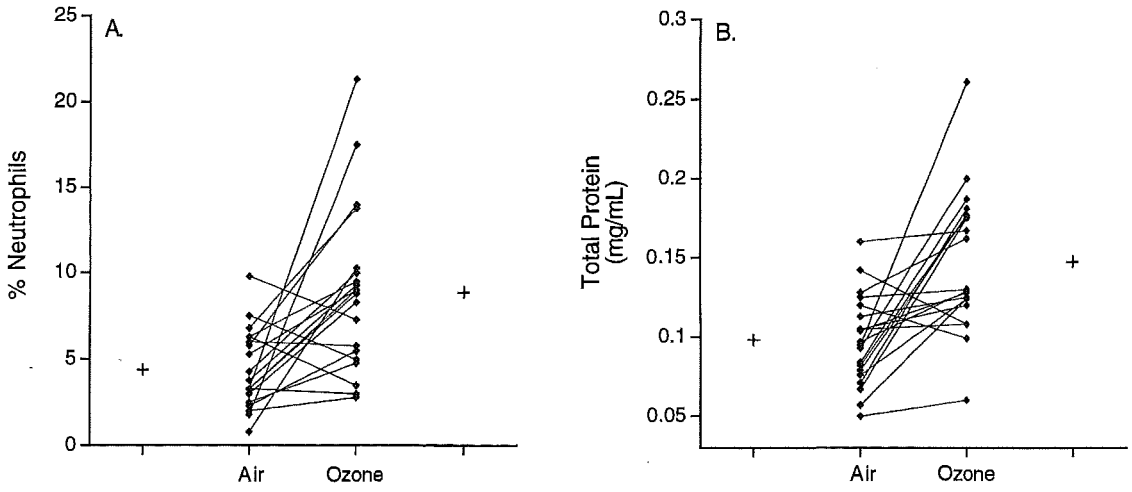

Figure 8. Phase II. Bronchoalveolar lavage results for normal subjects (n = 20) 18 hours after exposure to filtered air or 0.2 ppm O_3 for 4 hours. Panel A shows individual data for each subject for percentage of neutrophils in BAL fluid, and Panel B shows individual data for total protein concentration (mg/mL) in BAL fluid. Group means are noted by the + signs next to each column of data points.

after O_3 exposure for percentage of neutrophils in PAL (p = 0.01), bronchial fraction ($p < 0.001$), and BAL ($p = 0.001$) fluids for all subjects combined, but no significant differences between the least- and most-sensitive groups. Total protein concentrations in bronchial fraction and BAL fluids also showed a significant O_3 effect for all subjects combined ($p < 0.001$ and $p < 0.001$, respectively), but no significant differences between the least- and most-sensitive groups. Similarly, fibronectin and GM-CSF concentrations in the BAL fluid and IL-8 concentration in the bronchial fraction were significantly increased after O_3 exposure for all subjects combined ($p < 0.01$, $p = 0.05$, and $p < 0.01$, respectively), but again, no significant differences were found between least- and most-sensitive groups. Although these results indicate evidence of O_3-induced respiratory tract inflammation, the magnitude of the cellular and biochemical responses was not significantly different between the least- and most-sensitive subjects, as defined by spirometic measurements.

Figure 9 shows the data plots of O_3-induced physiologic change (FEV_1 or sRaw) against two inflammatory endpoints (percentage of neutrophils and total protein concentration) in BAL fluid. No significant correlations were found between either FEV_1 or FVC (data not shown) and these inflammatory endpoints. In contrast, significant correlations were found between the magnitude of the increase in sRaw and the increase in both percentage of neutrophils and total protein concentration in BAL fluid after O_3 exposure ($r = 0.67$ and $r = 0.46$, respectively).

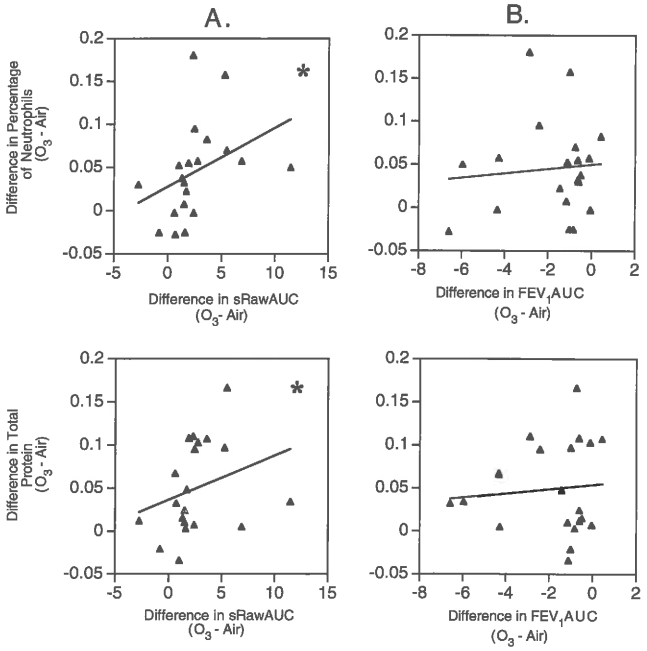

Figure 9. Phase II. Test of correlations between O_3-induced physiologic changes and two inflammatory end points for normal subjects ($n = 20$). The top graph of Panel A compares the air-corrected BAL neutrophilic response to O_3 (percentage of total cells that were neutrophils after O_3 – the percentage after air) with the air-corrected sRaw response to O_3 (sRawAUC after O_3 – sRawAUC after air). The bottom graph of Panel A compares the air-corrected BAL total protein response to O_3 (mg/mL after O_3 – mg/mL after air) with the air-corrected sRaw response to O_3. The top graph of Panel B compares the air-corrected BAL neutrophilic response to O_3 with the air-corrected FEV_1 response to O_3 (FEV_1AUC after O_3 – FEV_1AUC after air). The bottom graph of Panel B compares the air-corrected BAL total protein response to O_3 with the air-corrected FEV_1 response to O_3. The solid lines indicate the regression lines. Asterisks indicate significant correlations ($p < 0.05$).

Bronchial Biopsy Specimens: Immunohistochemistry, Morphometry, and Electron Microscopy

For all of the integrins evaluated, no clear difference in expression was found when O_3-exposed bronchial specimens were compared with the control samples. The expression of integrins that are constitutively present in the normal airway, that is, α2, α3, α6, α9 (Figure 10), and β5, was not clearly downregulated, upregulated, or redistributed in the epithelium after exposure to O_3. The expression of those integrins that are not constitutively expressed in the airway but are thought to be upregulated after injury, that is, α5, β3, and β6, also was not clearly upregulated in the epithelium after exposure to O_3.

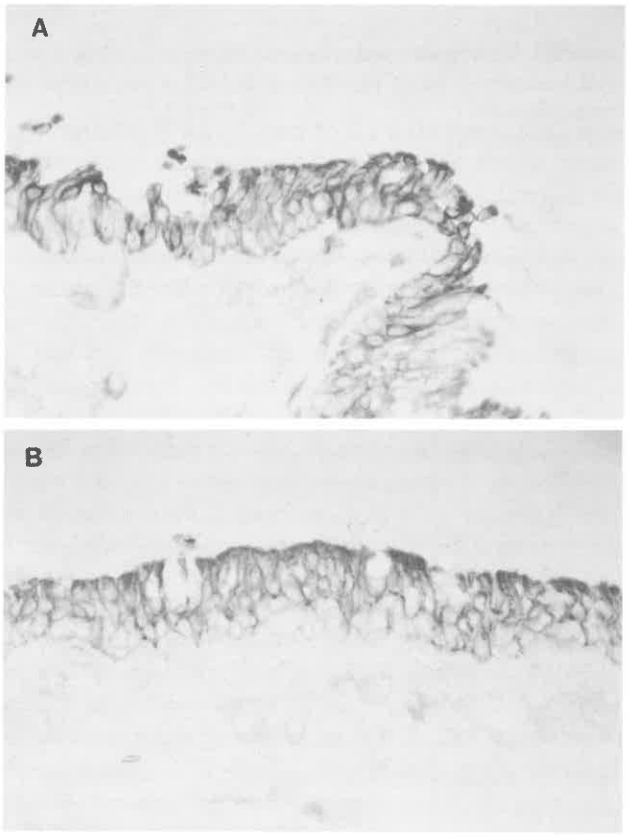

Figure 10. Phase II. Paired airway biopsy specimens from the same subject showing α9 integrin expression after exposure to (A) filtered air or (B) 0.2 ppm O_3 for 4 hours. Magnification is × 400.

Of the three cell adhesion molecules evaluated, only ICAM-1 showed any change in expression in the endothelium of blood vessels in the submucosa of the airway. ICAM-1 is constitutively expressed at low levels in the endothelium of normal airway tissue, but after exposure of the airway to O_3, ICAM-1 expression was clearly upregulated (Figure 11). Of six subjects with paired specimens, three had increased ICAM-1 expression after O_3 compared with air and three showed no difference ($p = 0.05$ for the O_3-air difference). Mean staining scores after O_3 and air, respectively, were 3.3 and 2.5. Nine subjects had adequate tissue sampling for immunohistochemistry after one of the two exposures, either air ($n = 5$) or O_3 ($n = 4$). Analysis of these specimens in unpaired fashion revealed a trend (specimens mean score for O_3 = 3.67 and air = 2.75 [$p = 0.20$]) toward significance and supported the results from the paired analysis. Although data from paired samples were not available to examine the effect of O_3 on epithelial ICAM-1 expression, basal cell expression of ICAM-1 was present on the tissues with preserved epithelium.

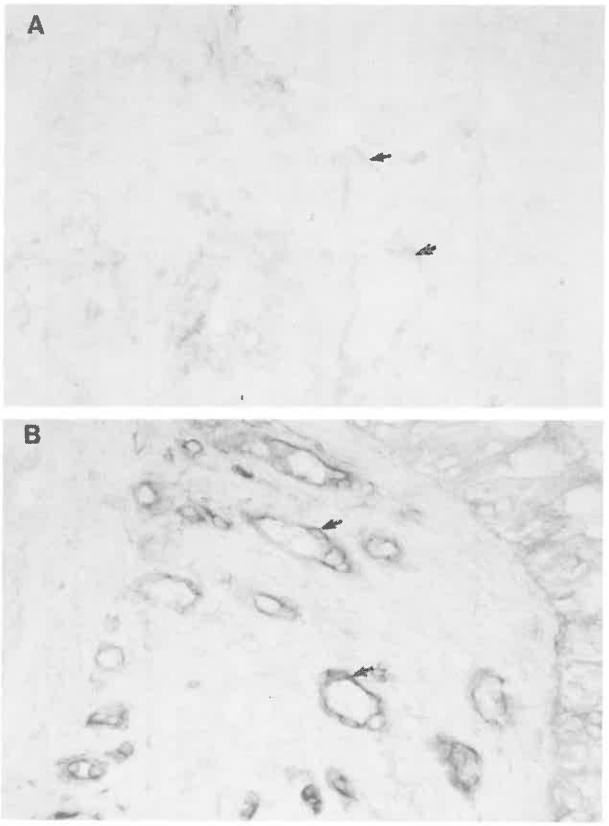

Figure 11. Phase II. Paired airway biopsy specimens from the same subject after exposure to (A) filtered air or (B) 0.2 ppm O_3 for 4 hours showing increased ICAM-1. Magnification is × 400. Arrows indicate bronchial vessel endothelium.

The other two molecules studied, VCAM and E-selectin, are not normally expressed in the endothelium of bronchial blood vessels. However, these molecules can be upregulated as a response to stimuli that can injure tissue and cause inflammation. No such upregulation of these two molecules was demonstrated after exposure of the airways to O_3.

From subjects in Phase II, 18 specimens were obtained with adequate tissue for leukocyte morphometry (11 after O_3 exposure and seven after air; no paired specimens). Nonsignificant trends were evident for increased neutrophil and eosinophil infiltration after O_3 exposure (Figure 12). The mean ± SE number of neutrophils/mm^2 was 14.0 ± 6.1 after O_3 exposure and 8.7 ± 2.5 after air exposure ($p = 0.6$). The mean ± SE number of eosinophils/mm^2 was 0.29 ± 0.08 after O_3 exposure and 0.14 ± 0.46 after air exposure ($p = 0.3$).

Transmission electron microscopy showed normal bronchial epithelium for all subjects exposed to air (Figure 13). Two subjects exposed for 4 hours to 0.2 ppm O_3 had no clear pathological alterations of their bronchial epithelium. The bronchial mucosa of four subjects exposed to O_3 revealed expansion of the intercellular spaces (Figure 13). In general, this change was limited to the basal and intermediate zones of the epithelium, but focally involved the full epithelial thickness. Ciliated cells remained intact, and no consistent changes occured in the goblet cells. Neither neutrophils nor eosinophils were detected within the dilated intercellular spaces of the airway epithelium exposed to O_3.

PHASE III. EFFECTS OF PROLONGED EXPOSURE TO 0.2 ppm OZONE ON PROXIMAL AIRWAYS AND DISTAL LUNG IN SUBJECTS WITH ASTHMA

METHODS AND STUDY DESIGN

Subjects

The aim of our Phase III study was to compare the lung function and inflammatory responses after exposure to O_3 in asthmatic subjects with those in normal subjects. We recruited 36 subjects with a history of physician-diagnosed asthma for the study; 6 were excluded because they did not have nonspecific airway hyperresponsiveness; 2 subjects dropped out after the first bronchoscopy; 9 dropped out because of scheduling difficulties or lack of interest; 19 subjects completed the study protocol. One of these 19 subjects was excluded from the data analysis because her asthma had been exacerbated by a respiratory tract infection that had not fully resolved at the time of her second (air) exposure (data from 18 asthmatic subjects were ultimately analyzed). In total, 81 nonasthmatic subjects participated in Phase I, or Phase II, or both. The entire group of 81

subjects was used as the comparison group for the lung function analyses (except that only 70 of these subjects had sRaw measurements), and the 20 subjects with good quality lavage data in Phase II served as the comparison group for the inflammatory endpoint analyses.

Characteristics of the 18 asthmatic subjects included in the Phase III data analysis are listed in Table 8. Predicted values for the spirometric parameters are those of Knudson and coworkers (1983). All of the subjects were nonsmoking (fewer than 50 cigarettes in the last year and none in the last 6 weeks) adults without active medical problems other than asthma. Each subject was informed of the risks of the experimental protocol and asked to sign a consent form approved by the Committee on Human Research of the University of California, San Francisco. No subject took prescription medications, inhaled beta-adrenergic agonists, inhaled steroids, antihistamines, nonsteroidal antiinflammatory agents, or vitamins C and E within 1 week of testing. No subject consumed caffeine within 4 hours of arriving at the laboratory for each experimental day. All subjects received financial compensation for their participation.

Pulmonary Function Measurements

Specific airway resistance, FEV_1, and FVC were measured in the same manner as described for Phase I.

Experimental Protocol

The exposure protocol was identical to that of Phase I.

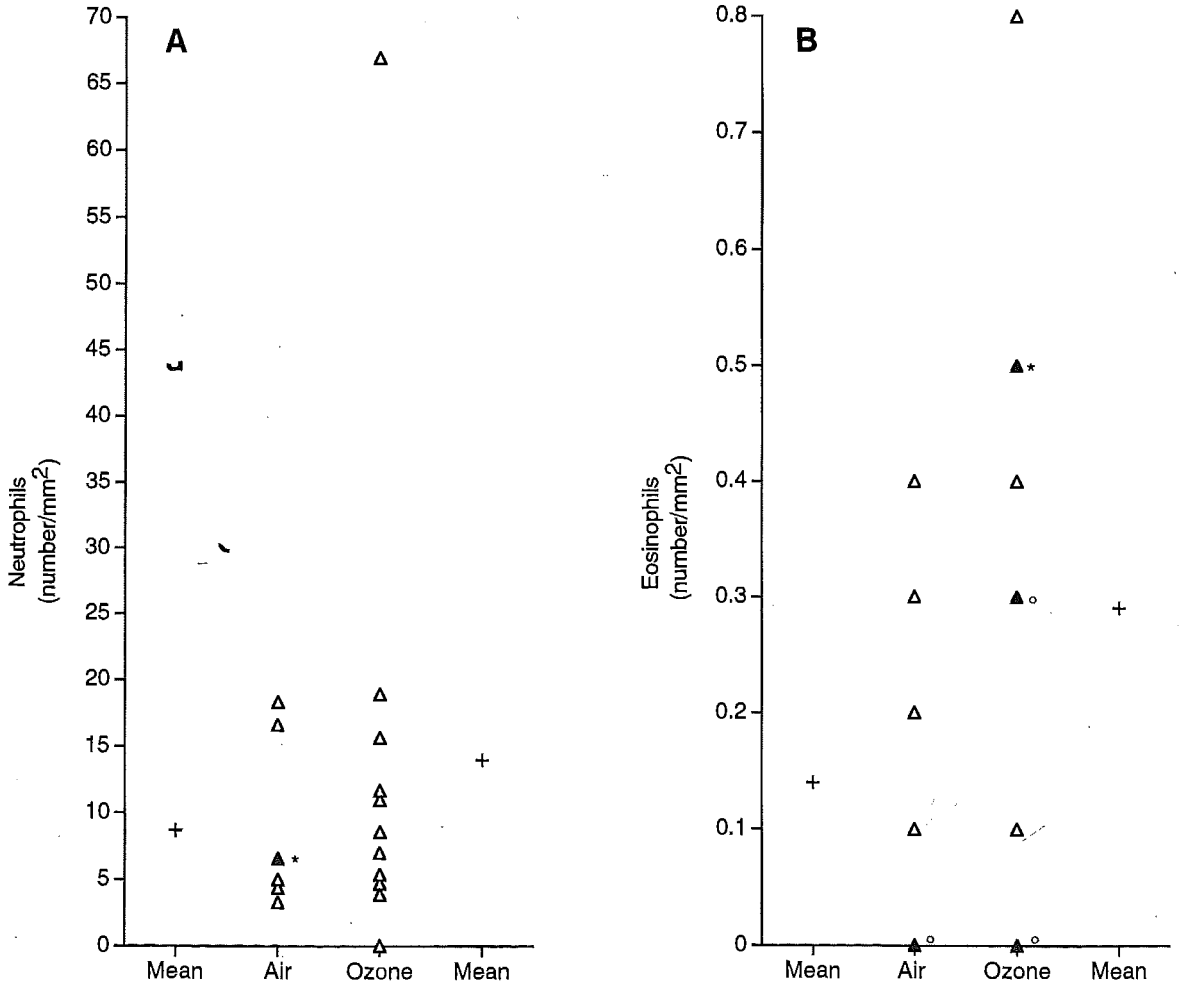

Figure 12. Phase II. Neutrophil (A) and eosinophil (B) counts per squared millimeter of bronchial mucosa after exposure of normal subjects to filtered air (n = 7) or 0.2 ppm O_3 (n = 11) for 4 hours. An open triangle (Δ) indicates a single subject; a filled triangle (▲) indicates n = > 1; an asterisk (*) indicates n = 2, and a circle (○) indicates n = 3. Group means are noted by the + signs next to each column of data points.

Exposure Chamber and Atmospheric Monitoring

All exposures took place in the same chamber and under the same conditions as described above for Phase I.

The environmental characteristics of the exposures are listed in Table 9. No statistically significant differences in relative humidity or temperature between the O_3 and air exposures were noted. Also, no significant difference in the subjects' mean ventilatory rate was found between the exposures.

Bronchoscopy, Lavage, and Biopsy Procedures

These procedures were identical to those described for Phase II.

Measurement of Biochemical Constituents of Lavage Fluids

Lavage fluid biochemical constituents were measured in aliquots of PAL, bronchial fraction, and BAL supernatants that had been frozen at –70°C, except for lactate dehydrogenase (LDH), which was measured within 30 minutes of each lavage with a commercially available kit (No. 228-10, Sigma Chemical Co., St. Louis, MO) and a spectrophotometer (DU 65, Beckman Instruments, Inc., Fullerton, CA). Lactate dehydrogenase was measured because it is a good marker of cell injury. The methods used for analyzing the frozen aliquots for total protein, fibronectin, IL-8, and GM-CSF were the same as those descibed above for Phase II. In addition, concentrations of myeloperoxidase (MPO), a neu-

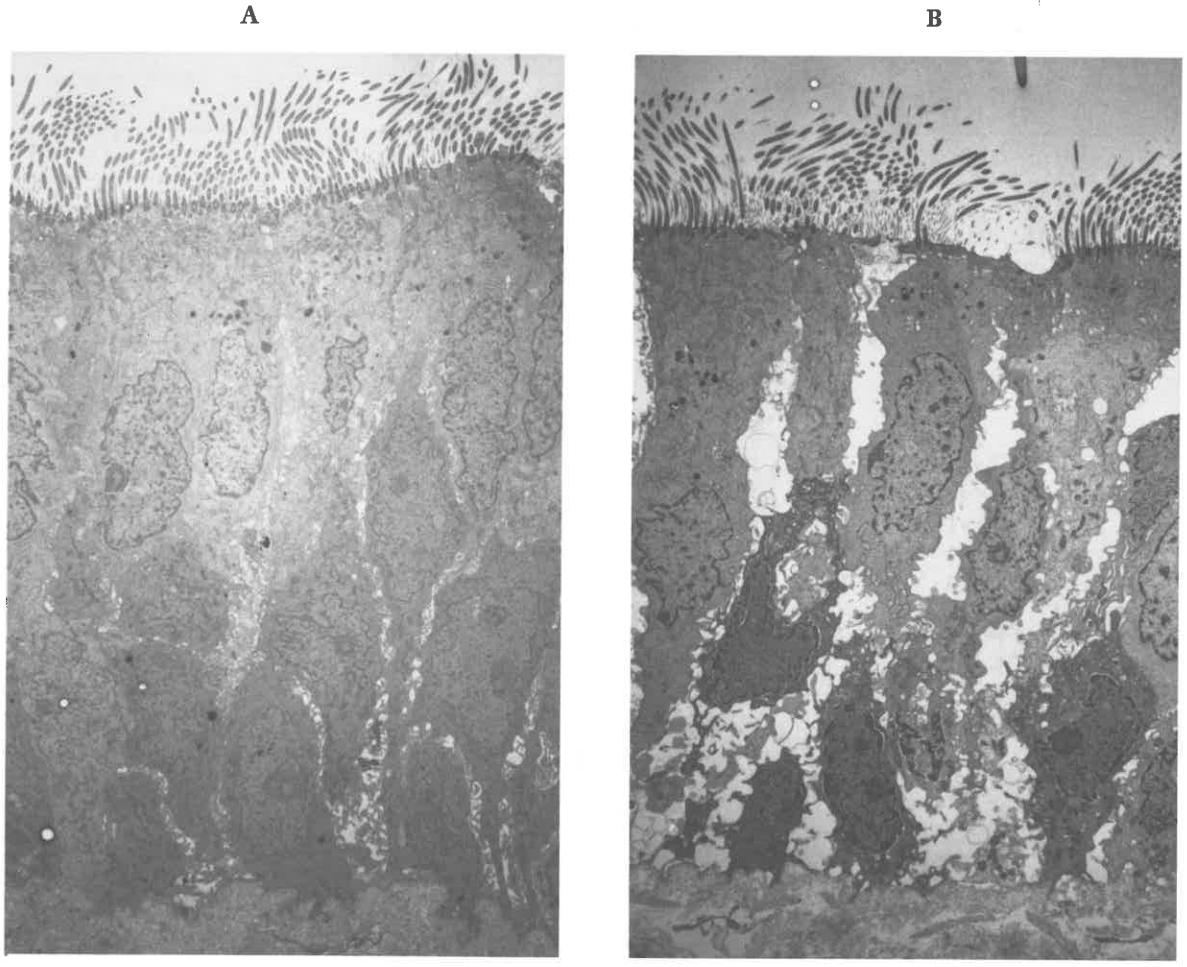

Figure 13. Phase II. Electron micrographic evidence of O_3-induced epithelial injury. A. Transmission electron microscopy showed normal bronchial epithelium for all subjects exposed to air and for two of six subjects exposed to 0.2 ppm O_3. B. Four of six subjects exposed to 0.2 ppm O_3 for 4 hours had expansion of the intercellular spaces, primarily in the basal and intermediate zones, of the epithelium.

trophil product, and β2 transforming growth factor (TGF-β2), a protein involved in injury repair, were measured by commercially available kits (Cayman Chemical, Ann Arbor, MI, and R & D Systems, Minneapolis, MN, respectively).

Processing of Biopsy Specimens, Immunohistochemistry, Neutrophil and Eosinophil Morphometry, and Electron Microscopy

These procedures were identical to those described for Phase II.

STATISTICAL METHODS AND DATA ANALYSIS

The physiologic responses (sRaw, FEV_1, and FVC) during the O_3 exposure were calculated as described for Phase II, and compared with those during the air exposure by two-tailed Wilcoxon signed rank tests. The responses during the O_3 exposure were corrected for those during the air exposure (i.e., exercise-induced bronchoconstriction) by subtracting the response to air from the response to O_3. The differences in air-corrected responses between the normal ($n = 81$) and asthmatic ($n = 18$) subjects were then compared by two-tailed Wilcoxon rank sum tests.

Cellular and biochemical endpoints in the PAL, bronchial fraction, and BAL fluids were first compared between the O_3 and air exposures by Wilcoxon signed rank tests to determine if there were differences between the two exposures for the asthmatic subjects. For each lavage fluid endpoint, the value after O_3 exposure was corrected for the air exposure by subtracting the value after air exposure from the value after O_3 exposure. Then, cellular and biochemical endpoints were compared between the normal ($n = 20$) and asthmatic ($n = 18$) subjects with two-tailed Wilcoxon rank sum tests.

Symptom scores were categorized as described for Phase II. For each exposure, the symptom score differences were determined by subtracting the preexposure symptom score from the postexposure symptom score. The symptom score differences after exposure to O_3 were compared with those after exposure to air using the Wilcoxon signed rank test.

Table 8. Phase III. Subject Characteristics

Gender	Age	Height (cm)	FEV_1a (L)	FEV_1 (%)	FVC[a] (L)	FVC (%)	sRaw[b] (L × cm H_2O/L/sec)	PC_{20} (mg/mL)	Medications[c]
M	31	174	3.64	92	4.94	101	3.3	0.49	BA
F	25	165	3.20	102	3.67	97	5.9	8.15	BA
M	33	180	3.62	85	4.93	93	4.8	1.95	BA
M	19	183	5.60	127	6.69	133	3.5	0.44	BA
M	23	164	3.31	88	4.70	105	3.5	0.89	BA
M	28	189	4.38	90	5.96	99	4.4	0.18	BA
M	27	188	4.79	100	6.55	110	6.2	2.63	BA, IS, C
M	22	171	3.69	91	4.38	92	3.8	2.28	BA
F	19	163	2.98	90	3.66	101	5.1	0.33	BA, IS
M	33	173	2.65	68	4.16	86	8.3	0.24	BA
M	23	171	3.40	83	4.90	101	8.2	0.60	BA
M	28	182	4.80	107	6.10	110	4.4	0.90	BA
F	26	160	3.05	102	4.20	118	6.6	1.29	BA
M	36	180	2.67	64	3.27	63	2.5	0.37	BA
F	18	168	3.53	105	3.86	103	4.9	0.28	None
F	29	170	3.25	102	4.41	114	6.6	0.66	BA, C
M	28	186	3.92	84	6.95	120	8.6	1.61	BA
F	19	170	3.29	94	3.83	98	1.4	0.44	BA, IS
Mean	26	174	3.65	93	4.84	102	5.1	1.32	
SD	5.4	9.0	0.79	14.4	1.14	15.0	2.0	1.86	

[a] Mean of best of six baseline values on exposure days.

[b] Mean of five baseline values on exposure days.

[c] BA = inhaled beta-agonist; IS = inhaled steroid; C = cromolyn sodium.

Immunohistochemical staining scores of bronchial specimens for various adhesion molecules and counts of neutrophils and eosinophils after exposure to O_3 were compared with those after exposure to air using the Wilcoxon signed rank test.

In all of these analyses, a p value of < 0.05 was considered statistically significant.

RESULTS OF PHASE III

Pulmonary Function Responses

The mean pre- and postexposure FEV_1, FVC, and sRaw data for the asthmatic subjects are shown in Table 10. There were significant O_3-induced changes in all three parameters ($p < 0.04$). The mean value for percentages of change in FEV_1 and sRaw during both air and O_3 exposures are also shown graphically in Figure 14.

Symptoms

There was a significant increase in lower respiratory symptom scores after O_3 for the asthmatic subjects (postexposure score – preexposure score [mean ± SD], 7.1 ± 4.9; $p < 0.001$).

Table 9. Phase III. Exposure Characteristics[a]

	Air	Ozone
O_3 (ppm)[b]	0.002 ± 0.002	0.197 ± 0.004
Temperature (° C)[c]	20.8 ± 1.6	20.5 ± 1.6
Relative humidity (%)[c]	50.3 ± 2.3	50.1 ± 2.7

[a] Values are presented as means ± SD.

[b] Number of observations per exposure = 67.

[c] Number of observations per exposure = 400.

Table 10. Phase III. Physiologic Changes in Lung Function After Exposure to Air or 0.2 ppm Ozone[a]

		Asthmatic Group (n = 18)	Normal Group (n = 81)
FEV_1	Air	−3.8 ± 1.9	−1.0 ± 0.6
	Ozone	−24.6 ± 2.8	−19.0 ± 1.6
FVC	Air	−4.0 ± 0.8	−1.3 ± 0.5
	Ozone	−18.2 ± 2.8	−15.2 ± 1.3
sRaw	Air	+10.9 ± 11.7	+3.3 ± 2.5[b]
	Ozone	+95.4 ± 36.7	+35.6 ± 3.7[b]

[a] All parameters are measured as percentage of change from baseline and values are presented as means ± SE.

[b] n = 70.

Cellular and Biochemical Responses

Mean PAL, bronchial fraction, and BAL data after both air and O_3 exposures for the asthmatic subjects are shown in Table 11. The total leukocyte counts in BAL fluid were not significantly different between the two exposures. The mean ± SD differential cell counts in BAL fluid after O_3 exposure were 69.1% ± 17.2% macrophages, 4.8% ± 2.1% lymphocytes, 16.1% ± 12.7% neutrophils, and 3.3% ± 4.4% eosinophils. After air exposure, these values were 85.0% ± 10.1% macrophages, 3.7% ± 2.8% lymphocytes, 4.1% ±

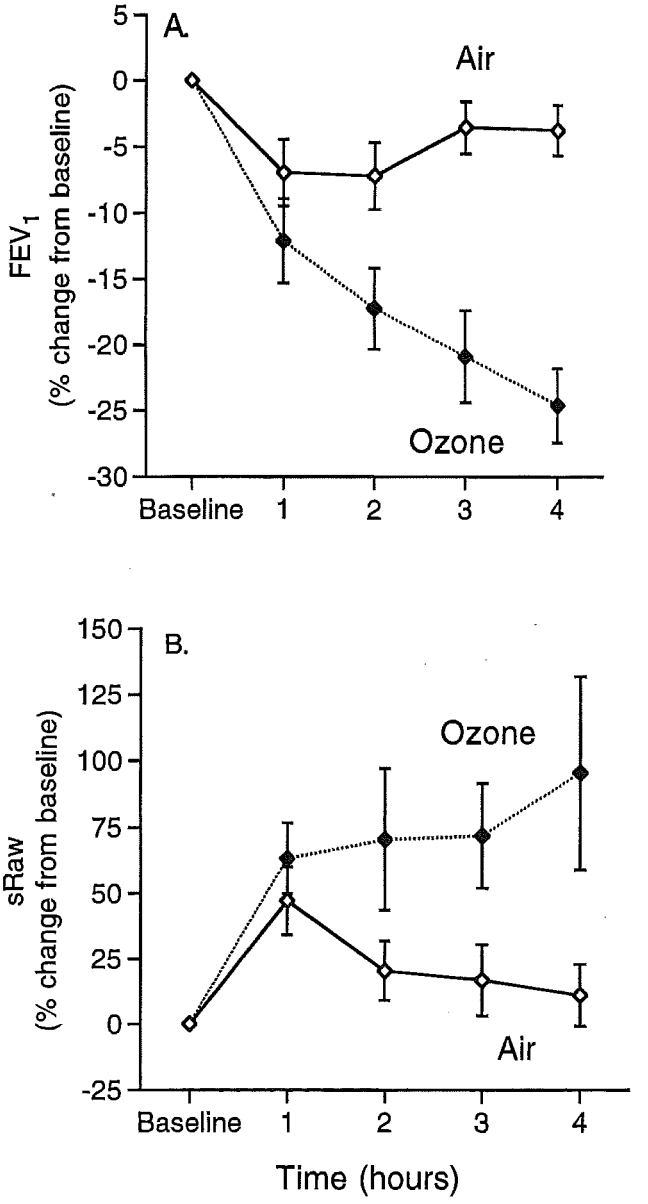

Figure 14. Phase III. Mean ± SE percentage of change from baseline in (A) FEV_1 or (B) sRaw at hourly intervals during exposure to filtered air or 0.2 ppm O_3 for asthmatic subjects (n = 18).

3.9% neutrophils, and 4.0% ± 6.6% eosinophils. The BAL results for percentage of neutrophils and total protein concentration for individual asthmatic subjects are shown in Figure 15. Significant increases were found after O_3 exposure in the percentage of neutrophils in PAL ($p < 0.01$), bronchial fraction ($p < 0.001$), and BAL ($p < 0.001$) fluids from the asthmatic subjects. Total protein concentrations in bronchial fraction and BAL fluids also showed a significant O_3 effect for the asthmatic subjects ($p < 0.05$ and $p < 0.01$, respectively). Similarly, bronchial fraction IL-8 concentration ($p < 0.01$) and BAL fibronectin, IL-8, GM-CSF, and MPO concentrations ($p < 0.001$, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively) were significantly increased after O_3 exposure for the asthmatic subjects. The BAL results for LDH and fibronectin, IL-8 and GM-CSF, and MPO and TGF-β2 concentrations are shown in Figures 16, 17, and 18, respectively.

The mean pre- and postexposure FEV_1 and FVC data for the asthmatic subjects are compared to those of the 81 normal subjects in Table 10. The mean pre- and postexposure sRaw data for the asthmatic subjects were compared to the same data for the 70 normal subjects for whom these data were available. No significant difference in O_3-induced lower respiratory symptoms was found between the two groups. Mean air-corrected lung function and selected lavage endpoint responses to O_3 for both asthmatic and normal subjects are shown in Table 12. The lung function responses to O_3 exposure of the asthmatic subjects were not significantly different from those of the normal subjects when adjustment was made for the effect of exercise-induced bronchoconstriction, although a trend was noted toward a greater O_3-induced increase in sRaw in the asthmatic subjects compared with the normal subjects ($p < 0.13$). In contrast, several inflammatory endpoints (percentage of neutrophils and total protein concentration in BAL fluid) after O_3 exposure were significantly greater ($p < 0.05$) in the asthmatic subjects than in the normal subjects ($n = 20$), even after adjusting for the values after air exposure with intermittent exercise (see Table 12). These data are presented graphically in Figure 19. A higher mean O_3-minus-air difference in IL-8 concentration in BAL fluids for the asthmatic subjects than for the normal subjects (36.1 vs. 11.6 pg/mL) did not reach statistical significance ($p < 0.2$).

Table 11. Phase III. Lavage Endpoints[a]

	Number of Subjects	Air		Ozone	
Bronchoalveolar Lavage					
Total leukocytes (× 10^4)	18	19.5 ± 2.9		26.9 ± 7.3	
Neutrophils (%)	18	4.1 ± 0.9		16.1 ± 3.0[b]	
Total protein (mg/mL)	18	0.11 ± 0.03	(1.0)	0.18 ± 0.01[b]	(1.0)
LDH (U/L)	15	6.1 ± 0.5	(55.5)	10.5 ± 1.7[b]	(58.3)
Fibronectin (ng/mL)	18	115.2 ± 29.6	(1047.3)	369.1 ± 38.8[b]	(2050.6)
IL-8 (pg/mL)	18	41.3 ± 4.5	(375.5)	75.7 ± 11.7[b]	(420.6)
GM-CSF (pg/mL)	18	1.8 ± 0.4	(16.4)	3.0 ± 0.5[b]	(16.7)
MPO (ng/mL)	14	1.8 ± 0.4	(16.4)	6.4 ± 0.9[b]	(35.6)
TGF-β2 (pg/mL)	16	4.9 ± 1.3	(44.5)	6.4 ± 1.2[b]	(35.6)
Bronchial Fraction					
Total leukocytes (× 10^4)	18	16.6 ± 1.2		22.4 ± 2.5	
Neutrophils (%)	18	5.7 ± 1.2		32.5 ± 4.4[b]	
Total protein (mg/mL)	18	0.14 ± 0.02	(1.0)	0.20 ± 0.03[b]	(1.0)
IL-8 (pg/mL)	18	138.2 ± 16.7	(987.1)	289.4 ± 53.6[b]	(1447.0)
Proximal Airway Lavage					
Total leukocytes (× 10^4)	17	6.7 ± 2.6		7.1 ± 2.2	
Neutrophils (%)	14	25.9 ± 6.4		39.9 ± 5.7[b]	
Total protein (mg/mL)	17	0.18 ± 0.06	(1.0)	0.12 ± 0.02	(1.0)
IL-8 (pg/mL)	15	286.4 ± 72.8	(1591.1)	347.7 ± 159.4	(2921.8)

[a] Values are presented as means ± SE, with ratio to total protein concentration given in parentheses where applicable.

[b] O_3 values > air values, $p < 0.05$.

Bronchial Biopsy Specimens: Immunohistochemistry, Morphometry, and Electron Microscopy

Immunohistochemical staining for the integrins and adhesion molecules in Phase III revealed two differences from the results obtained for the normal subjects studied in Phase II. Expression of ICAM-1 after air exposure was higher in most asthmatic subjects than in most normal subjects, and a patchy expression of integrin β6 was seen in some of the asthmatic subjects after air exposure. There were no differences in the asthmatic subjects due to O_3 exposure. With the use of new or resharpened forceps in Phase III, 34 biopsy specimens were obtained from asthmatic subjects (17 after O_3 exposure and 17 after air; 15 paired biopsies) with adequate tissue for leukocyte morphometry. Similar to the results for the normal subjects studied in Phase II, trends for increased neutrophil and eosinophil infiltration after O_3

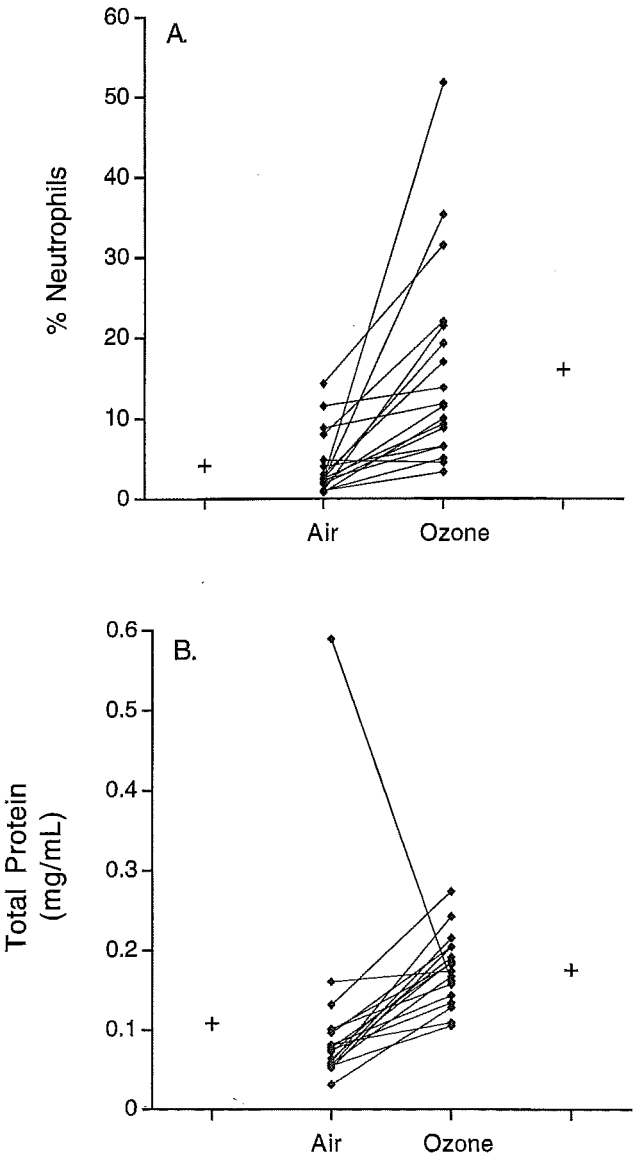

Figure 15. Phase III. Bronchoalveolar lavage results for asthmatic subjects (n = 18) 18 hours after exposure to filtered air or 0.2 ppm O_3 for 4 hours. Panel A shows individual data for each subject for percentage of neutrophils in BAL fluid, and Panel B shows individual data for total protein concentration (mg/mL) in BAL fluid. Group means are noted by the + signs next to each column of data points.

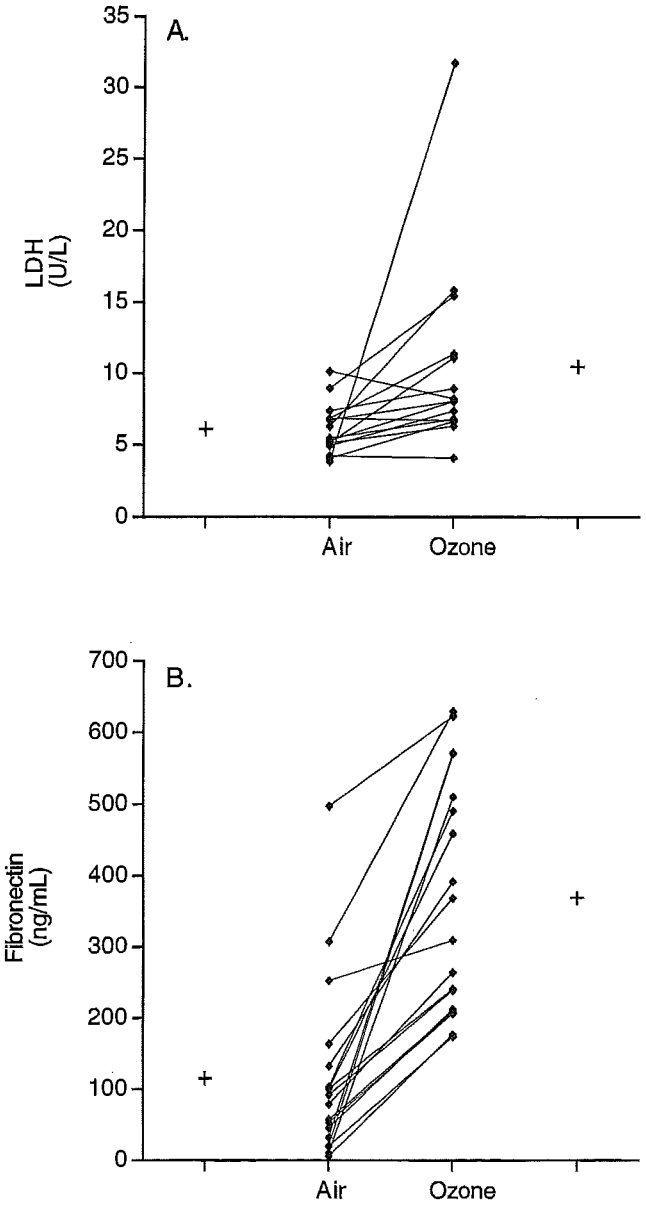

Figure 16. Phase III. Bronchoalveolar lavage results for asthmatic subjects (n = 18) 18 hours after exposure to filtered air or 0.2 ppm O_3 for 4 hours. Panel A shows individual data for each subject for LDH concentration (U/L) in BAL fluid, and Panel B shows individual data for fibronectin concentration (ng/mL) in BAL fluid. Group means are noted by the + signs next to each column of data points.

exposure were present in Phase III (Figure 20). For the asthmatic subjects in Phase III, the mean ± SE number of neutrophils/mm^2 was 6.5 ± 2.3 after O_3 exposure and 3.8 ± 1.2 after air exposure ($p = 0.8$). The mean ± SE number of eosinophils/mm^2 was 0.71 ± 0.26 after O_3 exposure and 0.64 ± 0.46 after air exposure ($p = 0.2$).

DISCUSSION

PHASE I

The results of our Phase I study indicate that baseline nonspecific airway responsiveness is not associated with

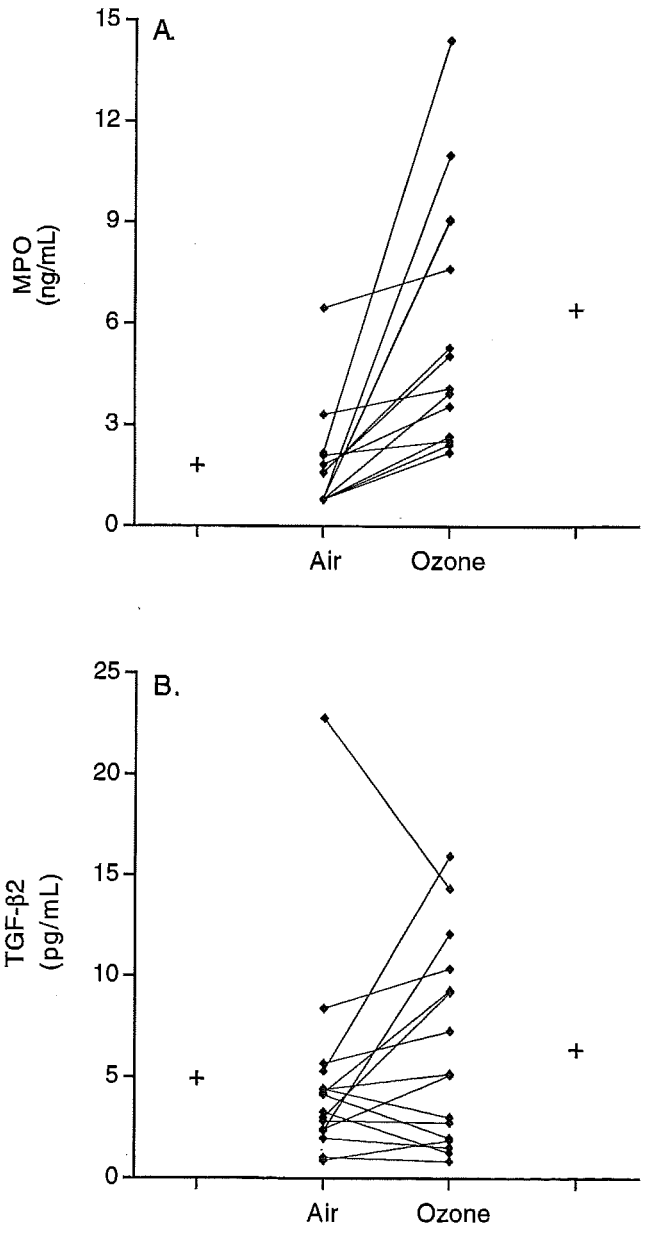

Figure 17. Phase III. Bronchoalveolar lavage results for asthmatic subjects ($n = 18$) 18 hours after exposure to filtered air or 0.2 ppm O_3 for 4 hours. Panel A shows individual data for each subject for IL-8 concentration (pg/mL) in BAL fluid, and Panel B shows individual data for GM-CSF concentration (pg/mL) in BAL fluid. Group means are noted by the + signs next to each column of data points.

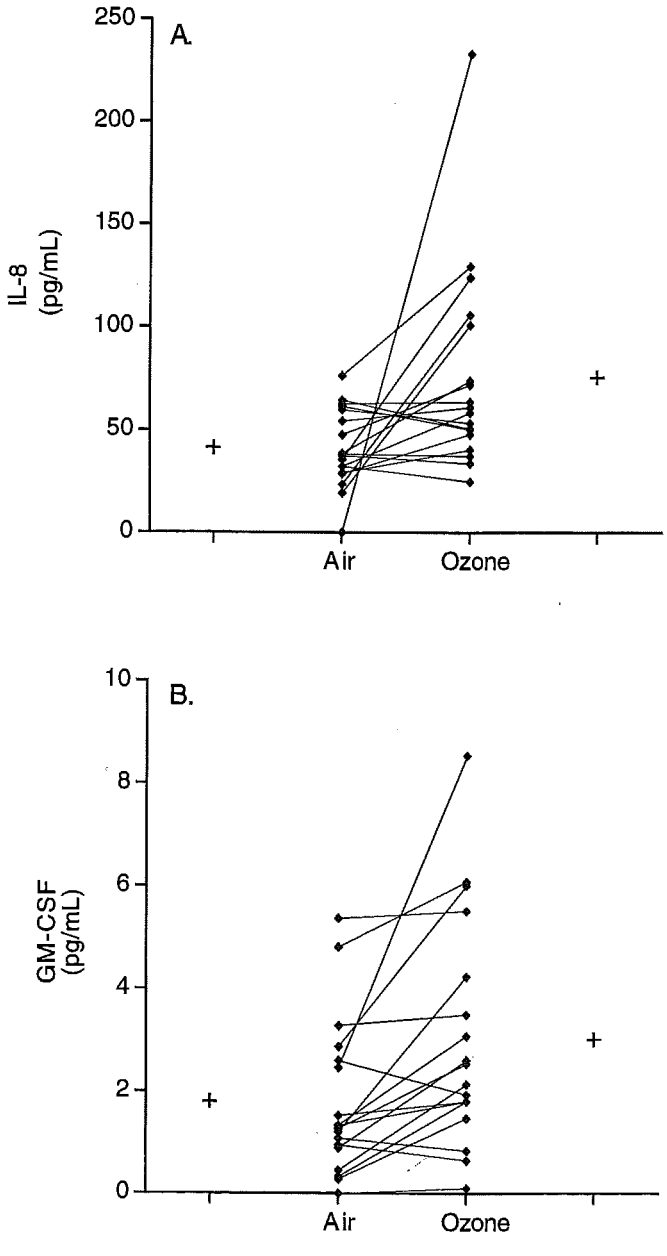

Figure 18. Phase III. Bronchoalveolar lavage results for asthmatic subjects 18 hours after exposure to filtered air or 0.2 ppm O_3 for 4 hours. Panel A shows individual data for each subject ($n = 14$) for MPO concentration (ng/mL) in BAL fluid, and Panel B shows individual data ($n = 16$) for TGF-β2 concentration (pg/mL) in BAL fluid. Group means are noted by the + signs next to each column of data points.

O_3-induced decreases in FEV_1 or FVC, but that it is weakly associated with O_3-induced increases in sRaw. The Phase I study was performed in a prospective fashion to test the hypothesis that nonspecific airway responsiveness would predict O_3 sensitivity as defined by decreases in FEV_1. Our results do not support this hypothesis. However, these results suggest that some individuals with nonspecific airway responsiveness are at risk of increases in airway resistance after exposure to O_3. Ozone-induced increases in airway resistance may be the result of airway narrowing due to inflammation (i.e., mucosal edema) rather than bronchoconstriction. Individuals with nonspecific airway hyperresponsiveness (i.e., those with asthma and allergic rhinitis) may develop greater airway inflammation after exposure to ambient levels of O_3 than normal individuals.

McDonnell and coworkers first proposed an association between nonspecific airway responsiveness and O_3 sensitivity (McDonnell et al. 1987). They exposed 26 nonasthmatic subjects with allergic rhinitis to 0.18 ppm O_3 for 2 hours after measuring histamine responsiveness. The mean provocative concentration of histamine that caused a 50% increase in sRaw (PC_{50}) and the decline in FEV_1 after O_3 were similar for the rhinitic (11.9 mg/mL and −0.37 L) and the normal (9.2 mg/mL and −0.29 L) subjects, respectively. One possible explanation for their failure to find an association between PC_{50} and O_3 sensitivity was that their subjects had a narrow range of PC_{50} values (2.7 to 20.0 mg/mL) and, therefore, the sample size may have been too small to detect a relationship. Hackney and coworkers were the first to report a relationship between baseline airway responsiveness and O_3-induced pulmonary function changes by noting that 8 of 12 subjects ("responders"), chosen for sensitivity to O_3 based on FEV_1 decreases, demonstrated increased responsiveness to methacholine prior to O_3 exposure (Hackney et al. 1989). Kreit and coworkers (1989) also provided data supporting the hypothesis that nonspecific airway hyperresponsiveness was a risk factor for developing O_3-induced decreases in lung function by reporting greater decreases in FEV_1 in subjects with asthma exposed to O_3 compared with those in normal subjects. We previously reported that a group of normal subjects selected for O_3 sensitivity as defined by a 10% or greater fall in FEV_1 had a significantly lower PC_{100} than did ozone-insensitive subjects (Aris et al. 1991).

As noted for the study conducted by McDonnell and coworkers, our Phase I study also may have had an insufficient number of individuals across the full range of methacholine responsiveness to detect a relationship between baseline nonspecific airway responsiveness and FEV_1 changes after exposure to O_3. Another potential problem with our Phase I study was that most of our subjects had large decreases in FEV_1 after O_3 exposure and, therefore, we may have had too few ozone-insensitive subjects to have adequate power to find a relationship between PC_{100} and FEV_1. More than likely, however, our original finding (Aris et al. 1991) was inherently biased since it was a retrospective observation made from data generated to test a different hypothesis. Thus, taken as a whole, the results of controlled human exposure studies that have looked at the correlation of baseline nonspecific airway responsiveness and FEV_1 response to O_3 show little evidence of an association.

The physiologic changes after O_3 exposure that we measured for our 66 subjects are similar to those reported by

Table 12. Phase III. O_3-minus-Air Differences[a]

	Normal Group[b]	Asthmatic Group[c]
FEV_1(AUC)	−1.65 ± 0.17	−1.56 ± 0.33
FVC(AUC)	−1.42 ± 0.14	−1.39 ± 0.37
sRaw(AUC)	2.24 ± 0.39	4.82 ± 2.43
Neutrophils (%)	4.5 ± 1.26	12.0 ± 2.95[d]
Total protein (mg/mL)	0.05 ± 0.01	0.07 ± 0.03[d]
IL-8 (pg/mL)	11.6 ± 8.4	36.1 ± 16.6

[a] Values are presented as means ± SE.

[b] For the normal group, for FEV_1 and FVC, n = 81; for sRaw, n = 70; for neutrophils, total protein, and IL-8, n = 20.

[c] For the asthmatic group, for all measurements, n = 18.

[d] $p < 0.05$.

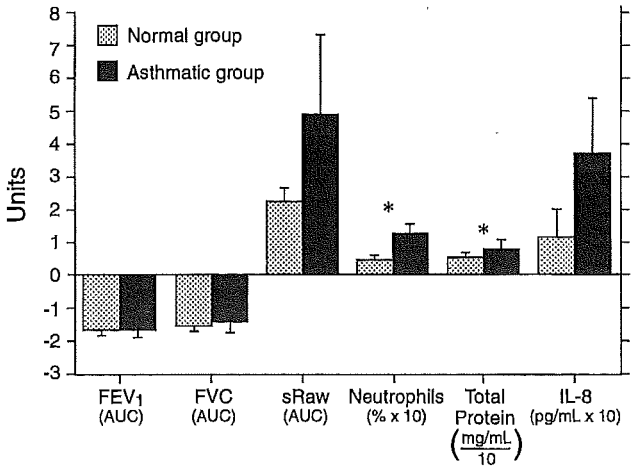

Figure 19. Phase III. Mean ± SE values for the O_3 − air differences in FEV_1AUC, FVCAUC, sRawAUC, percentage of neutrophils, total protein (TP) concentration (mg/mL), and IL-8 concentration (pg/mL) in BAL fluid for asthmatic subjects (solid columns) and normal subjects (stippled columns). Asterisks indicate significant differences ($p < 0.05$).

other investigators using comparable O_3 concentrations and exercise protocols (Kerr et al. 1975; Glinder et al. 1983; McDonnell et al. 1983; Horstman et al. 1990). Even after as little as 1 hour of exposure to 0.2 ppm O_3, a significant decrease in FEV_1 (–5.5%) was recorded. For the group as a whole, a mean decrease of 10% in FEV_1 was reached after about 1.5 hours of exposure. These results emphasize that short exposures with moderate exercise intensity can produce adverse effects at a level of O_3 that is reported in many large metropolitan areas in the U.S. (U.S. Environmental Protection Agency 1992). Since the O_3 concentration used in this study is the level at which many cities call a Stage I Alert, our results indicate that large numbers of people must be experiencing adverse health effects without adequate warning.

As expected, we also found that exposure to O_3 resulted in excess symptoms (in decreasing order of severity: cough, wheeze, chest pain on deep inspiration, chest discomfort or tightness, shortness of breath, and sputum production) compared with air exposure. Approximately one-half of the subjects not having an adverse physiologic response to O_3 (i.e., a decrease in FEV_1 greater than 10%) had an adverse symptomatic response (i.e., mild to moderate respiratory symptoms). Separate regression analysis of the different

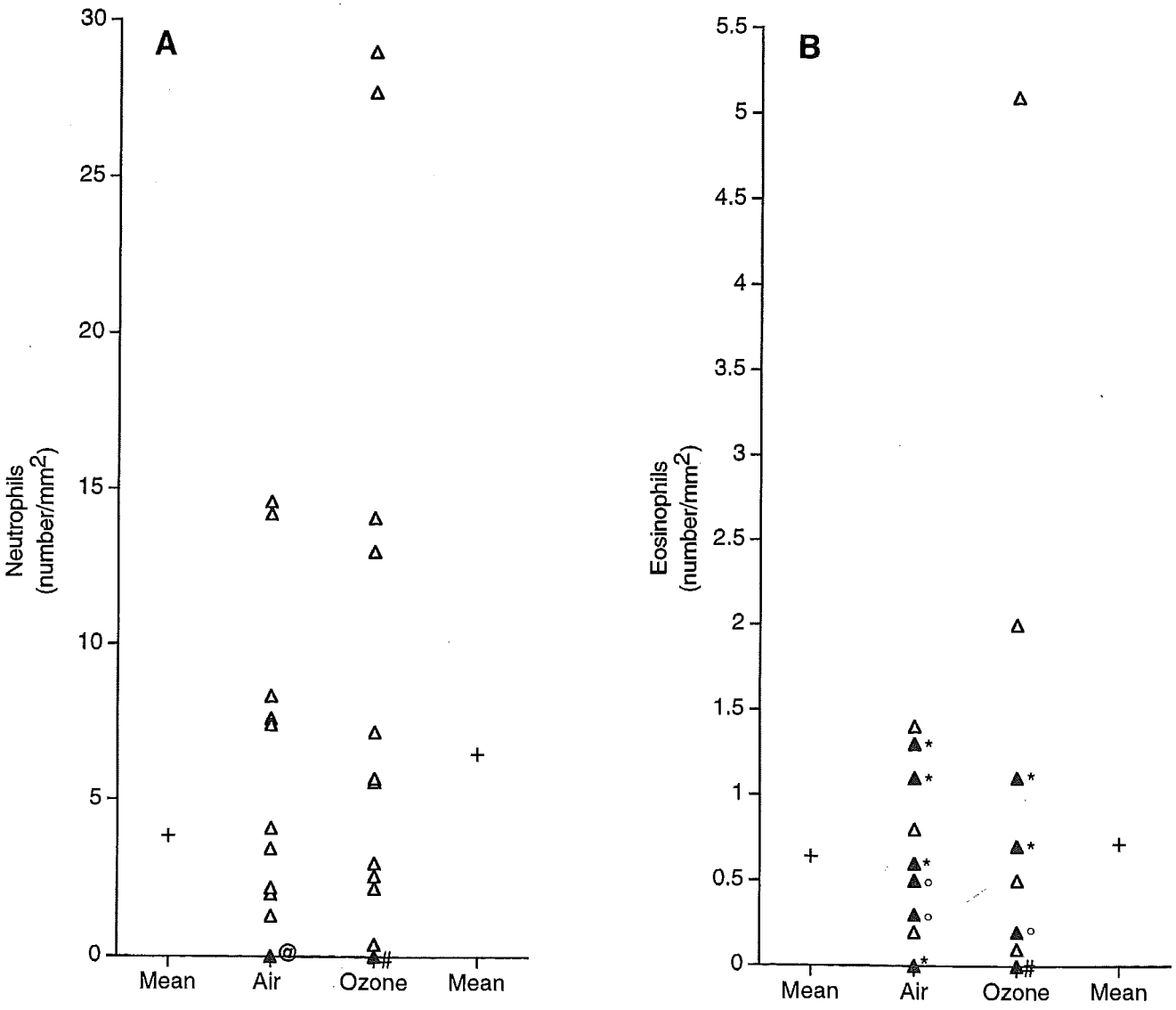

Figure 20. Phase III. Neutrophil (A) and eosinophil (B) counts per squared millimeter of bronchial mucosa after exposure of asthmatic subjects (n = 17) to filtered air or 0.2 ppm O_3 for 4 hours. An open triangle (Δ) indicates a single subject; a filled triangle (▲) indicates n = > 1; an asterisk (*) indicates n = 2, a circle (O) indicates n = 3, a pound sign (#) indicates n = 6, and an @ sign indicates n = 7. Group means are noted by the + signs next to each column of data points.

measures of lung function on each symptom added further support to the observation that symptoms and physiologic responses were not closely associated. This result differs from a number of previous studies that have reported a close relationship between group mean respiratory symptoms and physiologic responses in adults after short-term exposure to O_3 (Adams et al. 1981; Kulle et al. 1985; Horstman et al. 1990). However, these studies have attempted to quantify the relationship between symptoms and physiologic changes using individual data from relatively small numbers of subjects.

Hayes and coworkers were the first to analyze data from a group of clinical studies in order to achieve enough power to detect relationships between symptoms and lung function changes after O_3 exposure that are specific to a single individual (Hayes et al. 1987). They characterized the association between physiologic and symptomatic changes in adults after exposure to O_3 as weak to moderate by calculating the correlations across individuals at different O_3 doses. They did not analyze the relationships between changes in lung function and respiratory symptoms within the group at each O_3 dose. Lastly, Ostro and coworkers used logistic regression models to reanalyze the data from four controlled human exposure studies with a combined number of 214 subjects (Ostro et al. 1989). They found that a 10% decline in FEV_1 was associated with a 30% increase in the probability of a respiratory symptom occurring, and with a 15% increase in the probability of a moderate respiratory symptom occurring; they concluded that small declines in FEV_1 may be of concern because of their association with symptoms.

Our Phase I study, because of the larger sample size, was better able to examine relationships between symptoms and lung function than many previous studies. The relatively weak associations between individual-specific symptomatic and physiologic responses to O_3 are not particularly surprising because the decreases in FEV_1 are largely caused by pain on deep inspiration. Since pain is a highly variable symptom, it would not be expected, *a priori*, to be associated with other respiratory symptoms. Pain on deep inspiration is probably mediated by irritant receptors on vagal fibers in the large conducting airways (Hazucha et al. 1989). The interindividual variability in lung volume decreases after O_3 exposure may be due to differences in irritant receptors (e.g., number, density, or distribution), vagal responses, or in the perception of painful stimuli.

Our Phase I results, which indicate relatively weak associations between symptoms and lung function changes, are important for several reasons. First, they suggest that individuals may experience adverse symptoms without large decreases in lung function. Conversely, large decreases in lung function may occur without prominent symptoms and, therefore, affected individuals may remain in a potentially unhealthy environment. The relative discordance between respiratory symptoms and decreases in physiologic endpoints is of particular importance to regulatory agencies since the current NAAQS for O_3 is based predominantly on FEV_1 responses. If spirometry does not correlate with symptomatic responses, then the EPA will need to give this consideration when it reassesses the NAAQS for O_3.

In summary, our Phase I study did not find an association between baseline nonspecific airway responsiveness and O_3 sensitivity as defined by decline in FEV_1, but did find a weak association between baseline nonspecific airway responsiveness and O_3-induced increases in airway resistance. If O_3-induced increases in airway resistance reflect airway inflammation, sRaw may be a useful endpoint in the study of O_3. Since the decreases in FEV_1 after exposure to O_3 may be due to how much each individual's chest pain limits his or her ability to take a deep breath, FEV_1 may not be an optimal measure of the adverse effects of O_3. Our failure to find important relationships between the change in FEV_1 and a variety of respiratory symptoms in our current study is further evidence of the limitations of FEV_1 in predicting important clinical endpoints in the study of O_3.

PHASE II

The results of our Phase II study demonstrate no association between decrements in FEV_1 or FVC and lavage fluid inflammatory endpoints after exposure to O_3. These results strongly suggest that spirometric responses do not predict O_3-induced inflammatory changes. This issue is of importance because the current NAAQS for O_3 was largely established on the basis of spirometric responses to O_3 inhalation. If spirometry does not correlate with inflammatory indices of lung injury, then the U.S. EPA may need to reassess the NAAQS for O_3.

Although O_3-induced increases in airway resistance were small, we found a significant association between the magnitude of these increases and the percentage of neutrophils and the total protein concentration in BAL fluid. One possible explanation for this association is that an increase in sRaw reflects O_3-induced airway injury and inflammation better than a decrease in FEV_1. As noted in the introduction, the putative mechanism for decrements in FEV_1 and FVC after O_3 inhalation appears to be neurally mediated, involuntary inhibition of inspiratory effort (Hazucha et al. 1989). The magnitude of this physiologic response may have more to do with a subject's preexposure neural sensitivity to the inhalation of noxious stimuli than with the level of O_3-induced airway injury and inflammation. The mechanism for the mild increase in sRaw after O_3

inhalation is not entirely clear, but could involve a decrease in airway caliber due to mucosal edema, which would thus correlate better with the level of O_3-induced injury and inflammation. Because we only looked at indices of airway inflammation at 18 hours following exposure to O_3, we are unable to provide data to support our speculation that mucosal edema may have occurred during the 4-hour exposure period.

Although physiologic responses to O_3 have been extensively studied, the published data on the comparison between O_3-induced spirometric and inflammatory changes are limited. Schelegle and coworkers (1991) demonstrated a trend ($r = -0.813$, $p < 0.10$) toward an inverse relationship between maximal FEV_1 decrements and the peak neutrophilia observed in the bronchial fraction of BAL fluid obtained 6 hours after a 1-hour exposure to 0.3 ppm O_3 in five normal volunteers. In other words, the greater the decrease in FEV_1, the lower the increase in neutrophils. In an earlier study (Aris et al. 1993b) involving 12 subjects exposed to 0.2 ppm O_3 for 4 hours, we also found an inverse relationship ($r = -0.52$, $p < 0.05$) between the percentage of decrease in FEV_1 and the percentage of neutrophils in BAL fluid 18 hours after exposure. No significant associations were found, however, between other BAL fluid endpoints and the change in FEV_1. Although we did not confirm an inverse relationship between FEV_1 and BAL neutrophilic responses to O_3 in the current study, we did find a trend ($r = -0.16$) toward such a relationship with the bronchial fraction of BAL fluid. Thus, all three studies that have reported on the relationship between FEV_1 decrement and BAL inflammatory endpoints are consistent in finding no positive associations (i.e., the magnitude of the FEV_1 decrement has not been correlated with the magnitude of increase in BAL endpoints). Again, these results suggest the need to reconsider the standard-setting process for O_3.

The results of our Phase II study confirm several findings that we and others have previously reported. We have shown PAL to be one valuable technique in assessing the response of the airway epithelium to inhaled pollutants, including O_3 (Aris et al. 1993a,b). In the current study, we found significantly more neutrophils in PAL fluid after O_3 exposure, which provides further support for the concept that O_3 injures proximal airways as well as the distal lung. We chose to measure IL-8 and GM-CSF concentrations in lavage fluids because we hypothesized that both cytokines might be involved in mediating neutrophil recruitment to the airways following O_3 exposure. In contrast to our earlier study (Aris et al. 1993b), we did not find a significant increase in the PAL IL-8 concentration, although we did find such an increase in the bronchial fraction, another lavage fluid that samples relatively large airways. The cellular and biochemical evidence of O_3-induced inflammation in BAL fluid corroborates the results of previous investigations (Seltzer et al. 1986; Koren et al. 1989; Aris et al. 1993b), including our recently reported finding of an increase in GM-CSF (Aris et al. 1993b). The increase in GM-CSF that we have repeatedly observed after O_3 exposure suggests that neutrophil activation occurs in addition to the increase in neutrophil number (Takahashi et al. 1993).

The major limitation of our Phase II study is the relatively small sample size. This is a generic problem in controlled human exposure studies involving bronchoscopy. Our subject population ($n = 20$) is actually larger than is the rule for such studies, and was selected from an even larger population ($n = 81$) of normal subjects whose lung function responses to the O_3 dose were well characterized. Our sample size gave us adequate power to detect significant effects of the O_3 exposure on both FEV_1 and inflammatory responses. However, it is possible that our failure to detect any significant association between FEV_1 decrement and lavage fluid inflammatory endpoints is the result of a Type II error because an insufficient number of subjects were studied. On the other hand, if such an association does exist, it is likely to be weak. Another potential limitation of our study is that we may not have selected the appropriate parameters of airway inflammation for analysis. We focused on markers of inflammation that reflect epithelial permeability (total protein), possible injury (fibronectin), and neutrophil recruitment and activation (IL-8 and GM-CSF). It is possible that other lavage fluid endpoints, such as those related to neurogenic inflammation, might be better correlated with FEV_1 decrement.

The pathology component of our Phase II study demonstrated ultrastructural evidence of epithelial injury by electron microscopy, nonsignificant trends toward increased neutrophil infiltration of the bronchial mucosa by morphometry, and increased endothelial expression of ICAM-1 by immunohistochemistry after O_3 exposure. Taken together, these results confirm our previous finding that exposure to 0.2 ppm O_3 for 4 hours during moderate exercise can cause airway inflammation with histologic evidence that is visible by bronchial biopsies obtained 18 hours after exposure (Aris et al. 1993b). Previous animal studies have also demonstrated acute airway injury and inflammation after short-term O_3 exposure (Castleman et al. 1980; Holtzman et al. 1983; Murlas et al. 1985).

To our knowledge, this study is the first to demonstrate O_3-induced ultrastructural changes in the airway epithelium of human subjects with electron microscopy. This observation was not unexpected given the light microscopic changes that we had reported earlier (Aris et al.

1993b). The results of neutrophil morphometry were less definitive than we expected on the basis of our previous work. Why the trends that we observed toward increased subepithelial neutrophil counts after O_3 exposure did not reach statistical significance can be explained by several possible reasons. First, during each bronchoscopy, the bronchial biopsy specimens were obtained from only one site in the tracheobronchial tree, making sampling error highly likely. Although more histologic evidence of neutrophil infiltration into airway tissue would have lent support, the increased number of neutrophils observed in both PAL and BAL fluids after O_3 exposure clearly demonstrated an inflammatory response. These lavage fluids actually provide integrated samples from a much larger fraction of the airways than do the focal bronchial biopsy specimens. Second, the technical difficulties we encountered in obtaining adequate biopsy specimens for morphometry during Phase II led to an inability to compare paired specimens after both O_3 and air exposures, severely limiting the power of this aspect of the study. Third, in our earlier investigation (Aris et al. 1993b), we counted neutrophils in bronchial blood vessels as well as in the nonvascular subepithelial mucosa, whereas in this study we counted only extravascular neutrophils.

The increased expression of ICAM-1 after O_3 exposure that we observed is the first evidence that this adhesion molecule is involved in the recruitment of peripheral blood neutrophils to the human lungs in the O_3 model of acute airway injury. The lack of upregulation of VCAM is consistent with the absence of eosinophilia in both PAL and BAL fluids. The fact that we did not find clear immunohistochemical evidence of upregulation of any of the integrin subunits believed to be involved in injury repair may be due to the relatively mild airway injury that resulted from our O_3 exposure protocol.

In summary, our Phase II study indicates that the decrements in spirometric parameters of lung function and the lavage fluid evidence of respiratory tract inflammation that occur after short-term exposure to 0.2 ppm O_3 are not well correlated. The mild O_3-induced increase in airway resistance, however, does appear to be associated with the inflammatory response. Analysis of bronchial biopsy specimens confirms that short-term exposure to 0.2 ppm O_3 causes bronchial tissue injury in addition to an inflammatory response in the distal lung. Because the existing air quality standard was based largely on preventing spirometric responses to inhaled O_3 and because recurrent O_3-induced respiratory tract injury and inflammation could potentially lead to chronic lung damage, the EPA's regulatory strategy may need to be revised.

PHASE III

The results of our Phase III study demonstrate no enhanced sensitivity to O_3 in terms of lower respiratory symptoms or lung function responses in subjects with asthma. In contrast, these results do show evidence of an enhanced O_3-induced inflammatory response in the asthmatic groups. Both the percentage of neutrophils and the total protein concentration in BAL fluids were significantly greater in asthmatic than in nonasthmatic subjects. Given that airway inflammation appears to be a paramount feature of asthma (National Asthma Education Program Expert Panel 1991), it is reasonable to expect people with asthma to be at risk of developing a greater inflammatory response to O_3 than normal persons without preexisting airway inflammation. The mechanisms underlying this enhanced inflammatory response to a nonspecific irritant are not entirely clear, but the trends toward a higher concentration of IL-8 in BAL fluid after ozone exposure and the increased expression of ICAM-1 in the submucosal vessels evident by bronchial biopsy of asthmatic subjects after air exposure is consistent with the possibility that low-level inflammation of the airway epithelium in these subjects primes the inflammatory response to O_3.

Although the absolute O_3-induced changes in lung function of our asthmatic subjects were greater than those of our normal subjects, this difference was entirely due to a component of exercise-induced bronchoconstriction in the asthmatic group. When the lung function response to moderate exercise for 4 hours in filtered air was subtracted from that in O_3, no differences remained between the asthmatic and nonasthmatic subjects for FEV_1 and FVC. A trend toward a greater sRaw response to O_3 was noted among the asthmatic subjects, however. The mechanism by which O_3 induces decrements in FEV_1 and FVC appears to be neurally mediated, involuntary inhibition of inspiratory effort (Hazucha et al. 1989) involving stimulation of airway C-fibers (Coleridge et al. 1993). The magnitude of this physiologic response may have more to do with a subject's preexposure neural reflex sensitivity to inhaling noxious stimuli than with the level of O_3-induced airway injury and inflammation. Asthma appears not to confer greater neural reflex sensitivity to inhaled O_3. The mechanism for the mild increase in sRaw after O_3 inhalation is not entirely clear, but may involve a decrease in airway caliber due to mucosal edema, which correlates better with the level of O_3-induced injury and inflammation than decrements in FEV_1 and FVC, thus explaining the trend toward a greater sRaw response among the asthmatic subjects.

When we designed this study, no data had been published regarding findings in BAL fluids from asthmatic subjects after O_3 exposure. Recently, however, Basha and colleagues

reported the results of a study with a small sample size (five asthmatic and five normal subjects) (Basha et al. 1994). These results are similar to our own. Although the physiologic responses to O_3 of the asthmatic subjects were not significantly different from those of the normal subjects, evidence of greater O_3-induced inflammation was apparent for the asthmatic subjects. Thus, the findings of the two studies involving postexposure bronchoscopy that have been reported to date are reasonably consistent.

The major limitation of our Phase III study is the relatively small sample size. As noted above, our subject population for the bronchoscopy endpoints (18 asthmatic and 20 normal subjects) is actually larger than is the rule for such studies. The 20 normal subjects were selected from an even larger population ($n = 81$) that was used for comparing lung function responses to O_3. Our sample size gave us adequate power to detect significant effects of the O_3 exposure on lung function and inflammatory responses in both asthmatic and nonasthmatic subjects. Although it is possible that our failure to detect any significant difference in O_3-induced lung function responses between the asthmatic and nonasthmatic groups could be due to a Type II error because an insufficient number of subjects were studied, it is unlikely that we missed an effect of large magnitude.

In summary, our Phase III study indicates that although asthmatic persons may not have greater sensitivity to O_3 in terms of lower respiratory symptoms and lung function responses, they appear to be at risk of enhanced O_3-induced airway inflammation. This finding may help to explain the increased asthma morbidity associated with O_3 pollution episodes observed in epidemiologic studies.

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APPENDIX A. Telephone Interview Form

v-1-7/91

TELEPHONE INTERVIEW FORM

IDENTIFICATION NUMBER: ____ ____ ____ 1-3

DATE OF TELEPHONE CALL: ____ ____ ____ (month / day / year) 4-9

NAME: _____ 10-24
(record full first name but code first initial and full last name)

PHONE NUMBERS: WORK: (___ ___ ___) ___ ___ ___ - ___ ___ ___ ___ 25-38
(area code + #) HOME: (___ ___ ___) ___ ___ ___ - ___ ___ ___ ___ 35-48

Our laboratory is conducting a study to assess the effects of air pollution on people, so that we may be able to determine what level of pollution may be harmful. We are seeking volunteers, who we will pay, to exercise for four hours in a large chamber filled with a level of ozone that would occur on a day with moderate air pollution. During the exercise, we will measure the function of the lungs of the volunteers.

If you are eligible to volunteer, you will be paid $40 at your first visit, which will last about 2 hours and will involve filling out paper work, having tests of your lung function and exercise capacity. Two weeks and five weeks later, you will be asked to return to exercise in the chamber filled with clean air and ozone. You will be paid $15 per hour for each visit, which will last about 4 to 5 hours

Are you interested in participating? NO YES

IF THE SUBJECT AGREES TO PARTICIPATE, COMPLETE THE ENTIRE QUESTIONNAIRE.

IF THE SUBJECT DOES NOT WISH TO PARTICIPATE, ASK THE SUBJECT:
"Would you be willing to answer a few questions about your health? All of your answers will be kept strictly confidential."
AND
OBTAIN ZIP CODE AND ASK: EXERCISE, COUGH, PHLEGM, ASTHMA AND SMOKING QUESTIONS.

*****CIRCLE CHOICES OR FILL IN LINES, AS REQUIRED*****

ADDRESS: street _____ 45-64

city _____ (65-79) zip code ___ ___ ___ ___ ___ 80-84

DATE OF BIRTH: ____ ____ ____ (month / day / year) (85-90) SEX 1-F 2-M 91

HOW DID YOU HEAR ABOUT THE STUDY? _____

EXERCISE:

1. DO YOU EXERCISE 3 OR MORE TIMES A WEEK FOR 45 MINUTES AT A TIME? 1-NO 2-YES 92

IF 'YES', ASK REMAINDER OF Q1.

1B. DOES THIS EXERCISE INVOLVE:
JOGGING? 1-NO 2-YES 8-NA 93
SWIMMING? 1-NO 2-YES 8-NA 94
ROWING? 1-NO 2-YES 8-NA 95
OTHER? _____ 1-NO 2-YES 8-NA 96

2. ARE YOU LIMITED FROM EXERCISING BY ANY CONDITION? 1-NO 2-YES 97

ASK ALL REMAINING QUESTIONS EVEN IF SUBJECT FAILS EXERCISE CRITERIA

COUGH:

3. DURING THE PAST YEAR, DID YOU USUALLY HAVE A COUGH? 1-NO 2-YES 98

IF 'YES', ASK REMAINDER OF Q3.

3A. HAVE YOU COUGHED LIKE THIS ON MOST DAYS FOR 3 CONSECUTIVE MONTHS OR MORE DURING THE PAST YEAR? 1-NO 2-YES 8-NA 99

4. DURING THE PAST YEAR, DID YOU USUALLY COUGH AT ALL DURING OR AFTER EXERCISE? 1-NO 2-YES 100

5. DURING THE PAST YEAR, DID YOU USUALLY COUGH AT ALL WHEN FIRST GOING OUT OF DOORS IN THE COLD WEATHER? 1-NO 2-YES 101

PHLEGM:

6. DURING THE PAST YEAR, DID YOU USUALLY BRING UP PHLEGM OR MUCUS FROM YOUR CHEST? 1-NO 2-YES 102

IF 'YES', ASK REMAINDER OF Q6.

6B. HAVE YOU BROUGHT UP PHLEGM OR MUCUS LIKE THIS FOR 3 CONSECUTIVE MONTHS OR MORE DURING THE PAST YEAR? 1-NO 2-YES 8-NA 103

ASTHMA:

7. HAVE YOU EVER BEEN TOLD BY A DOCTOR THAT YOU HAD ASTHMA? 1-NO 2-YES 104

IF 'NO', ASK REMAINDER OF Q7.

7B. DO YOU THINK THAT YOU HAVE EVER HAD ASTHMA? 1-NO 2-YES 8-NA 105

SMOKING:

8. HAVE YOU EVER SMOKED AS MUCH AS 1 CIGARETTE PER DAY OR 20 PACKS IN YOUR LIFETIME? 1-NO 2-YES 106

IF 'YES', ASK Q8B.

8B. ARE YOU CURRENTLY SMOKING? 1-NO 2-YES 8-NA 107

IF 'NO', ASK Q8C AND Q8D.

8C. IN THE PAST 12 MONTHS, HAVE YOU SMOKED AS MUCH AS 1 CIGARETTE PER DAY? 1-NO 2-YES 8-NA 108

8D. WHEN YOU WERE SMOKING, WHAT WAS THE USUAL NUMBER OF CIGARETTES THAT YOU SMOKED EACH DAY? ____ ____ 109-110
(1 pack= 20 cigarettes)

9. HAVE YOU EVER SMOKED AS MUCH AS 1 POUCH OF PIPE TOBACCO OR 5 CIGARS? 1-NO 2-YES 111

IF 'YES', ASK Q9B.

9B. AT ANY TIME IN THE PAST 12 MONTHS, HAVE YOU SMOKED A PIPE CIGARS? 1-NO 2-YES 8-NA 112

10. HAVE YOU EVER SMOKED MORE THAN 1 MARIJUANA CIGARETTE (JOINT) PER WEEK ON A REGULAR BASIS? 1-NO 2-YES 113

IF 'YES', ASK Q10B.

10B. AT ANY TIME IN THE PAST 12 MONTHS, HAVE YOU SMOKED AS MUCH AS 1 JOINT PER WEEK? 1-NO 2-YES 8-NA 114

CARDIOVASCULAR DISEASE:

11. HAS A DOCTOR EVER TOLD YOU THAT YOU HAD A PROBLEM WITH YOUR HEART? 1-NO 2-YES 115

11B. IN THE PAST 12 MONTHS, HAVE YOU TAKEN ANY MEDICINE FOR YOUR HEART? 1-NO 2-YES 116

IF 'YES', SPECIFY _____

12. HAVE YOU EVER BEEN TOLD THAT YOU HAD HIGH BLOOD PRESSURE? 1-NO 2-YES 117

12B. IN THE PAST 12 MONTHS HAVE YOU TAKEN ANY MEDICINES FOR HIGH BLOOD PRESSURE? 1-NO 2-YES 118

IF 'YES', SPECIFY _____

OTHER:

13. DO YOU HAVE ANY REASON TO THINK THAT YOU ARE NOW PREGNANT? 1-NO 2-YES 8-NA 119

14. DO YOU PLAN TO MOVE FROM THE BAY AREA WITHIN THE NEXT 6 MONTHS? 1-NO 2-YES 120

Because it is essential that we are as certain as we can be that you have healthy lungs, we must ask you some personal questions about health problems or practices that can affect your lungs without you feeling ill. Let me remind you that your answers to these next questions are strictly confidential and cannot be obtained by any one who might look at the questionnaire that I am reading.

15. 1-NO 2-YES 121

16. 1-NO 2-YES 122

REFUSED 1-NO 2-YES(P) 3-YES(P&Q) 123
REJECTED 1-NO 2-YES 124
reason for rejection (record Q #) ____ ____ 125-126

APPENDIX B. Subject Interview Form

v1-8/91

SUBJECT INTERVIEW FORM

office use only

IDENTIFICATION NUMBER: ___ ___ ___ 1-3

DATE OF INTERVIEW: ___ month ___ day ___ year 4-9

NAME: _____
(RECORD FULL FIRST AND LAST NAME)

PLEASE READ DIRECTIONS BEFORE COMPLETING THIS FORM

Read each question completely before answering it. Do not leave any blanks. Circle the 1 choice that BEST reflects your answer to the question. If you are asked to provide a number or a date, make sure that you write legibly. If you do not know the answer to a question, circle the choice "DK" or write "DK" on the appropriate line. If you do not understand a question, ask for help. DO NOT SKIP THE QUESTION without getting help, because your ability to answer the next question may depend on your understanding of questions that came before it. THANK YOU.

EPISODES OF COUGH AND PHLEGM (MUCUS):

1. DURING THE PAST 12 MONTHS, HAVE YOU HAD ANY PERIODS OR EPISODES OF COUGH AND PHLEGM (OR INCREASED COUGH OR PHLEGM IF YOU USUALLY HAVE COUGH AND PHLEGM) THAT LASTED AT LEAST 3 WEEKS?
1-NO 2-YES 7-DK 10

IF YOU ANSWERED 'YES,' ANSWER Q 1B.

1B. HOW MANY SUCH EPISODES HAVE YOU HAD?
1) 1 episode 2) 2 to 3 3) 4 or more 7-DK 11

WHEEZING:

2. IN THE PAST 12 MONTHS, HAS YOUR CHEST EVER SOUNDED WHEEZY OR WHISTLING?
1-NO 2-YES 7-DK 12

IF YOU ANSWERED 'YES', ANSWER THE REMAINDER OF Q 2.

2B. IN THE PAST 12 MONTHS, HAS YOUR CHEST EVER SOUNDED WHEEZY OR WHISTLING: (check best response for each question)

	1 NEVER	2 SOMETIMES	3 MOST DAYS	7 DK	
WITH COLDS?	( )	( )	( )	( )	13
APART FROM COLDS?	( )	( )	( )	( )	14
MOST DAYS & NIGHTS?	( )	( )	( )	( )	15

2C. IN THE PAST 12 MONTHS, HAS YOUR CHEST EVER SOUNDED WHEEZY OR WHISTLING: (check best response for each question)

	1 NEVER	2 SOMETIMES	3 MOST DAYS	7 DK	
WITH EXERCISE OR EXERTION?	( )	( )	( )	( )	16
WITH EXPOSURE TO POLLEN?	( )	( )	( )	( )	17
WITH EXPOSURE TO GRASSES?	( )	( )	( )	( )	18
WITH EXPOSURE TO CATS?	( )	( )	( )	( )	19
AFTER TAKING ASPIRIN?	( )	( )	( )	( )	20

2D. IN THE PAST 12 MONTHS, HAS YOUR CHEST EVER SOUNDED WHEEZY OR WHISTLING: (check best response for each question)

	1 NEVER	2 SOMETIMES	3 MOST DAYS	7 DK	
WITH EXPOSURE TO DUST AT HOME OR AT WORK?	( )	( )	( )	( )	21
WITH EXPOSURE TO FUMES AT WORK?	( )	( )	( )	( )	22
WITH EXPOSURE TO SPRAYS AT HOME OR AT WORK?	( )	( )	( )	( )	23

2E. HOW RECENTLY HAS YOUR CHEST SOUNDED WHEEZY OR WHISTLING?

1-IN THE PAST WEEK 2-IN THE LAST MONTH 3-MORE THAN 1 MONTH AGO 7-DK 24

2F. IN THE PAST 12 MONTHS, HAVE YOU TAKEN ANY MEDICINE OR TREATMENT FOR THIS WHEEZING OR WHISTLING?
1-NO 2-YES 7-DK 25

IF YOU ANSWERED 'YES', PLEASE LIST MEDICINES.

_____
_____
_____

ALLERGY SYMPTOMS:

3. DO YOU EVER GET EPISODES OF:(check best response for each question)

	1 NO	2 YES	7 DK	
RUNNY OR ITCHY EYES APART FROM COLDS?	( )	( )	( )	26
SNEEZING OR RUNNY NOSE APART FROM COLDS?	( )	( )	( )	27

IF YOU ANSWERED 'YES' TO EITHER OF THE ABOVE, ANSWER Q 3B.

3B. ARE THESE SYMPTOMS WORSE IN ANY SEASON?
(place the number of your choice next to each season)

1-NO 2-YES 7-DK

SPRING ___ 28
SUMMER ___ 29
FALL ___ 30
WINTER ___ 31

4. HAVE YOU EVER BEEN TOLD BY A DOCTOR OR NURSE THAT YOU HAVE (OR HAVE HAD) "HAY FEVER" OR "ALLERGIC RHINITIS"?
1-NO 2-YES 7-DK 32

IF YOU ANSWERED 'YES', ANSWER Q 4B.

4B. HAVE YOU EVER RECEIVED MEDICINE FOR THIS? 1-NO 2-YES 7-DK 33

5. HAVE YOU EVER RECEIVED ALLERGY SHOTS?
1-NO 2-YES 7-DK 34

IF YOU ANSWERED 'YES', ANSWER Q 5B.

5B. DO YOU STILL GET ALLERGY SHOTS?
1-NO 2-YES 7-DK 35

OTHER ILLNESSES:

6. IN THE PAST 12 MONTHS, HAS A DOCTOR TOLD YOU THAT YOU HAD:

BRONCHITIS? 1-NO 2-YES 7-DK 36

PNEUMONIA? 1-NO 2-YES 7-DK 37

7. IN THE PAST 12 MONTHS, HAVE YOU HAD ANY CHEST OPERATIONS?
1-NO 2-YES 7-DK 38

IF YOU ANSWERED 'YES', ANSWER Q 7B.

7B. PLEASE SPECIFY THE OPERATION(S):

RESIDENTIAL HISTORY:

To complete the residential history, begin with your CURRENT residence and work your way backwards in time. If you do not remember all of the information for any place that you have lived, write "DK" in the appropriate space.

	CITY/TOWN	STATE	ZIPCODE	FROM MON	FROM YEAR	TO MON	TO YEAR
current:	(39-53)	(54-55)	(56-60)	(61-2)	(63-4)	(65-6)	(67-8)
	(69-83)	(84-85)	(86-90)	(91-2)	(93-4)	(95-6)	(97-8)
	(99-113)	(114-5)	(116-20)	(121-2)	(123-4)	(125-6)	(127-8)
	(129-143)	(144-5)	(146-50)	(151-2)	(153-4)	(155-6)	(157-8)
	(159-173)	(174-5)	(176-80)	(181-2)	(183-4)	(185-6)	(187-8)

OCCUPATIONAL HISTORY:

To complete the Occupational history, start with your current job and work backwards in time. If you do not remember all of the information for any job that you have had, write "DK" in the appropriate space. Include only jobs that you have held in the last 5 years for at least 6 months.

	JOB TITLE	INDUSTRY	MATERIALS EXPOSED TO	FROM MON	FROM YEAR	TO MON	TO YEAR
current							
2.				189-0	191-2	193-4	[illegible]
3.				197-8	199-0	201-2	[illegible]
4.				205-6	207-8	209-0	[illegible]
5.				213-4	215-6	217-8	[illegible]
				221-2	223-4	225-6	[illegible]

office use

	Industry	job	materials	duration
c.	___ ___(229-30)	___ ___(231-32)	___ ___(233-34)	___ ___(235-36)
2.	___ ___(237-38)	___ ___(239-40)	___ ___(241-42)	___ ___(243-44)
3.	___ ___(245-46)	___ ___(247-48)	___ ___(249-50)	___ ___(251-52)
4.	___ ___(253-54)	___ ___(255-56)	___ ___(257-58)	___ ___(259-60)
5.	___ ___(261-62)	___ ___(263-64)	___ ___(265-266)	___ ___(267-68)

APPENDIX C. Symptom Questionnaire

SYMPTOM QUESTIONNAIRE

Name _____ Subject # _____ Date _____
Time _____

Please rate the severity of your symptoms on a scale indicated below.

0 = None	
1 = Minimal	symptom is barely noticeable
2 = Mild	symptom is present by not annoying
3 = Moderate	symptom is somewhat annoying
4 = Severe	symptom is very annoying and/or limits performance

anxiety	0	1	2	3	4
chest discomfort or chest tightness	0	1	2	3	4
chest pain on deep inspiration	0	1	2	3	4
cough	0	1	2	3	4
eye irritation	0	1	2	3	4
headache	0	1	2	3	4
nasal irritation	0	1	2	3	4
nausea	0	1	2	3	4
phlegm or sputum production	0	1	2	3	4
shortness of breath	0	1	2	3	4
throat irritation	0	1	2	3	4
wheezing	0	1	2	3	4

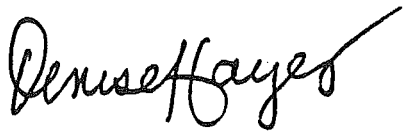
a.) Did you smell an unusual odor in the chamber? Y N
b.) Do you think you were exposed to ozone today? Y N

6/24/91 v.1

APPENDIX D. External Quality Assurance Report

The conduct of this study has been subjected to periodic audits by the Quality Assurance Officer from BioDevelopment Laboratories. The audits have included in-process monitoring of study activities and audits of the data. The dates of audits and nature of the visit are listed in Table D.1. The results of the inspections were reported to the Director of Research of the Health Effects Institute, who was responsible for transmitting the reports to the Principal Investigator.

Observations made during these visits indicate that the study is well documented and that the report describes the methods used and reflects the raw data. The effect of deviations from the protocol and standard operating procedures on the results of the study have been considered and addressed as appropriate in the data or final report.



Denise Hayes, M.S.
Quality Assurance Officer
BioDevelopment Laboratories

Table D.1. Audits by Quality Assurance Officer

Date	Procedure or Data Reviewed
December 7, 1990	Pre-study visit
March 4-5, 1992	Observe activities and audit data for Phase I
September 9-10, 1996	Audit data and review final report

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PUBLICATIONS RESULTING FROM THIS RESEARCH

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ABBREVIATIONS

AUC	area under the curve
BAL	bronchoalveolar lavage
ELAM	E-selectin or endothelial leukocyte adhesion molecule
EPA	U.S. Environmental Protection Agency
FEF_{25-75}	forced expiratory flow from 25% to 75% of the vital capacity
FEV_1	forced expiratory volume in one second
FVC	forced vital capacity
GMA	glycolmethacrylate
GM-CSF	granulocyte–macrophage colony–stimulating factor
ICAM-1	intercellular adhesion molecule-1
IL-8	interleukin-8
LDH	lactate dehydrogenase
MANOVA	multivariate analysis of variance
MPO	myeloperoxidase
O_2	oxygen
O_3	ozone
NAAQS	National Ambient Air Quality Standard
PAL	proximal airway lavage
PBS	phosphate-buffered saline
PC_{100}	concentration of methacholine that produced a 100% increase in sRaw
sRaw	specific airway resistance
TGF-β2	transforming growth factor β2
VCAM	vascular cell adhesion molecule
$\dot{V}_E$	ventilatory rate

Effects of Ozone on Normal and Potentially Sensitive Human Subjects

Part II: Airway Inflammation and Responsiveness to Ozone in Nonsmokers and Smokers

Mark W. Frampton, Paul E. Morrow, Alfonso Torres, Karen Z. Voter, John C. Whitin, Christopher Cox, Donna M. Speers, Ying Tsai, and Mark J. Utell

ABSTRACT

Exposure to ozone at levels near the National Ambient Air Quality Standard causes respiratory symptoms, changes in lung function, and airway inflammation. Although ozone-induced changes in lung function have been well characterized in healthy individuals, the relationship between airway inflammation and changes in pulmonary function have not been prospectively examined. The purpose of this study was to determine whether individuals who differ in lung function responsiveness to ozone also differ in susceptibility to airway inflammation and injury. A secondary goal was to determine whether ozone exposure induces airway inflammation in smokers, a population known to have airway inflammation and an increased burden of toxic oxygen species.

Healthy nonsmokers (n = 56) and smokers (n = 34) were exposed to 0.22 parts per million (ppm)* ozone for 4 hours, with intermittent exercise, for the purpose of selecting ozone "responders" (decrement in forced expiratory volume in 1 second [FEV_1] > 15%) and "nonresponders" (decrement in FEV_1 < 5%). Selected subjects then were exposed twice to ozone (0.22 ppm for 4 hours with exercise) and once to air (with the same exposure protocol), each pair of exposures separated by at least 3 weeks, in a randomized, double-blind fashion. Nasal lavage (NL) and bronchoalveolar lavage (BAL) were performed immediately after one ozone exposure and 18 hours after the other, and either immediately or 18 hours after the air exposure. Indicators of airway effects in lavage fluid included changes in inflammatory cells, proinflammatory cytokines, protein markers of epithelial injury and repair, and generation of toxic oxygen species.

In the classification exposure, fewer smokers than nonsmokers were responsive to ozone (11.8% vs. 28.6%, respectively); an insufficient number of smoker-responders were identified to study as a separate group. In the BAL study, all groups developed a similar degree of airway inflammation, consisting of increases in interleukins 6 and 8 (maximal immediately after exposure), and increases in polymorphonuclear leukocytes (PMNs), lymphocytes, and mast cells (maximal 18 hours after exposure). The increase in PMNs was inversely correlated with age (p = 0.013), but gender, nonspecific airway responsiveness, and allergy history were not predictive of inflammation. Alveolar macrophage production of toxic oxygen species decreased after ozone exposure in nonsmokers; however, not in smokers. Findings from nasal lavage did not mirror lower airway inflammatory responses in these studies. We conclude that, in response to ozone exposure, smokers experienced smaller decrements in lung function and fewer symptoms than nonsmokers; however, the intensity of the airway inflammatory response was independent of smoking status or airway responsiveness to ozone. Furthermore, the burden of toxic oxygen species following ozone exposure was greater for smokers than for nonsmokers. Subjects were young, healthy, and able to sustain exercise; the results may not be representative of nonsmokers or smokers in general. Nevertheless, the findings indicate that measuring symptoms and spirometric changes is not sufficient to assess the potential risks associated with ozone exposure.

INTRODUCTION

The potential health effects of exposure to ozone are of major interest to the public, industry, and regulators. Recently, controlled exposures to ozone at levels at or below

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

This Investigators' Report is Part II of Health Effects Institute Research Report Number 78, which also includes *Part I: Airway Inflammation and Responsiveness to Ozone in Normal and Asthmatic Subjects*, by John R. Balmes and associates; *Part III: Mediators of Inflammation in Bronchoalveolar Lavage Fluid from Nonsmokers, Smokers, and Asthmatic Subjects Exposed to Ozone: A Collaborative Study*, by Mark W. Frampton, John R. Balmes, and colleagues; a Commentary by the HEI Health Review Committee, and an HEI Statement about the research project. Correspondence concerning this Investigators' Report may be addressed to Dr. Mark W. Frampton, University of Rochester School of Medicine, 601 Elmwood Avenue, Box 692, Rochester, NY 14642-8692.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award R824835 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

the National Ambient Air Quality Standard (NAAQS) of 0.12 ppm have resulted in respiratory symptoms and decrements in pulmonary function in healthy volunteers. In some individuals FEV_1 decreased as much as 50% following exposure with exercise to ozone in the ambient range of concentrations (McDonnell et al. 1985, 1995; Folinsbee et al. 1988). Individuals have been consistent in their responses to subsequent ozone exposures (McDonnell et al. 1985), which suggests that determinants of susceptibility do exist; the nature of these determinants is largely unknown. Changes in lung function in response to ozone exposure, which have been well characterized in humans, have historically driven the setting of air quality standards for ozone.

In addition, exposures to ozone at levels near the NAAQS cause cellular and biochemical changes in the upper and lower respiratory tract characteristic of an acute inflammatory response. Human studies using BAL have shown ozone-induced increases in PMNs, soluble markers of inflammation and repair, and markers of epithelial permeability (Seltzer et al. 1986; Kehrl et al. 1987; Koren et al. 1989a,b, 1991; Devlin et al. 1991). Using nasal lavage, inflammation and permeability changes have been observed in the upper airway after ozone exposure (Graham and Koren 1990). The increase in permeability may result from direct epithelial injury, or indirectly from release of toxic oxygen species (TOS) and proteolytic enzymes by PMNs, or alveolar macrophages (AMs), or both.

The relative role of PMNs and AMs in contributing to epithelial injury after ozone exposure is unclear. Both cells release TOS as part of the oxidative burst; this is an important component of host defense against microorganisms, but also may contribute to lung injury. Oxidant release has been shown to injure epithelial cells (Cantin et al. 1987) and fibroblasts (Simon et al. 1981). Alveolar macrophages are more numerous than PMNs, even after ozone exposure, and may therefore present the largest burden of TOS to the respiratory epithelium. However, studies in mice indicate that acute exposures to ozone decrease AMs' release of superoxide anion (Amoruso et al. 1981; Ryer-Powder et al. 1988), which provides a possible explanation for this species' increased susceptibility to infectious challenge after ozone exposure. Koren and coworkers (1989b) found no difference in the release of superoxide anion by human alveolar macrophages obtained by BAL 18 hours after air or ozone (0.4 ppm for 2 hours) exposure. However, neither the responses immediately after exposure nor the relative contribution of PMNs to the total burden of TOS has been assessed in humans.

Additional gaps persist in our understanding of the inflammatory response to ozone in humans. First, the time course of the response is not clearly defined. Koren and coworkers (1991), comparing two separate studies that had examined the effects of exposure to 0.40 ppm ozone for 2 hours, found the influx of PMNs was 2.3-fold greater 1 hour after exposure than it was 18 hours after exposure. Schelegle and coworkers (1991) performed a time-course study in five subjects, and found that the peak recovery of PMNs by BAL occurred at 6 hours after exposure, rather than at less than 1 hour or 24 hours after exposure. Do changes in AMs precede the influx of PMNs in response to ozone exposure? Early events after exposure could include altered expression of cell surface receptors on AMs that play a role in host defense, or oxidant injury, or both.

Second, the relationship between pulmonary function decrements and airway inflammation has not been determined. Airway inflammation could result from ozone-induced stimulation of nerves that contain neuropeptides within the airways; increased levels of substance P have been found in BAL fluid of human subjects immediately after a 1-hour exposure to 0.25 ppm ozone (Hazbun et al. 1993). If lung function changes and inflammation are both mediated via intraepithelial nerve stimulation, inflammation intensity should correlate with lung function responsiveness. An alternative mechanism is the release of chemotactic cytokines such as interleukins 6 and 8 (IL-6, IL-8) from airway epithelial cells through nonneurogenic pathways, such as activating the nuclear regulatory element NF-κB (Bromberg and Koren 1995). If the mechanisms are unrelated, it is possible that individuals who do not experience symptoms or lung function changes would be at greatest risk of airway inflammation from failure to avoid exposure or limit inspiration. A recent study (Schelegle et al. 1991) found an inverse correlation between lung function changes and the airway inflammatory response to ozone, suggesting the possibility that changes in airway geometry may reduce ozone delivery to sites of injury.

Third, little is known about the airway inflammatory response to ozone in individuals with underlying respiratory disease. Many chronic respiratory diseases are characterized by inflammation; ozone exposure could intensify manifestations of disease by acutely increasing the underlying inflammatory processes.

Approximately 48 million Americans smoke cigarettes, and smoking remains the single most common cause of respiratory morbidity and mortality in the United States (Fiore et al. 1994). Data from the Harvard Six-Cities Study (Dockery et al. 1993) suggest that the mortality risk associ-

ated with exposure to air pollution is greater in current and former smokers than in nonsmokers. Furthermore, smoking and ozone exposure have caused some similar pulmonary responses. For example, smoking has been shown to cause a respiratory bronchiolitis with similarities to the lesions found in animals after prolonged exposure to ozone (Adesina et al. 1991). Cigarette smoke and ozone exposure both cause airway inflammation and increased epithelial permeability (Kehrl et al. 1987; Mason et al. 1983). Both may cause lipid peroxidation; increased levels of lipid peroxidation products have been found in the blood of smokers (Morrow et al. 1995). Inflammatory cells producing TOS in the airways and alveoli may inactivate antiprotease enzymes, thereby contributing to the destruction of elastic tissue that is characteristic of the effects of cigarette smoking (Hubbard et al. 1987). Finally, smokers have increased numbers of AMs in distal airspaces that may respond to stimuli by releasing increased quantities of TOS (Lin et al. 1989). If exposure to ozone causes an increase in airway inflammation or stimulates release of TOS by resident inflammatory cells, smokers may be at increased risk of adverse effects.

The few studies of ozone exposure in smokers are limited to examining pulmonary function effects. Kerr and coworkers (1975) found that smokers experienced smaller decrements in forced vital capacity (FVC) and FEV_1 and fewer symptoms than nonsmokers after exposure to 0.5 ppm ozone for 6 hours. In contrast, Hazucha and coworkers (1973) found greater decrements in FEV_1 and larger increases in residual volume in smokers than in nonsmokers exposed to 0.75 ppm ozone for 2 hours, with intermittent exercise. Effects of ozone exposure on airway inflammation, release of TOS by inflammatory cells, and epithelial injury have not been studied in smokers.

Improved understanding of the airway inflammatory response to ozone exposure is needed to assess potential health risks. In the current study, we proposed to address several of the deficiencies in our knowledge of those effects.

SPECIFIC AIMS

The primary purpose of this study was to determine whether individuals who differ in lung function responsiveness to ozone also differ in susceptibility to airway inflammation and injury. The four specific aims of this project were as follows:

1. In smokers and nonsmokers determined to be responsive or nonresponsive to ozone exposure, using FEV_1 as the indicator of response, examine the relationship between decrements in FEV_1 and lower airway inflammation.
2. Characterize the effects of in vivo ozone exposure on AMs and on the in vitro generation of TOS by alveolar cells; compare the findings from ozone-responsive and -nonresponsive smokers and nonsmokers.
3. Determine the reproducibility of symptoms and decrements in FEV_1 after repeated identical exposures to ozone in smokers and nonsmokers.
4. Determine to what extent findings from NL reflect inflammation of the lower airways, as evaluated by BAL, in ozone-responsive and -nonresponsive smokers and nonsmokers.

Subjects selected were either responsive or nonresponsive to ozone exposure in terms of lung function (FEV_1). Indicators of airway effects included changes in inflammatory cells, proinflammatory cytokines, and protein markers of epithelial injury and repair. Alveolar macrophages were characterized with regard to changes in size and in their expression of selected surface markers important to the host's defense. The generation of TOS was determined for the whole cell population and for AMs separately. The experimental design allowed for measurements at two time points after exposure, thus providing information on the time course of the inflammatory response.

METHODS AND STUDY DESIGN

SUBJECTS

Volunteers of both genders, ages 18 to 40 years, were recruited from the local community through bulletin board and newspaper advertisements; most were college students. Informed consent was obtained, and the study was approved by the Research Subjects Review Board of the University of Rochester. (The consent form is Appendix F, which is available on request from the Health Effects Institute.) Only subjects who consented to the entire study, including the BAL phase, were recruited, even though it was clear that not all subjects would be asked to participate in the BAL phase of the study. The considerable time and effort required of volunteers for the overall study was emphasized at the time of screening in order to ensure commitment to complete the study. Subjects were evaluated with the following methods: a respiratory questionnaire, modified from Lebowitz and coworkers (1975) (shown in Appendix A), which included demographic information, history of allergies, use of medications and vitamins, smoking history, and passive smoke exposure; physical exami-

nation; spirometry; exercise testing on a bicycle ergometer; and inhalation challenge with methacholine. Subjects were required to be free of cardiorespiratory disease, without symptoms of respiratory infection within 3 weeks of the study, able to complete the exercise protocol, and have normal spirometry without exercise-induced bronchoconstriction (decrease in FEV_1 < 5% after 20 minutes of exercise at a minute ventilation [$\dot{V}_E$] of 25 L/min/m^2). Subjects with a history of asthma (based on previous physician diagnosis or a history of episodic wheezing and dyspnea) were excluded, but subjects with allergy, hay fever, or allergic rhinitis were not excluded. Nonsmokers had never smoked regularly and denied any tobacco use in the 3 years prior to the study. Smokers were currently smoking at least one pack per day, with at least three pack-years of smoking history.

STUDY DESIGN

A primary goal of these studies was to determine the nature and intensity of the airway inflammatory responses in both nonsmokers and smokers who we had determined to be responsive or nonresponsive to ozone based on decrements in pulmonary function. The experimental protocol is summarized in Figure 1, and the detailed exposure protocol is provided in Appendix B. The overall protocol required a minimum of 5 days for completion, and each subject completing the entire protocol underwent four exposures (three to ozone and one to air) and three BAL and NL procedures.

Day one was devoted to subject screening and baseline measurements. Day two involved an initial 4-hour exposure to 0.22 ppm ozone to classify subjects as "responders" or "nonresponders" based on their decrement (> 15%) or lack of decrement (< 5%) in FEV_1, respectively. Subjects selected on the basis of the classification exposure then were invited to participate in the BAL portion of the study. On days three through five, subjects were exposed once to air and twice to ozone, with NL and BAL performed immediately after one of the ozone exposures (referred to subsequently as "ozone early") and 18 hours after the other ozone exposure ("ozone late"). Exposures were separated by at least three weeks. Because each subject was exposed to filtered air only once, half of the subjects underwent lavage directly after exposure ("air early") and the other half 18 hours after exposure ("air late").

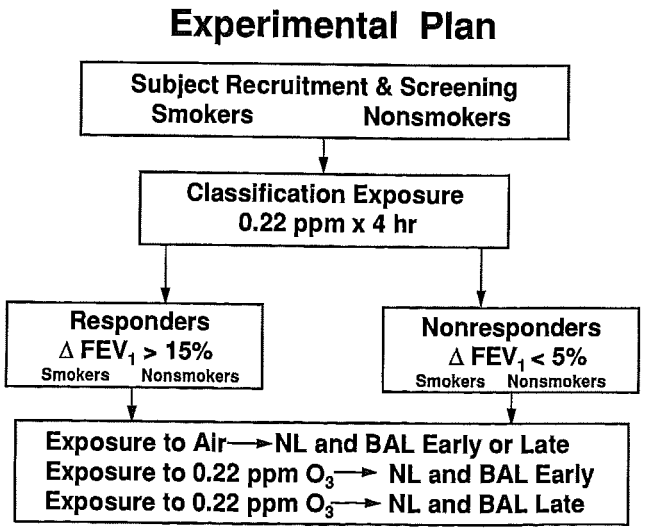

Figure 1. Experimental protocol.

All ozone exposures were to 0.22 ppm ozone for 4 hours, with exercise for 20 minutes of each 30 minutes, which was sufficient to achieve a $\dot{V}_E$ of approximately 25 L/min/m^2 body surface area. During the BAL phase of the study, the order of the exposures was randomized, and neither subjects nor investigators were informed of the exposure atmosphere. Pulmonary function was measured immediately before exposure, and after 2 hours and 4 hours of exposure. Symptoms were assessed by questionnaire following each exposure; subjects ranked the severity of each symptom on a scale from 0 ("not present") to 5 ("incapacitating"). The symptom questionnaire is shown in Appendix C.

Smokers were not permitted to smoke during exposure, but were not advised to abstain from smoking prior to exposure. In order to confirm the accuracy of subject classification with regard to smoking, saliva samples were collected prior to exposure and stored at –70 °C; samples from the 38 subjects completing the BAL phase of the study were sent to the laboratory of Dr. Neal Benowitz (University of California, San Francisco) for cotinine determination by gas chromatography.

EXPOSURE FACILITIES

All exposures were undertaken in a 45-m^3 environmental chamber in the General Clinical Research Center at the University of Rochester, the characteristics of which have been described (Utell et al. 1984). Exercise bicycle ergometers and pulmonary function testing equipment are housed within the chamber, so that subjects were not required to exit the chamber for physiologic testing. For comfort, temperature and relative humidity were maintained at 21.2 °C ± 0.92°C (SD) and 37.1% ± 3.0%, respectively.

All ozone exposures were conducted at a target concentration of 0.22 ppm (430 mg/m^3). Ozone generation was accomplished by passing breathing-quality oxygen through a flow meter into a water-cooled, high-voltage discharge

ozonator (Model 03V5, Ozone Research and Equipment Corp., Phoenix, AZ). A portion of the ozonator output (1 L/min) was connected to the chamber air intake (10 m^3/min) through a Venturi mixer.

An ozone analyzer (Model 8810, Monitor Labs Inc., Englewood, CO) continuously sampled the ozone concentration in the chamber atmosphere through a Teflon tubing connection. By means of feed-back circuitry, this Monitor Analyzer regulated the ozonator output. A Dasibi ozone analyzer (Model 1003-AH, Dasibi Environmental Corp., Glendale CA), designated as an E.P.A. Transfer Standard, was used to calibrate the Monitor Analyzer. Before each ozone exposure, the calibration procedure required that the Monitor Analyzer be compared to the Transfer Standard while both instruments were sampling the identical ozone concentration produced by a portable ozone generator (Stable Ozone Generator, Model SOG-2, Ultraviolet Products Inc., San Gabriel, CA). The output of the UV ozone generator, in turn, was validated at least bimonthly against a certified ozone standard at the Air Quality Control Station of New York State Department of Environmental Conservation (Avon, NY).

Air exposures, and the diluent air for the ozone exposures, used environmental air passed through an air intake purification system (Utell et al. 1984). The quality of the purified air as to background ozone, nitrogen oxides, and sulfur dioxide concentrations was established by using the Dasibi Model 8810 ozone analyzer, an NO_x analyzer (Model 8840, Monitor Labs Inc., Englewood, CO), and a Meloy SO_2 analyzer (Model SA285E, Columbia Scientific Instruments, Jollyville, TX), respectively.

PHYSIOLOGIC TESTING

Spirometry was performed using a pneumotachograph interfaced with a computer (Microloop, Medical Graphics, St. Paul, MN). Forced vital capacity, FEV_1, and forced expiratory flow between 25% and 75% of FVC (FEF_{25-75}) were recorded from the best of three determinations based on FEV_1. Airway resistance and thoracic gas volume were measured during panting using an integrated-flow, pressure-corrected body plethysmograph. Specific airway conductance (sGaw) was determined as the reciprocal of airway resistance, corrected for thoracic gas volume. Minute ventilation was measured initially at rest and during exercise using inductive plethysmography (Respigraph Model PN SY01, NonInvasive Monitoring Systems, Miami Beach, FL), calibrated with a rolling seal spirometer (Model 840, Ohio Medical Products, Houston, TX).

METHACHOLINE CHALLENGE

Airway challenge with methacholine was performed at the time of subject screening; subjects were not excluded on the basis of airway responsiveness. Increasing concentrations of methacholine (0.0, 0.625, 1.25, 2.50, 5.00, 10.00, 20.00, 40.00 mg/mL) in normal saline were administered at 4-minute intervals using a nebulizer (Model 646, Devilbiss Co., Somerset, PA) with a dosimeter (Rosenthal-French Model D-2A, Laboratory for Applied Immunology Inc., Fairfax, VA) calibrated to deliver 0.01 mL/breath. Subjects were instructed to take five breaths each lasting 6 seconds, and sGaw was measured 30 seconds after the last breath. Challenge was stopped if sGaw decreased more than 50% from the baseline value. The concentration of methacholine that produced a 50% decrease in sGaw (PD_{50}) was determined by interpolation using the regression line of the methacholine dose response. For subjects who did not experience a 50% decrease in sGaw after inhaling 40 mg/mL methacholine, a value of 40 mg/mL was assigned. The dose-response slope was calculated according to the method of O'Connor and coworkers (1987).

BRONCHOALVEOLAR LAVAGE

We performed BAL on each subject immediately after or 18 hours after separate but identical exposures to ozone, and after air exposure. Lavage was performed using fiberoptic bronchoscopy in both the lingula and the right middle lobe. Subjects were premedicated with 0.75 to 1.0 mg intravenous atropine, and topical anesthesia of the upper airway was established using lidocaine spray. Oxygen by nasal cannula was administered and cardiac rhythm monitored throughout the procedure. The fiberoptic bronchoscope (FB-19H, 6.3 mm o.d., Pentax, Orangeburg, NY) was passed orally, and topical lidocaine was administered through the bronchoscope to suppress cough. The bronchoscope was gently wedged in a subsegmental airway of the inferior segment of the lingula. Four 50-mL aliquots of sterile normal saline were sequentially instilled and immediately withdrawn under gentle suction, and collected into a siliconized Erlenmeyer flask on ice. The bronchoscope was then withdrawn and gently wedged in a subsegmental airway of the right middle lobe and the lavage was repeated. For these studies, return from the two lung lobes were analyzed separately for cell counts in order to assess the influence of regional variability in the inflammatory response. The same lingular and middle lobe subsegments were entered during each subject's three bronchoscopies. After completing the first four subjects, the BAL protocol was modified so that the return of the first 50-mL aliquot instilled into the right middle lobe was identified as a bronchial lavage (BL) sample and counted separately, be-

cause this portion of the lavage has been shown to be more representative of changes in the proximal airways (Rennard et al. 1990). The remaining three 50-mL aliquots were pooled as the alveolar lavage (AL) sample.

NASAL LAVAGE

In order to determine whether the nasal inflammatory response correlates with airway inflammatory responses in these groups, NL was performed just prior to BAL following each exposure. Warmed normal saline (5 mL) was instilled in each nostril, held for 15 seconds, and then discharged into a sterile container. The procedure was then repeated, and the discharged fluids were pooled, placed on ice, and transported to the laboratory for total and differential cell counts.

CELL QUANTIFICATION AND CHARACTERIZATION

Analysis of cells recovered by BAL was designed to detect influx of inflammatory cells or changes in the distribution of alveolar cell subpopulations in response to the exposure. Total cell counts were performed separately on the combined BAL sample obtained from the lingula, and on the BL and AL samples obtained from the right middle lobe, using a hemocytometer. Viability was assessed using trypan blue dye exclusion. Cytospin slides (Shandon Inc., Pittsburgh, PA) were prepared from aliquots of BAL, BL, and AL of sufficient volume to contain 5×10^4 cells. Slides were stained with Diff-Quick (American Scientific Products, McGraw Park, IL) for differential counts; at least 500 cells from each slide were counted. Total and differential cell counts for BAL were expressed as averages of the results from the two lavaged lung segments. A separate slide of cells from the right middle lobe (AL) was stained with Mayer's hematoxylin and toluidine blue for enumeration of mast cells.

Flow cytometry was a sensitive method of evaluating changes in AMs following ozone exposure, separately from other cells in BAL. Measurements included autofluorescence, light-scattering properties, and surface marker expression. After a single wash in cold phosphate-buffered saline (PBS), BAL cells were pooled and run immediately on a FACStar Plus (Becton Dickinson, Mountain View, CA) equipped with a 15-mW argon laser at 488 nm. Data (forward scatter, linear scale; wide-angle light scatter, log scale; and fluorescence emission at 500–560 nm and 543–627 nm, log scale) were collected in list mode for subsequent analysis. In order to standardize detection of fluorescence from run to run, a premixed preparation of fluorescent calibration beads (Quantum 25 Premixed, Flow Cytometry Standards Corporation, San Juan, Puerto Rico) was analyzed with each experiment. The fluorescence intensity of these beads, which ranged from 5×10^4 to 2×10^6 molecules of equivalent soluble fluorochrome (MESF), was used to construct a standard curve, as described previously (Gavras et al. 1994). Fluorescence intensity is therefore expressed as MESF in these studies.

Alveolar macrophage surface receptor expression was quantitated by indirect immunofluorescence using standard techniques as described previously (Gavras et al. 1994). Briefly, lavaged cells were incubated for 45 minutes on ice in excess concentrations of the following monoclonal antibodies: MOPC-21 (control, obtained from Sigma Chemicals, St. Louis, MO), 32.2 (FcRI, CD64), IV.3 (FcRII, CD32), 3G8 (FcRIII, CD16), OKM1 (complement receptor 3, CD11b, obtained from Ortho Diagnostics, Raritan, NJ), or anti–Leu-M3 (CD14, obtained from Becton Dickinson, Mountain View, CA). Antibodies against FcR were provided courtesy of Dr. R. John Looney (University of Rochester). After three washes at 4°C in PBS containing 1% bovine serum albumin and 0.1% azide, cells were stained (45 minutes, 4°C) with excess FITC-conjugated $F(ab)_2$ goat antimouse immunoglobulin (Tago, Burlingame, CA). Stained cells were fixed in 1% paraformaldehyde and kept at 4°C prior to analysis, which was performed within 5 days. A murine IgG1 monoclonal antibody (MOPC-21) was used as an irrelevant control; previous experiments showed no differences between murine IgG1 and IgG2b control antibodies in labeling AMs, so MOPC-21 was used with each run. For analysis on the flow cytometer, the macrophage population was identified using its characteristic pattern of forward and wide-angle light scatter. Mean fluorescence emission at 530 nm was obtained from these cells and converted to MESF using the standard curve calculated from the calibration beads. The MESF of fixed, unstained AMs was subtracted from the value obtained for each antibody to correct for autofluorescence.

As a measure of changes in AM size in response to ozone exposure, an aliquot of fresh BAL fluid was analyzed using a Coulter channelyzer (Coulter Diagnostics, Hialeah, FL). Gates were set to exclude lymphocytes and debris from the analyzed population, and mean cell diameter was recorded.

TOXIC OXYGEN SPECIES

The generation of TOS by airway cells may play a role in mediating injury after exposure to oxidants, and is an important component of intrinsic defense against microorganisms. Two methods were used to measure production of TOS in these studies: generation of superoxide anion (O_2^-) by the whole BAL cell population using superoxide dismutase (SOD)–inhibitable cytochrome C reduction, and AM production of TOS using flow cytometry.

For measurement of O_2^- generation, BAL cells were centrifuged at 300 × *g* for 10 minutes, then resuspended in Krebs Ringer Phosphate with 5-mM glucose. Two × 10^5 cells were added to warmed mixtures of 20 nmol cytochrome C with and without 4 μg SOD, with and without 0.2 μg phorbol myristate acetate (PMA) in wells of a 96-well microtiter plate. Absorbance readings at 550 nm were taken at 30-second intervals for 30 minutes at 37°C in a microtiter plate spectrophotometer (MAXline, Molecular Devices Corporation, Menlo Park, CA) equipped with a 1-nm bandpass 550-nm filter. Kinetic absorbance curves were generated and stored on floppy disks. The maximal rate of O_2^- generation was determined by visually selecting the computer-generated line that best matched the steepest portion of the absorbance curve. The final absorbance reading after 30 minutes of incubation represented the total O_2^- generated. Measurements were made in triplicate and the mean of the values was used in calculations.

The rate of O_2^- generation was calculated using the following equation:

$$\text{nmol } O_2^-/\text{min} = \frac{\Delta(\text{mOD/min})}{0.021 \times 0.54 \times 1000}$$

where Δ(mOD/min) represents the difference in the rate of change with and without SOD in milli-optical density units per minute, from the spectrophotometer; 0.021 is the nanomolar extinction coefficient for superoxide; and 0.54 is the light path correction factor for the volumes used in these plates in this spectrophotometer.

Total O_2^- generation was similarly calculated from the difference in absorbance at 30 minutes with and without SOD, using the following equation:

$$\text{nmol } O_2^-/\text{min} = \frac{\Delta\text{OD}}{0.021 \times 0.54}$$

Alveolar macrophage production of TOS was determined using flow cytometric measurement of oxidation of 2′-,7′-dichlorofluorescin. Cells were centrifuged at 300 × *g* for 10 minutes, resuspended at 1 × 10^6/mL in RPMI with 10% fetal bovine serum, and then incubated in a shaking water bath at 37°C for 15 minutes with and without 5 μM 2′-,7′-dichlorofluorescin diacetate (DCFD, Molecular Probes, Eugene, OR). Phorbol myristate acetate (1 μg) was added to half the tubes, incubation continued for an additional 30 minutes, and the tubes were then placed on ice. Propidium iodide (0.025 μg) was added and flow cytometric analysis was performed immediately, gating on the AM population using forward scatter and wide-angle light scatter. Cells containing propidium iodide (585-nm fluorescence-positive cells) were eliminated from the analysis. Data were collected in list mode for subsequent analysis. Mean fluorescence of the AMs was converted to MESF using the standard curve obtained from the calibration beads. Baseline TOS production was calculated by subtracting MESF of cells incubated without DCFD from cells with DCFD (both without stimulation by PMA). Stimulated TOS production was calculated by subtracting MESF of PMA-treated cells not stained with DCFD from cells treated with both DCFD and PMA.

MEASUREMENTS OF PROTEINS AND CYTOKINES IN BRONCHOALVEOLAR LAVAGE FLUID

Concentrations of total protein, albumin, and IgM were determined to provide indices of changes in epithelial permeability. α_2-Macroglobulin (a2M) is a large molecule present in BAL that may inhibit macrophage production of O_2^- (Hoffman et al. 1983), and has been shown to increase in BAL fluid following exposure to nitrogen dioxide (Frampton et al. 1989). Interleukin-6 and IL-8 were measured to determine their potential role in recruiting inflammatory cells following ozone exposure.

Bronchoalveolar lavage fluids were stored at −70°C prior to analysis; determinations were done simultaneously on all samples from each subject. Immunoassays were validated for BAL fluid using serial dilutions and "add back" of purified antigen to confirm accurate recovery. Total protein was determined using the method of Lowry and associates (Lowry et al. 1951), with crystalline bovine serum albumin as the standard. Albumin was measured using a modified antibody-capture ELISA as described previously (Frampton et al. 1989). IgM was measured using a sandwich ELISA with sensitivity in the range of 5 to 200 ng/mL. Human IgM (standard), murine anti–human IgM (primary antibody), peroxidase-conjugated goat anti–human IgM (secondary antibody), and orthophenylene-diamine-dihydrochloride (color indicator) were obtained from Sigma Chemicals (St. Louis, MO). α_2-Macroglobulin was determined using Western blotting. Samples and standards (human α_2-macroglobulin, Sigma Chemicals, St. Louis, MO) were transferred to nitrocellulose paper using a Millipore 96-well filtration manifold (Schleicher and Schuell, Keene, NH). Rabbit anti–human α_2-macroglobulin was added, followed by biotin-conjugated goat anti–rabbit IgG (both from Sigma Chemicals, St. Louis, MO). Avidin horseradish peroxidase (Vectastain ABC, Vector Labs, Burlingame, CA) and commercially obtained Western blotting reagents (ECL Western detection reagents, Amersham Life Sciences, Arlington Heights, IL) were used in detection. Blots were exposed on radiographic film (X-omat, Kodak Corp., Rochester, NY) and read by densitometer.

Interleukin-6 and IL-8 were determined in unconcentrated BAL fluid using commercially obtained immunoassay kits (R&D Systems, Minneapolis, MN). Samples were assayed in duplicate and read on a microtiter plate reader (Model EL312, Bio-Tek Instruments, Winooski, VT).

DATA HANDLING AND STATISTICAL METHODS

Sample Size

Koren and coworkers (1989b) found an eight-fold increase in PMNs recovered by BAL after 2-hour exposures to 0.4 ppm ozone. Based on estimates of the total intake of ozone in the study by Koren and colleagues, we estimated a roughly sixfold increase in PMNs in our study. A sample size computation for this effect indicated that eight subjects per group would be required to achieve 80% power at the 5% level of significance. A group size of 12 was selected to provide a sufficient margin of statistical power.

Randomization

Subjects were run in pairs whenever possible. In the BAL phase of the study, subject pairs were randomized so that one subject was scheduled to undergo BAL immediately after exposure and the other 18 hours after exposure. A restricted randomization scheme was employed to balance the various treatment assignments over time. Pairs of subjects were assigned to all three treatments (air, ozone early, ozone late) using a single randomization. Treatment assignments were randomly selected from lists prepared by Dr. Christopher Cox using a call-in scheme.

Data Handling and Analysis

Data collection and storage were performed in accordance with guidelines established by a quality assurance plan. Data books were custom made, numbered, and bound. All entries were made in ink, dated, signed by the responsible person(s), and verified periodically by the project's quality assurance (QA) officer (Harriet E. Beiter). All records were identified by coded numbers and treated as confidential information.

Data were entered using a Digital Pro 350 computer (Boston, MA) and RS1 software (BBN, Cambridge, MA) by a technician trained in biostatistics (Donna M. Speers). Data sets were then created using SAS (SAS Institute Inc., Cary, NC) and transferred to the Department of Biostatistics Local Area VAX Cluster. The primary analyses were all performed in SAS using this system, under the direction of Dr. Christopher Cox.

Pulmonary function data from smokers and nonsmokers at three time points (before exposure, and after 2 hours and 4 hours of exposure) were analyzed by repeated measures analysis of variance (ANOVA). The Wilcoxon rank sum test was used to compare symptom scores in different groups of subjects. Paired *t* tests were used to compare means for the same subjects tested under different conditions. Rates or percentages were compared using chi-squared tests, and the odds ratio was used as a measure of the difference between two groups. In addition a multiple logistic regression was performed, using responder status as the dependent variable. Association between continuous variables was assessed by correlation analysis.

The primary analyses for the data from BAL were based on a two-way mixed model or repeated measures ANOVA, which included one between-subjects factor and two within-subject factors. The between-subjects effect was the subject's group. The primary within-subject effect in the model was treatment: air, ozone early, or ozone late (three conditions). For these analyses the air exposure was considered as a single treatment, regardless of whether subjects underwent lavage early or late. This assumption was checked in a separate two-way ANOVA, which compared the air-early group with the air-late group. For any variable for which air-early was different from air-late, the ANOVA was run separately for each group of subjects.

The second within-subject factor was a period effect. Because subjects were run at different times of the year, period effects were not expected, and the effect was included as a check on this assumption. As in any repeated measures ANOVA, a random subject effect was also included, which was nested within groups. The analysis also included a test of interaction between the treatment effect and the subject group. If this interaction was significant, then we concluded that differences among the three treatments were different among the three groups of subjects. If the interaction was not significant, then the individual or main effects were examined for statistical significance. No terms were included in the model for carry-over effects because of the relatively long interval between repeated measurements on the same subject.

Each analysis included an examination of residuals as a check on the assumptions of normally distributed errors with constant variance. In many instances the residual analysis indicated that the variance was not constant. In these cases a log transformation proved effective in stabilizing the variance. In some cases the data contained more than 10% zeros (for example, BAL eosinophil counts), and in this case the ANOVA could not be used. In such cases paired and unpaired Wilcoxon tests were used to make pairwise comparisons among groups and treatments. In other cases, *t* tests were used to make similar comparisons.

A level of 5% was required for statistical significance. Because of the multifaceted nature of these studies, a fairly large number of significance tests were performed. Our main strategy in interpreting these results was to rely on the pattern of significance tests, and on concordant effects among biologically related variables, rather than individual p values. In addition, outliers were not removed in these analyses, both because of the number of analyses and the strategy for interpretation that was adopted. The number of outliers was never more than two.

Finally, for a small number of endpoints analysis of covariance (ANCOVA) was performed. The dependent variables for these analyses were differences between ozone-late and air exposure. Covariates included age and similar differences using other lavage parameters. A residual analysis was included and outliers were removed for these analyses.

QUALITY ASSURANCE

The QA methods for this study included validating ozone exposure levels using the EPA reference methods for ozone analysis, and adjunctive standardization procedures using EPA-equivalent methods. We monitored the performance of the air purification system concurrently, which required EPA reference methods for NO_x, sulfur oxides, and ozone. Therefore, the basic QA requirements directed at achieving reliable, fully documented ozone exposure conditions were performed according to EPA-recommended procedures, but we extended our calibration and standardization procedures to include environmental instruments and all physiologic testing instrumentation, for which there are no specific EPA guidelines.

The QA program, under the direction of H.E. Beiter, includes provisions for spare parts inventories, service records, preventive maintenance schedules, the design and use of data books, analog and digital printouts, data storage, and methods of calibration and standardization. Standard operating procedures are maintained in a manual.

RESEARCH TEAM

This three-year project involved many individuals with complementary roles. The personnel and their designated responsibilities are given in Appendix D.

RESULTS

For clarity of presentation, significant p values from statistical comparison testing are found in the tables and figure legends.

SUBJECT CHARACTERISTICS

An objective at the outset of these studies was to compare the airway inflammatory response among four groups: nonsmoker-responders, nonsmoker-nonresponders, smoker-responders, and smoker-nonresponders. Because responders were fewer than nonresponders (see the Pulmonary Function section below), the number of subjects screened and classified was determined by the number of responders identified. Classification of 34 smokers identified only four responders. Of these four, only two subjects completed the subsequent BAL phase of the study. It became clear that studying smoker-responders as a separate group was not feasible. The findings from BAL are therefore presented as comparisons among three groups: nonsmoker nonresponders (n = 13), nonsmoker responders (n = 12), and smokers (n = 13). The group of smokers includes the two ozone responders.

Table 1 shows the number of subjects participating at each phase of the study. A total of 90 subjects (56 nonsmokers, 34 smokers) completed the classification exposure, and their screening data are shown in Table 2. Other than smoking history, smokers differed significantly from nonsmokers only in the ratio of FEV_1:FVC and the mean value for FEF_{25-75}, although these values were within the normal predicted range for all subjects.

In order to assess the accuracy of the smoking histories for the 38 subjects (13 smokers, 25 nonsmokers) who completed the BAL phase of the study, measurements of salivary cotinine were performed on saliva samples obtained at the time of the classification exposure. The mean (± SD) cotinine level for smokers was 264 ± 41 ng/mL. One smoker had a level lower than generally found in active smokers (31.7 ng/mL), and this subject was a responder to ozone. Nonsmoking status was confirmed in all nonsmokers with salivary cotinine measurements as well.

EXPOSURE DATA

Sixty-eight exposure sessions were required to determine ozone responsiveness in the 90 subjects because some subjects were studied singly. Ozone concentration (mean ± SD) for these exposures was 0.219 ± 0.004 ppm. The subsequent study of responders and nonresponders required a total of 47 ozone exposures, with a mean concentration of 0.219 ± 0.005 ppm. Minute ventilation during exposure was similar for nonsmokers and smokers, as well as responders and nonresponders. During exercise, $\dot{V}_E$ ranged from 39.4 to 45.3 L/min in nonsmokers, and from 42.6 to 45.6 L/min in smokers. Ambient outdoor ozone levels during the study were obtained from the Department of Environmental Conservation of New York State, and did not exceed 0.10 ppm within the 24 hours prior to any exposure session.

Table 1. Subject Recruitment and Participation

	Smokers		Nonsmokers	
Phase	Number Participating	Withdrawals and Exclusions	Number Participating	Withdrawal and Exclusions
Screening	Screened: 72	Unable to complete exercise: 4 Abnormal pulmonary function tests: 1	Screened: 74	Unable to complete exercise: 1 Withdrew, no reason given: 1
Classification Exposure	Scheduled: 67 Started exposure: 39 Completed exposure: 34	Missed appointment: 28 Unable to maintain exercise: 4 (ΔFEV_1 0 to +1%)[a] Withdrew to attend job interview: 1 (ΔFEV_1 −1.7%)	Scheduled: 72 Started exposure: 58 Completed exposure: 56	Missed appointment: 14 Unable to maintain exercise: 1 (ΔFEV_1 −0.3%) Hypertension with exercise: 1
BAL Study	Invited: 28 (24 NR, 4 R) Started BAL study: 15 (12 NR, 3 R) Completed BAL study: 13 (11 NR, 2 R)	Refused: 5 (All NR) Left area: 6 (5 NR, 1 R) Quit smoking: 1 (NR) Missed appointment: 1 (NR) Withdrew during first bronchoscopy: 1 (NR) Unable to establish intravenous access on third bronchoscopy: 1 (R)	Invited: 32 (16 NR, 16 R) Started BAL study: 25 (12 NR, 13 R) Completed BAL study: 25 (12 NR, 13 R)	Refused: 4 (2 NR, 2 R) Left area: 2 (1 NR, 1 R) Pregnant: 1 (NR)

[a] ΔFEV_1 = percentage of change from baseline FEV_1 during exposure.

PULMONARY FUNCTION: CLASSIFICATION EXPOSURES

Figure 2 shows the percentage of change from baseline in FEV_1 during and after the classification exposure for nonsmokers and smokers. Changes after exposure were significantly less for smokers than for nonsmokers (ANOVA test for interaction, $p = 0.0013$). Decrements in FVC paralleled those for FEV_1, which is characteristic of the lung's functional response to ozone. Nonsmokers demonstrated a wide range of responsiveness to ozone (Figure 3), consistent with previous studies. Of 56 nonsmokers, 16 (28.6%) were responders to ozone (decrement in $FEV_1 > 15\%$), and 22 (39.3%) were nonresponders (decrement in $FEV_1 < 5\%$); the remaining 18 (32.1%) showed intermediate levels of responsiveness. For smokers, fewer subjects were responders (4 of 34, 11.8%). If the single smoker-responder with a salivary cotinine level below the range associated with active cigarette smoking is excluded, 9.0% of smokers were responders. Some smokers demonstrated small increases in FEV_1 following exposure (Figure 3), likely as a result of exercise-related bronchodilation.

No evidence for significant airway narrowing was found in either nonsmokers or smokers following ozone exposure. The ratio of FEV_1:FVC, although slightly lower at baseline

Table 2. Information Obtained at Screening for Subjects That Completed the Classification Exposure[a]

	Smokers ($n = 34$)	Nonsmokers ($n = 56$)
Age (years)	28 ± 1	25 ± 4
Gender (M/F)	28/6	36/20
Body surface area (m^2)	1.94 ± 0.20	1.86 ± 0.18
Pack-years	12.8 ± 9.2	0
History of allergies	17 (50%)	34 (61%)
FVC (% predicted)	106 ± 11	105 ± 12
FEV_1 (% predicted)	103 ± 12	106 ± 14
FEV_1/FVC (%)	81 ± 6	85 ± 6[b]
FEF_{25-75} (L/sec)	3.93 ± 1.04	4.48 ± 1.16[c]
PD_{50}	12.9 ± 13.4	14.9 ± 14.0
Slope[d]	−43.9 ± 74.7	−22.6 ± 28.3

[a] Values are presented as means ± SD; numbers in parentheses indicate percentage of subjects.

[b] $p < 0.01$.

[c] $p < 0.05$.

[d] Slope of the methacholine challenge–response curve (see the Methods section).

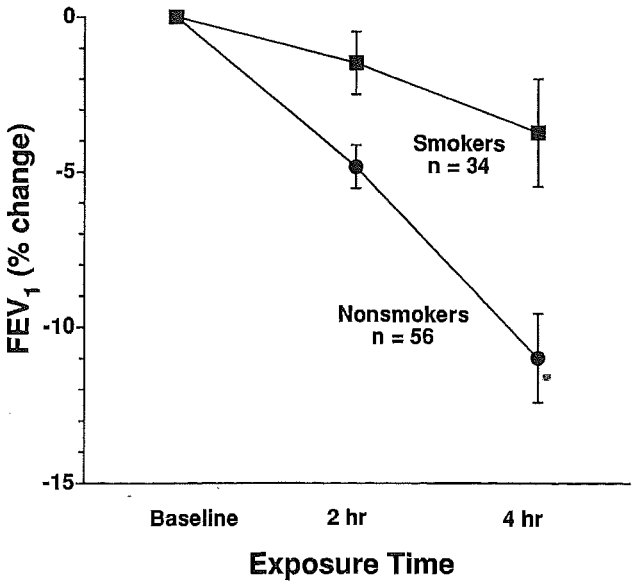

Figure 2. Percentage of change in FEV_1 after 2 and 4 hours of exposure to 0.22 ppm ozone (means ± SE). Smokers were less responsive to ozone than nonsmokers (ANOVA interaction between group and ozone effect, $p = 0.0013$).

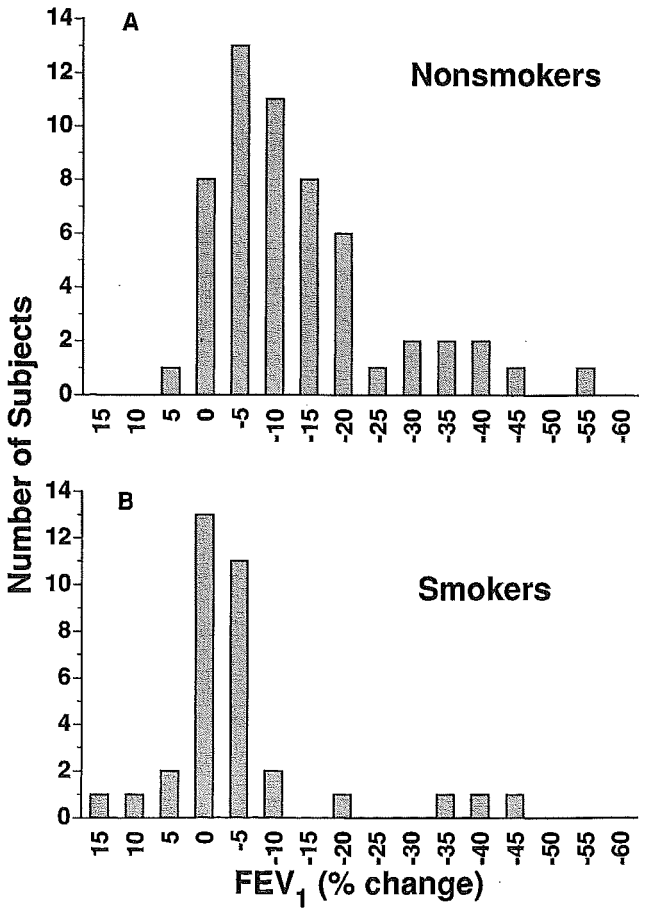

Figure 3. Distribution of ozone responsiveness in nonsmokers (Panel A) and smokers (Panel B). Fewer smokers than nonsmokers were responsive to ozone.

in smokers, did not change significantly following exposure (Figure 4A). Percentage decrements in FEF_{25-75} during and following exposure were similar in magnitude to the changes in FEV_1 (mean ± SE; nonsmokers: −12.2% ± 2.2%; smokers: −0.5% ± 3.3%). Nonsmokers experienced only modest decrements in sGaw (−17.3% ± 2.7%), whereas smokers demonstrated a small increase (+1.3% ± 6.2%) (Figure 4B).

In order to determine predictors of lung function responsiveness to ozone, we performed multiple logistic regression analyses with responder status as the dependent variable, and the following as predictors: age, gender, history of allergies, pack-years of smoking, and airway responsiveness (PD_{50} and slope of the dose-response curve). Pack-years of smoking was associated with decreased ozone responsiveness (odds ratio 0.87, $p = 0.017$). Among cigarette smokers, salivary cotinine levels correlated inversely with decrements in FEV_1 ($r = 0.65$, $p = 0.02$), whereas pack-years of smoking did not correlate with decrements in FEV_1 ($r = 0.44$, $p = 0.13$). This suggested that current intensity of tobacco use was more closely related to ozone responsiveness than lifetime total use.

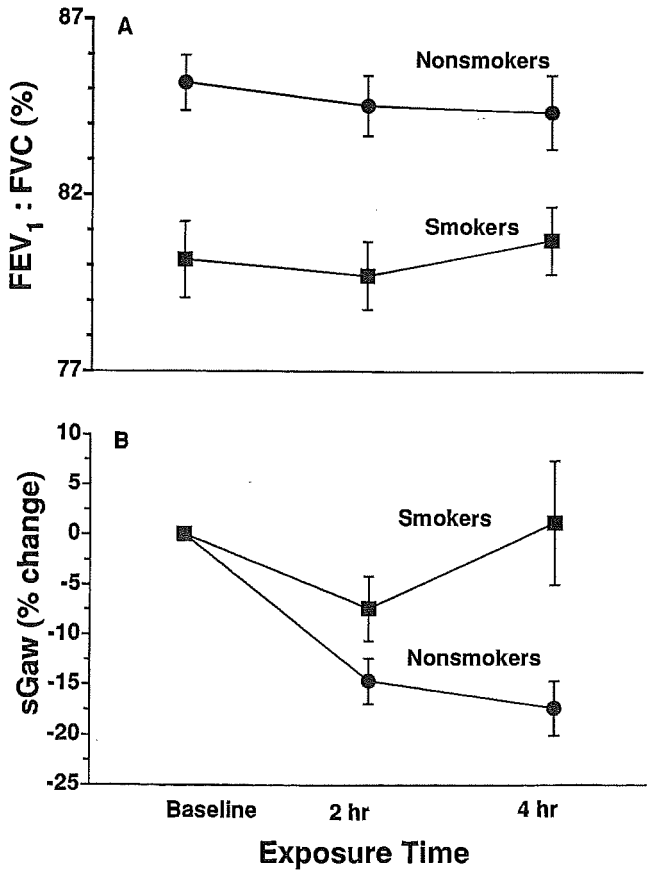

Figure 4. Changes in the ratio of FEV_1:FVC (Panel A) and sGaw (Panel B) after 2 and 4 hours of exposure to 0.22 ppm ozone.

Of 90 subjects, 51 reported allergies; 31% of subjects without allergies and 14% of those with allergies were responders (odds ratio 3.3, $p = 0.036$). This was also the case when data from nonsmokers, 34 of whom reported allergies, were analyzed separately: 41% of nonsmokers without allergies and 18% with allergies were responders (odds ratio 3.5, $p = 0.052$). Decrements in FEV_1 after ozone exposure were greater in those without allergies (9.6% with allergies, 13.1% without). Age, gender, and methacholine responsiveness were not predictive of ozone responder status.

CONSISTENCY OF RESPONSIVENESS

A total of 14 smokers (11 nonresponders, 3 responders) and 25 nonsmokers (12 nonresponders, 13 responders) were subsequently exposed once to air and twice to 0.22 ppm ozone during the phase involving BAL; exposures were separated by at least three weeks. Ozone responsiveness on each of the two subsequent exposures was significantly correlated with the response to ozone during the classification exposure (first subsequent exposure, $r = 0.90$, $p < 0.001$; second subsequent exposure, $r = 0.89$, $p < 0.001$). Smokers as well as nonsmokers were consistent in their subsequent responsiveness (or lack of responsiveness) to ozone (Figure 5). No subject experienced a decrease of more than 2% in FEV_1 following air exposure.

RECOVERY AFTER EXPOSURE

Of the 16 ozone responders who entered the BAL portion of the protocol, 15 (12 nonsmokers, 3 smokers) underwent spirometry 18 hours after one of the follow-up ozone exposures, prior to BAL. All showed improvement in FVC, FEV_1, and FEF_{25-75} in comparison with measurements at the end of exposure. Mean (± SE) decrement in FEV_1 from baseline was −4.9% ± 1.5% at 18 hours compared with a mean of −28.5% at the end of exposure. For all but two subjects, FEV_1 was within 10% of the preexposure baseline, and was within 5% for the three smoker-responders. One nonsmoking subject, for whom the postexposure decrement in FEV_1 was −23%, showed a persistent decrement of −20% 18 hours after exposure. Improvements in FEF_{25-75} paralleled those for FEV_1 and FVC; (mean ± SE) FEF_{25-75} was −6.1% ± 3.2% 18 hours after exposure compared with −32.3% ± 2.6% at the end of exposure.

SYMPTOMS

Symptom scores following the classification ozone exposure were analyzed to determine differences between study groups and the relationship of symptoms to pulmonary function changes. The most commonly reported symptoms were cough, dyspnea, chest tightness, chest pain, and throat irritation. Smokers reported fewer respiratory symptoms than nonsmokers following ozone exposure, but the difference was significant only for cough (symptom score, mean ± SE; nonsmokers: 1.93 ± 0.16; smokers: 1.09 ± 0.19; p = 0.001). Among the nonsmokers, nonresponders reported significantly fewer respiratory symptoms than responders (Table 3). However, symptoms were not strongly associated with changes in lung function. For example, many subjects reported a mild cough during exposure, but the correlation with decrements in FEV_1 was weak ($r = 0.46$, $p < 0.001$). Other symptoms were even less predictive of lung function changes.

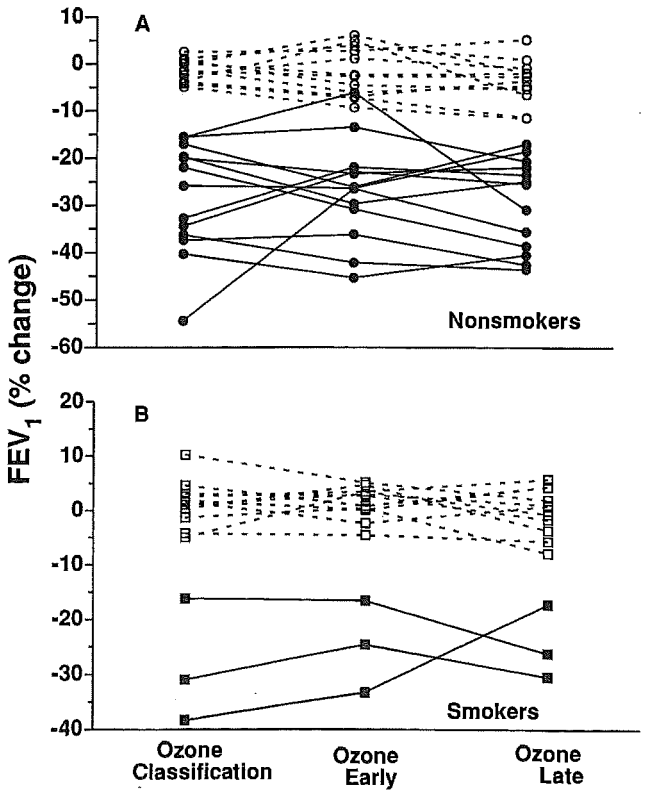

Figure 5. Consistency of responsiveness to ozone. Percentage of change in FEV_1 is shown for subjects who completed all three ozone exposures. Panel A: nonsmokers; Panel B: smokers. Open symbols and hatched lines represent nonresponders (decrement in FEV_1 < 5% following the classification exposure); filled symbols and solid lines represent responders (decrement in FEV_1 > 15%).

When 39 subjects were subsequently exposed to filtered air during the BAL phase of the study, smokers reported all symptoms, including nonrespiratory symptoms, more frequently than nonsmokers, and the differences were significant ($p < 0.05$) for chest pain, chest tightness, wheeze, sputum production, eye irritation, headache, fatigue, and anxiety. Thus, smokers were generally more symptomatic than nonsmokers in response to exercise in air, but experienced a smaller increase in respiratory symptoms following exposure to ozone than did nonsmokers.

AIRWAY INFLAMMATION AND INJURY

Bronchoalveolar Lavage Fluid and Cell Recovery

No effects of ozone exposure on the recovery of lavage fluid were noted, either from the BL or AL components of the lavage procedure. Tables 4 and 5 show the recovery of fluid and cells from the AL and BL components of BAL, respectively. Fluid recovery was slightly less in smokers for AL but not BL.

Alveolar cell recovery after air exposure was twofold greater in smokers than in nonsmokers. In all groups, cell recovery in both AL and BL decreased early after ozone exposure and increased at 18 hours with a high degree of statistical significance (Tables 4 and 5). Cell recovery at 18 hours after ozone exposure was slightly less than that 18 hours after air exposure in smokers, but higher than that 18 hours after air exposure in nonsmokers. When total BAL cell recovery was analyzed, including the four subjects in whom a separate BL had not been performed, smokers differed significantly from the other two groups in the effects of ozone on cell recovery ($p = 0.026$). The pattern of response in nonsmoker-nonresponders and nonsmoker-responders was similar.

The viability of recovered cells was generally greater than 90%. In comparison with air exposure, viability of BL cells decreased approximately 2% early after ozone exposure in all groups ($p = 0.026$).

Differential Cell Recovery

Differential cell counting from cytospin slides was performed both for total mixed BAL cells in 38 subjects (Figure 6) and for BL and AL cells separately in 34 subjects (Figure 7). Polymorphonuclear leukocytes showed the largest change in response to ozone exposure in all groups. For BAL, the increase in PMNs was progressive with time, and greatest at 18 hours after exposure, with a high degree of statistical significance. The concentration of PMNs was slightly greater in smokers after air and early after ozone

Table 3. Symptoms Following the Classification Exposure to Ozone[a]

	Smokers ($n = 34$)	Nonsmoker-Nonresponders ($n = 22$)	Nonsmoker-Responders ($n = 16$)
Cough	1.09 ± 0.19	1.09 ± 0.22	2.63 ± 0.18[b]
Sputum	0.56 ± 0.15	0.32 ± 0.18	0.94 ± 0.27[c]
Dyspnea	1.29 ± 0.23	0.73 ± 0.19	1.88 ± 0.36[c]
Chest pain	0.79 ± 0.23	0.41 ± 0.18	1.56 ± 0.43[c]
Chest tightness	0.85 ± 0.19	0.68 ± 0.24	1.44 ± 0.32[c]
Wheeze	0.56 ± 0.17	0.32 ± 0.15	0.50 ± 0.27
Throat irritation	1.00 ± 0.22	0.68 ± 0.23	1.31 ± 0.31
Nasal congestion	0.56 ± 0.15	0.50 ± 0.24	0.50 ± 0.24
Eye irritation	0.24 ± 0.07	0.05 ±0.05	0 ± 0
Headache	0.50 ± 0.17	0.18 ± 0.11	0.56 ± 0.27
Fatigue	0.56 ± 0.19	0.09 ± 0.06	0.88 ± 0.34
Nausea	0.12 ± 0.06	0.05 ± 0.05	0.50 ± 0.22[c]
Anxiety	0.24 ± 0.10	0 ± 0	0.50 ± 0.20[b]

[a] Data are presented as means ± SEM for symptom scores from 0 to 5, with 5 being the most severe.

[b] $p < 0.01$ compared with nonsmoker-nonresponders using Wilcoxon rank sum test.

[c] $p < 0.05$.

Table 4. Fluid and Cell Recovery from the Alveolar Component of Bronchoalveolar Lavage

	Smokers[a] ($n = 12$)		Nonsmoker-Nonresponders ($n = 11$)		Nonsmoker-Responders ($n = 11$)	
	Volume Returned (mL)	Cells ($\times 10^4$/mL)	Volume Returned (mL)	Cells ($\times 10^4$/mL)	Volume Returned (mL)	Cells ($\times 10^4$/mL)
Air	118.4 ± 3.1	64.4 ± 11.7	129.8 ± 1.9	23.3 ± 2.9	126.8 ± 3.4	19.3 ± 1.3
Ozone early[b]	116.7 ± 2.1	44.5 ± 7.5	125.4 ± 4.0	19.3 ± 2.1	120.0 ± 2.5	15.6 ± 2.1
Ozone late	117.3 ± 3.4	59.8 ± 10.5	129.2 ± 1.7	30.1 ± 4.4	123.7 ± 3.3	25.3 ± 3.6

[a] Smokers differed significantly from other groups with regard to volume returned and cells recovered, independent of ozone exposure (ANOVA group effect, $p = 0.0028$ and $p < 0.0004$, respectively).

[b] Cell recovery decreased when BAL was performed early after ozone exposure, and increased when it was performed late after exposure in all groups (ANOVA ozone effect, $p < 0.0001$).

Table 5. Fluid and Cell Recovery from the Bronchial Component of Bronchoalveolar Lavage

	Smokers ($n = 12$)		Nonsmoker-Nonresponders ($n = 11$)		Nonsmoker-Responders ($n = 11$)	
	Volume Returned (mL)	Cells ($\times 10^4$/mL)	Volume Returned (mL)	Cells ($\times 10^4$/mL)	Volume Returned (mL)	Cells ($\times 10^4$/mL)
Air	19.9 ± 0.9	21.7 ± 3.8	19.5 ± 1.0	17.7 ± 3.1	19.5 ± 1.3	17.8 ± 2.4
Ozone early[a]	17.2 ± 1.0	12.3 ± 2.8	19.2 ± 1.0	14.1 ± 3.2	17.8 ± 1.4	9.5 ± 1.1
Ozone late	18.4 ± 1.0	17.9 ± 1.3	20.4 ± 1.0	28.1 ± 4.8	18.6 ± 1.6	24.7 ± 2.8

[a] Cell recovery decreased when BAL was performed early after ozone exposure, and increased when it was performed late after exposure in all groups (ANOVA ozone effect, $p < 0.0001$).

exposure, compared with nonsmokers. However, the concentration of PMNs at 18 hours after ozone exposure was similar among all groups.

As shown in Figure 7, recovery of PMNs increased following ozone exposure in both BL and AL components. For PMNs recovered in AL fluid, the ozone response was similar among groups. In BL fluid, nonsmoker-nonresponders had fewer PMNs after air exposure ($0.57 \pm 0.12 \times 10^4$ cells/mL) than either nonsmoker-responders ($1.22 \pm 0.24 \times 10^4$ cells/mL) or smokers ($0.71 \pm 0.24 \times 10^4$ cells/mL) (ANOVA group difference, $p = 0.039$). However, no difference was found among the groups in the response to ozone.

Recovery of AMs in BAL and AL fluids was greater in smokers than in nonsmokers. Recovery of AMs in BL fluid was similar among groups. In all groups, AM recovery decreased early after ozone exposure, and returned to baseline 18 hours later. No significant differences among groups were found in the effects of ozone on AM recovery for any lavage component.

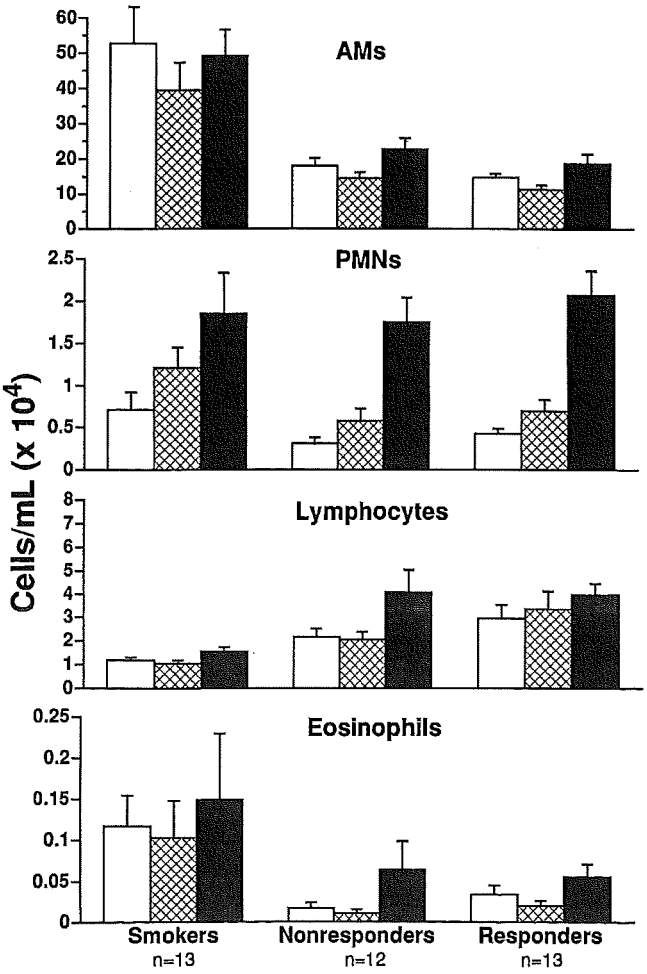

Figure 6. Cell recovery from BAL fluid taken from smokers and nonsmokers (nonresponders and responders). (Open bars: air exposure; cross-hatched bars: ozone early; solid bars: ozone late. Data are expressed as means ± SE.) AMs were more numerous in smokers than nonsmokers (group effect, $p < 0.0001$), and decreased early after ozone in all groups (ozone effect, $p < 0.0001$). PMNs progressively increased after ozone exposure for all groups (ozone effect, $p < 0.0001$), and were increased in smokers compared with nonsmokers after air and early after ozone (interaction between group and ozone effects, $p = 0.011$). Lymphocytes were decreased in smokers compared with nonsmokers (group effect, $p < 0.0001$), and increased late after ozone in all groups (ozone effect, $p < 0.0001$). Eosinophils showed no significant ozone effect.

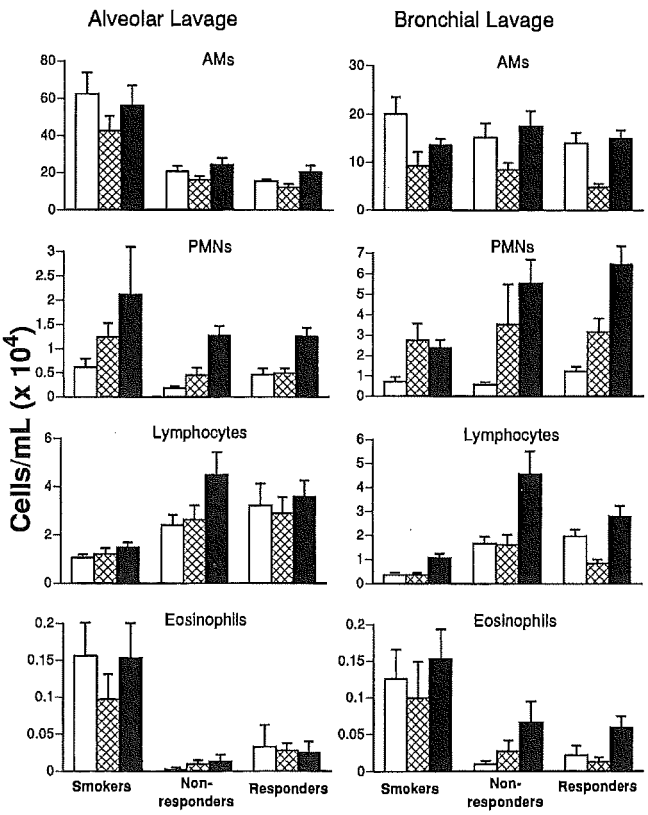

Figure 7. Cell recovery from alveolar and bronchial lavage components. (Numbers of subjects: smokers 12, nonsmoker-nonresponders 11, nonsmoker-responders 11. Open bars: air exposure; cross-hatched bars: ozone early; solid bars: ozone late. Data are expressed as means ± SE.) AMs were increased in smokers compared with nonsmokers in AL (group effect, $p < 0.0001$), but not BL, and decreased early after ozone in both fractions (ozone effect, $p < 0.0001$). PMNs progressively increased after ozone exposure in both lavage fractions for all groups (ozone effect, $p < 0.0001$). Lymphocytes were decreased in smokers compared with nonsmokers in both AL (group effect, $p < 0.0001$) and BL ($p = 0.0007$), and increased late after ozone in all groups for AL (ozone effect, $p < 0.0001$) and BL ($p = 0.0096$). Eosinophils were increased in smokers compared with nonsmokers in both AL (Wilcoxon rank sum test, $p = 0.0015$) and BL ($p = 0.011$), and increased late after ozone in BL for all nonsmokers (paired t test, $p = 0.0098$).

Lymphocyte recovery increased 18 hours following ozone exposure in all components of lavage in all groups. No significant differences between groups were seen in the effect of ozone on lymphocyte recovery, although fewer lymphocytes were recovered from smokers.

Eosinophils were more numerous in smokers than in nonsmokers after air exposure (Figure 7). Eosinophil concentration in BL fluid increased 18 hours after ozone exposure in all groups, but was significant only for nonsmokers considered as a single group (paired t test, $p = 0.0098$). Changes in BAL and AL eosinophils were not significant.

Metachromatic cells in BAL fluid were assessed by staining of cytospin smears with Mayer's hematoxylin and toluidine blue, and the results are shown in Figure 8. The number of metachromatic cells increased early after ozone exposure in all groups. A further increase was seen at 18 hours in nonresponders, but not in responders or smokers. Analysis of variance indicated the main effect of ozone was marginally significant ($p = 0.045$), with no significant differences between groups.

Epithelial cell recovery in BL fluid was assessed as an indicator of airway epithelial injury. Figure 9 shows both percentage and concentration of epithelial cells in BL. No significant change was noted in epithelial cell concentration in BL fluid following ozone exposure. The percentage of epithelial cells increased early after ozone exposure in all groups, possibly because of the decrease in recovery of AMs and total cells at this time point (see Table 4).

Inflammatory Cytokines

Levels of the inflammatory cytokines IL-6 and IL-8 were measured in BL and AL components of lavage fluid to examine their potential role in modulating the inflammatory response following ozone exposure. Both IL-6 (Figure 10) and IL-8 (Figure 11) increased early after ozone exposure and decreased toward baseline at 18 hours, and similar effects were seen in BL and AL. The largest change was in IL-6, which increased more than 10-fold in nonsmokers. The increase was greater in nonsmokers than in smokers in both AL and BL components. For IL-8, the response to ozone was not significantly different between groups.

To examine the possible role of IL-6 and IL-8 in the recruitment of PMNs following ozone exposure, ANCOVA

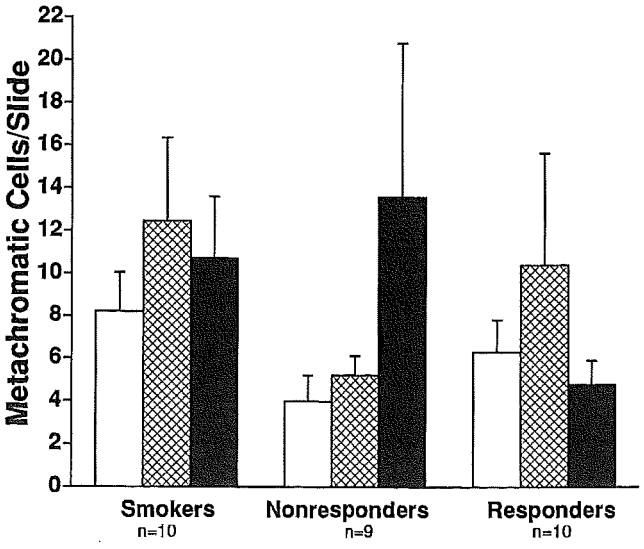

Figure 8. Metachromatic cells in BAL. Cytospin slides were prepared from equal numbers of cells obtained from BAL, stained with Mayer's hematoxylin and toluidine blue, and the total number of cells per slide were counted. Stained cells increased early after ozone exposure in all groups (ozone effect, $p = 0.045$). Nonresponders showed a further increase late after exposure, but there was no significant difference between groups. Open bars: air exposure; cross-hatched bars: ozone early; solid bars: ozone late. Data are expressed as means ± SE.

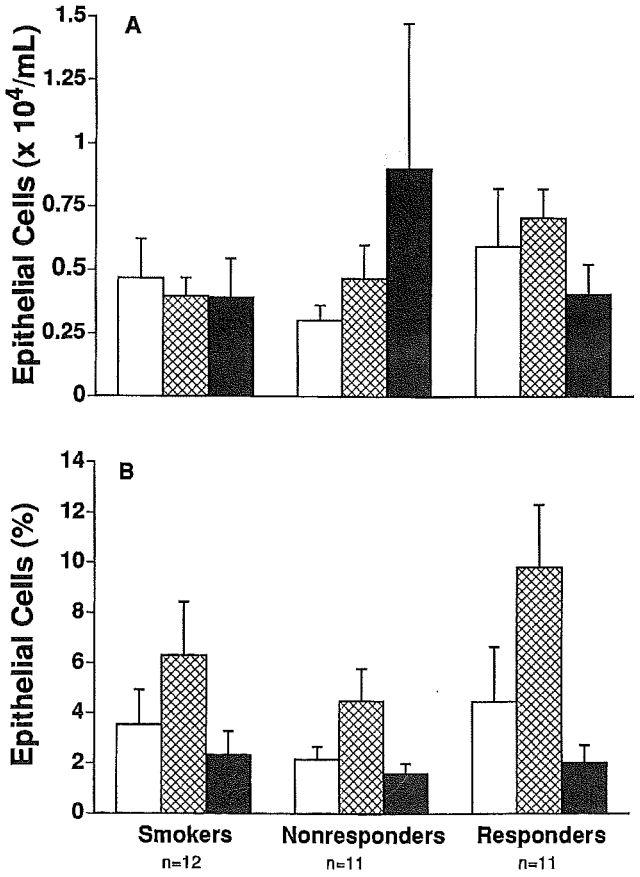

Figure 9. Epithelial cell recovery from BL. Panel A shows the concentration of epithelial cells; Panel B shows epithelial cells as a percentage of the total cells recovered from BL. Percentage, but not concentration, increased early after ozone exposure in all groups (ozone effect, $p = 0.0002$). Open bars: air exposure; cross-hatched bars: ozone early; solid bars: ozone late. Data are expressed as means ± SE.

was performed using changes in these inflammatory cytokines in AL fluid early after ozone exposure as independent variables, and changes in PMNs in BAL fluid 18 hours after ozone as the dependent variable. Early increases in both IL-6 and IL-8 correlated significantly with the late increase in PMNs ($p = 0.002$, $p = 0.007$, respectively). A marginally significant difference was found between groups for the IL-8 association ($p = 0.046$), but not for IL-6. These findings support the hypothesis that both IL-6 and IL-8 may play a role in recruiting PMNs following ozone exposure.

Predictors of Inflammation

In order to determine predictors of susceptibility to airway inflammation in response to ozone exposure, ANCOVA was performed with the change in PMNs in BAL fluid 18 hours after ozone exposure as the dependent variable and the following as independent variables: age, gender, PD_{50}, history of allergies, and subject group. Subject group was marginally predictive of increase in PMNs using this model ($p = 0.045$). When controlling for other factors, including subject group, age was inversely correlated with the inflammatory response ($p = 0.013$). The relationship between age and increase in PMNs is shown in Figure 12. Gender, PD_{50}, and allergy history were not predictive of an ozone-induced increase in PMNs.

Respiratory symptoms during exposure were not predictive of the intensity of airway inflammation. No significant correlation was found between the increase in PMNs in BAL fluid 18 hours after exposure to ozone and the increase in cough ($r = 0.14$, $p = 0.42$) or shortness of breath ($r = 0.01$, $p = 0.94$).

ALVEOLAR MACROPHAGE CHARACTERIZATION

Alveolar macrophages obtained by BAL were characterized to determine whether ozone exposure caused an influx of less-differentiated, monocyte-like macrophages

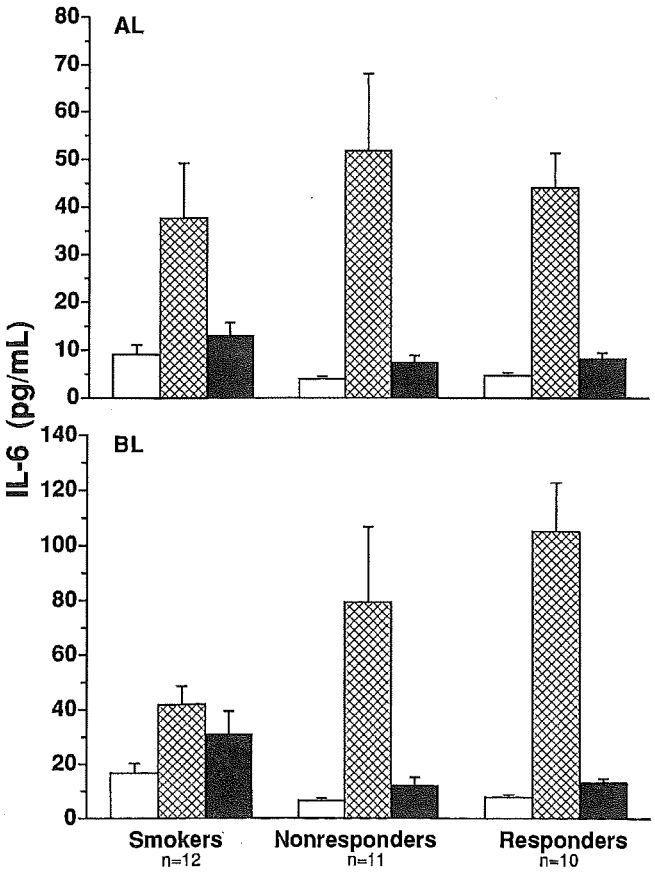

Figure 10. Concentration of IL-6 in AL and BL fluids. IL-6 increased early and returned to baseline late in both AL and BL fluids (ozone effect, $p < 0.0001$). The effect of ozone was greater in nonsmokers than in smokers for both AL and BL fluids (interaction between group and ozone effects, $p = 0.024$ and $p = 0.0002$, respectively). Open bars: air exposure; cross-hatched bars: ozone early; solid bars: ozone late. Data are expressed as means ± SE.

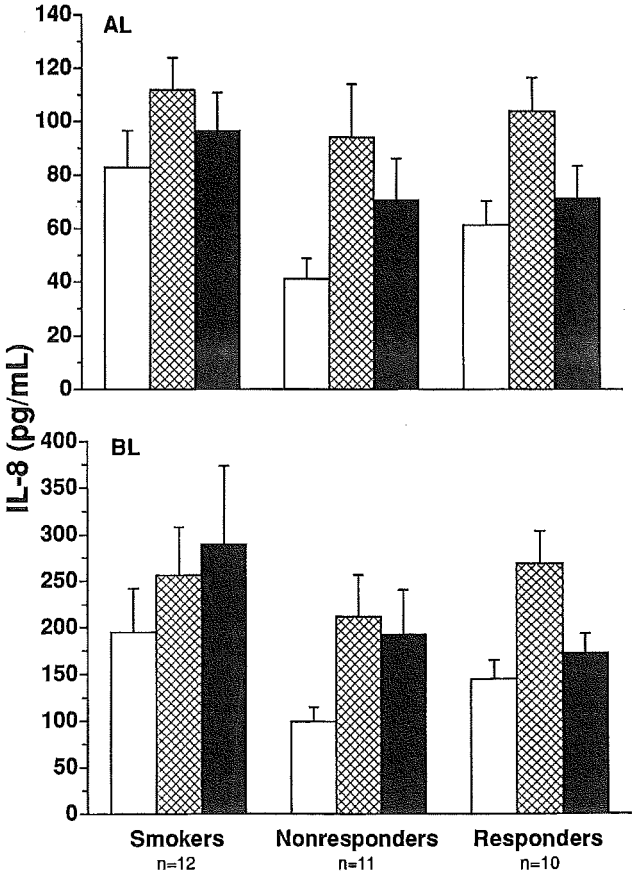

Figure 11. Concentration of IL-8 in AL and BL fluids. IL-8 increased early in all groups in both AL and BL (ozone effect, $p < 0.0001$). Open bars: air exposure; cross-hatched bars: ozone early; solid bars: ozone late. Data are expressed as means ± SE.

into the alveolar space, or caused activation of resident AMs. Changes in AM size were assessed by Coulter channelyzer. Flow cytometric measurement of forward scatter provided an additional index of cell size, as well as membrane changes associated with cell activation (Shapiro 1988). Wide-angle light scatter and autofluorescence were determined as markers of cell activation and metabolic activity (Shapiro 1988). Phenotypic expression of selected cell surface markers was used to detect an influx of less-differentiated monocyte macrophages.

Figure 13 shows the results of alveolar macrophage characterization using flow cytometry. Forward light scatter (Figure 13A) increased late after ozone exposure in smokers and decreased in nonsmokers, but the effect was not significant by ANOVA. Wide-angle light scatter (Figure 13B) and autofluorescence (Figure 13C) were significantly greater in smokers than nonsmokers under all conditions, but the effect of ozone exposure was not significant by ANOVA. No significant differences were noted between responders and nonresponders in AM light-scattering properties.

After subtracting autofluorescence, expression of FcRIII (CD16) decreased early after ozone exposure and increased 18 hours later in all groups, and the changes were significant by ANOVA (Figure 13D). The intensity of staining with monoclonal antibodies recognizing FcRI (CD64), FcRII (CD32), complement receptor 3 (CD11b), and CD14 was similar among groups and did not change significantly with ozone exposure. Coulter channelyzer measurements revealed no difference in AM size among the groups, and no significant effect of ozone exposure on AM size (data not shown).

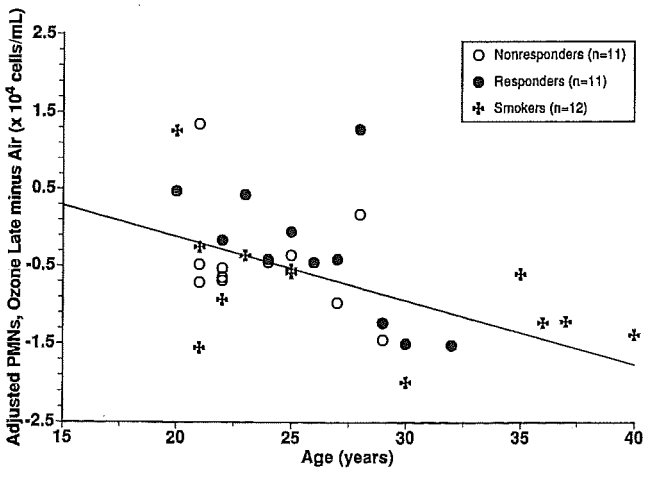

Figure 12. ANCOVA with age in years as the independent variable and the increase in PMNs 18 hours after exposure to ozone as the dependent variable. Shown is the association between the increase in PMNs and age as indicated in the ANCOVA, after adjusting for other independent variables (see text).

PRODUCTION OF TOXIC OXYGEN SPECIES

Two methods were used to assess production of TOS following ozone exposure in these studies. First, release of O_2^- by the whole BAL cell population was measured over 30 minutes, with and without PMA stimulation. Second, oxidation of DCFD was determined using flow cytometry, gating on AMs. This provided a measure of the effect of ozone exposure on production of TOS by AMs separate from other BAL cells. Figure 14 shows unstimulated and PMA-stimulated O_2^- production by the whole BAL cell population. Stimulation with PMA resulted in an approximately fourfold increase in O_2^- release by BAL cells. In nonsmoker-responders and nonsmoker-nonresponders, stimulated O_2^- production increased slightly but progressively, and the increase was statistically significant 18 hours after exposure (Figure 14B). In smokers, unstimulated O_2^- release nearly doubled early after ozone exposure (Figure 14A), although the difference did not reach statistical significance (paired t test, $p = 0.070$). A similar response was seen for stimulated O_2^- release in smokers. Smokers differed from nonsmokers both early and late after ozone exposure (Wilcoxon rank sum test, $p = 0.012$ and $p = 0.046$, respectively).

Incubation of AMs with DCFD caused a considerable increase in cellular fluorescence measurements, suggesting substantial constitutive production of TOS by AMs. For

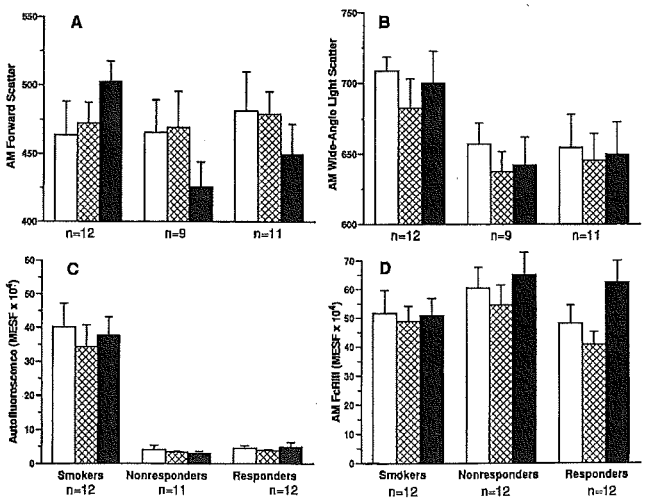

Figure 13. Characterization of AMs. Panels A, B, and C show forward scatter, wide-angle light scatter, and autofluorescence of AMs, respectively, obtained using flow cytometry. Forward scatter increased after ozone exposure in smokers but decreased in nonsmokers; difference between smokers and nonsmokers 18 hours after ozone exposure was significant by unpaired t test ($p = 0.0043$), but the ozone effect was not significant by ANOVA. Wide-angle light scatter and autofluorescence were increased in smokers relative to other groups (ANOVA group effect, $p = 0.010$ and $p < 0.0001$, respectively); ozone effect was not significant. Panel D shows AM expression of FcRIII (CD16) determined by immunofluorescence techniques. Expression of FcRIII decreased early and increased late after ozone (ANOVA ozone effect, $p = 0.037$), without significant group differences. Open bars: air exposure; cross-hatched bars: ozone early; solid bars: ozone late. Data are expressed as means ± SE.

example, fluorescence (mean ± SE) of AMs from nonsmokers obtained after air exposure increased from 4.41 ± 0.76 to 54.26 ± 8.20 × 10^4 MESF after staining with DCFD. For smokers after air exposure, AMs increased from 40.29 ± 6.95 to 66.46 ± 14.64 × 10^4 MESF. Stimulation with PMA produced little further increase.

Figure 15 shows the effects of ozone exposure on AM oxidation of DCFD, without PMA stimulation. Following ozone exposure, production of TOS in AMs from nonsmokers progressively decreased, but increased in AMs from smokers. The pattern was similar to that seen for AM forward scatter (Figure 13A), but effects were not significant by ANOVA ($p = 0.21$).

Analysis of covariance revealed no significant relationship between increases in PMNs and unstimulated production of TOS, either by the whole BAL cell population or by AMs using DCFD, at 18 hours after exposure.

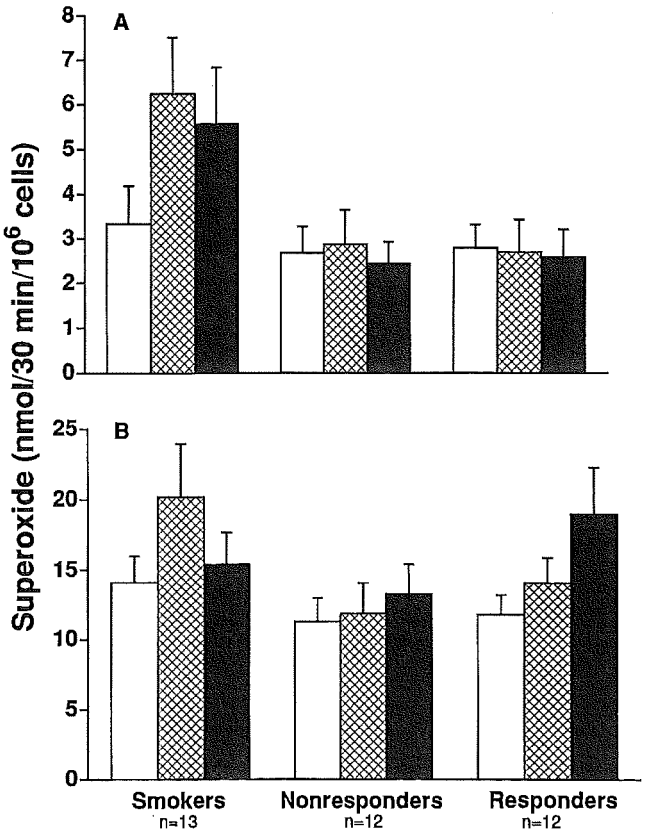

Figure 14. Unstimulated (Panel A) and PMA-stimulated (Panel B) O_2^- production by BAL cells. Unstimulated O_2^- release increased early after ozone in smokers but not nonsmokers, but changes were not significant by ANOVA. Smokers differed from nonsmokers both early and late after ozone exposure (Wilcoxon rank sum test, $p = 0.012$ and $p = 0.046$, respectively). Stimulated O_2^- production increased significantly for all nonsmokers 18 hours after ozone exposure (paired t test, $p = 0.0081$). Open bars: air exposure; cross-hatched bars: ozone early; solid bars: ozone late. Data are expressed as means ± SE.

EPITHELIAL PERMEABILITY

Protein influx into BAL fluid was measured as an index of changes in epithelial permeability following ozone exposure. In addition to total protein, albumin and IgM were selected as molecules of divergent size whose sources are from plasma exclusively. As shown in Figure 16, total protein, albumin, and IgM increased in response to ozone exposure in all subject groups, reaching maximal levels 18 hours after exposure. Albumin showed the greatest increase and IgM the least, consistent with a permeability effect. The increase in albumin following ozone exposure was delayed for smokers compared with nonsmokers (Figure 16B).

Analysis of covariance revealed that increases in albumin levels in BAL fluid were associated with increases in PMNs 18 hours after exposure (Figure 17). However, the strength of the association differed among groups. Smokers and nonsmoker-nonresponders showed the strongest association between increases in PMNs and increases in albumin ($r = 0.71$, $r = 0.73$, respectively); no correlation was found in nonsmoker-responders ($r = 0.04$).

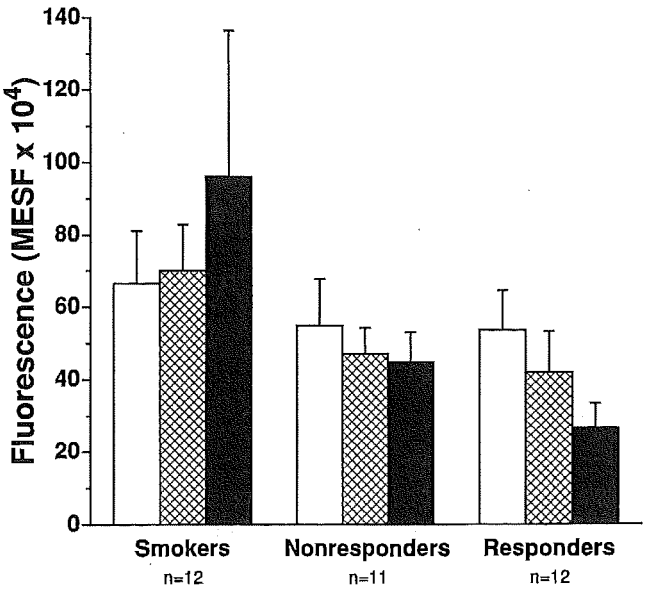

Figure 15. Production of TOS by unstimulated AMs. BAL cells were incubated with DCFD, and flow cytometry was performed gating on AMs. Data represent the increase in AM fluorescence (expressed as MESF) associated with DCFD staining of cells. Fluorescence increased following ozone exposure in smokers, but decreased in nonsmokers; effects were not significant by ANOVA. Open bars: air exposure; cross-hatched bars: ozone early; solid bars: ozone late. Data are expressed as means ± SE.

α_2-MACROGLOBULIN

The concentration of a2M in BAL fluid increased more than threefold 18 hours after exposure (Figure 18), in a pattern similar to that seen for increases in PMNs in this study. The increase was much larger than that seen for IgM, suggesting the source was not translocation from plasma. Plasma levels of a2M were measured and found to be unchanged by ozone exposure in these studies (data not shown). Analysis of covariance showed a significant relationship between increase in PMNs and increases in a2M 18 hours after exposure (p = 0.0001), with no differences among groups.

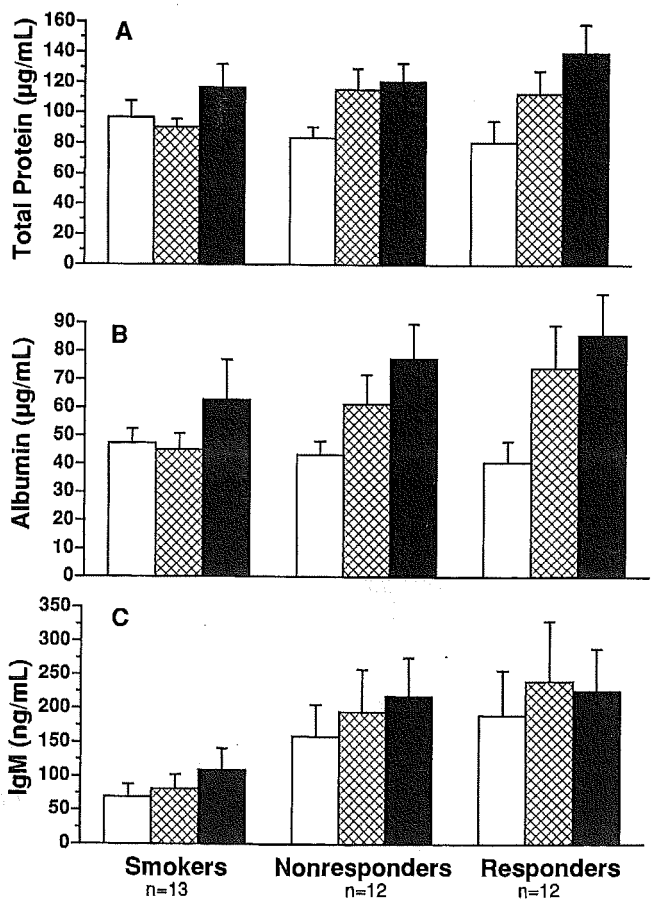

Figure 16. Concentration of total protein (Panel A), albumin (Panel B), and IgM (Panel C) in BAL fluid. Total protein, albumin, and IgM increased after ozone exposure (ANOVA ozone effect, $p < 0.0001$, $p < 0.0001$, and $p = 0.0022$, respectively). The increase in albumin was delayed in smokers relative to nonsmokers (ANOVA interaction between group and ozone effect, $p = 0.033$). Open bars: air exposure; cross-hatched bars: ozone early; solid bars: ozone late. Data are expressed as means ± SE.

NASAL LAVAGE

Cell recovery using NL proved variable under all exposure conditions. Although some subjects showed increases in PMN recovery associated with ozone exposure, other subjects had high numbers of PMNs recovered after air exposure. Mean PMN recovery varied depending on timing of nasal lavage, independent of ozone exposure: mean PMN recovery was greater when NL was performed 18 hours after air exposure than early after air exposure (Figure 19). No statistically significant differences were found between groups or significant effects of ozone exposure on PMN recovery (concentration or percentage of cells) by NL. No significant correlation was seen between NL PMNs and BAL PMNs.

DISCUSSION

For these studies, we chose an ozone exposure concentration and exercise level relevant to performing manual labor or exercising outdoors on a summer day in a major city such as Los Angeles. The findings are applicable to young, healthy individuals with normal lung function who are able to perform repeated exercise.

PHYSIOLOGIC RESPONSES

Our findings confirm previous studies indicating that a subset of young, healthy, nonsmoking individuals will ex-

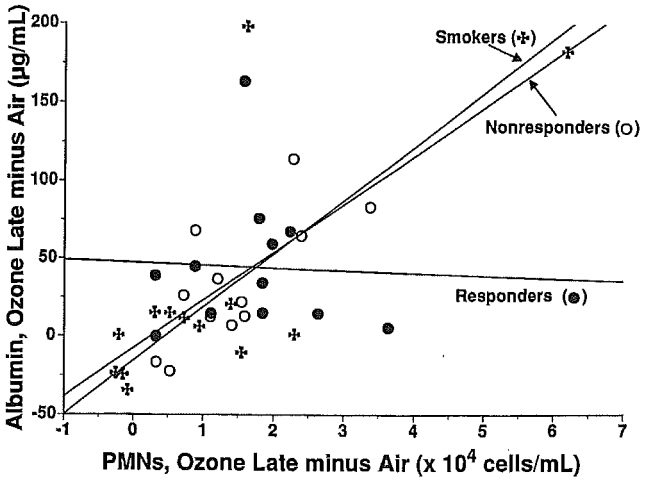

Figure 17. ANCOVA with the increase in PMNs 18 hours after ozone exposure as the independent variable and the increase in albumin at the same time point as the dependent variable. Increases in albumin and PMNs were significantly correlated for all subjects (p = 0.0026). The associations differed among groups (group effect, p = 0.0064), with smokers and nonsmoker-nonresponders showing the strongest correlation (r = 0.71 and r = 0.73, respectively) and nonsmoker-responders showing no correlation (r = 0.04).

perience substantial decrements in lung function when exposed to ozone under the conditions of this study (McDonnell et al. 1995; Bascom et al. 1996). Both FVC and FEV_1 were affected, which is consistent with previous studies that indicate inspiratory capacity is reduced following ozone exposure (Hazucha et al. 1989). Decrements in sGaw were generally small, suggesting an absence of significant bronchoconstriction. For subjects classified as responders to ozone, FEV_1 measured 18 hours after exposure had improved from the level measured immediately after exposure.

Smokers in our study experienced significantly smaller decrements in lung function than nonsmokers. Fewer smokers were responsive, and some subjects experienced slight increases in FEV_1, which suggests that exercise may have induced bronchodilation or clearance of airway mucus. Changes in sGaw and FEF_{25-75} also were smaller in smokers. The ratio of FEV_1:FVC tended to increase slightly during exposure (Figure 4A), indicating that smokers did not experience bronchoconstriction. Differences between smokers and nonsmokers were not explained by differences in levels of exercise or $\dot{V}_E$. Smokers remained consistent in their level of responsiveness on subsequent exposures, similar to nonsmokers (Figure 5).

Smokers also experienced fewer respiratory symptoms in response to ozone exposure. In the 39 subjects exposed to filtered air, smokers reported higher scores than nonsmokers for all symptoms. Nevertheless, following ozone exposure, smokers reported respiratory symptoms less frequently than nonsmokers.

It is interesting to speculate on possible explanations for the decreased responsiveness of smokers. More smokers than nonsmokers (28 vs. 14) failed to attend on the initial classification exposure day, and more smokers than nonsmokers (8 vs. 2) were unable to complete the exercise required (Table 1). However, no smokers failed to complete the study because of ozone responsiveness or symptoms, making significant selection bias unlikely. Nevertheless, smokers may be a self-selected group that is relatively tolerant of the effects of exposure to both cigarette smoke and ozone.

Lung function decrements in response to ozone exposure appear to be mediated by irritant receptor stimulation (Hazucha et al. 1989); the response diminishes with repeated exposures (Farrell et al. 1979). It is possible that oxidant components of cigarette smoke attenuate the irritant fiber responsiveness, thus explaining the reduced sensitivity to ozone. Alternatively, increases in the mucous layer lining the airways in smokers (Fiore et al. 1994) could serve as a "sink" for ozone and its reactive products, reducing effects on epithelial cells and irritant receptors. Emmons and Foster (1991) attempted to determine whether

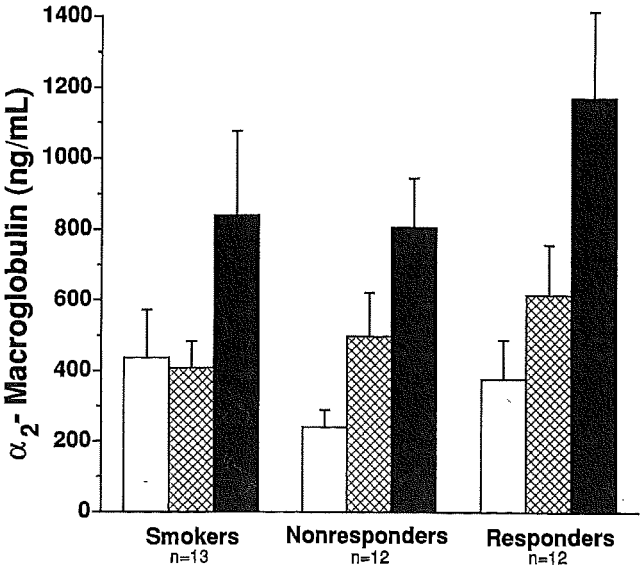

Figure 18. Concentration of a2M in bronchoalveolar lavage fluid. a2M increased progressively in all groups (ANOVA ozone effect, $p < 0.0001$). Open bars: air exposure; cross-hatched bars: ozone early; solid bars: ozone late. Data are expressed as means ± SE.

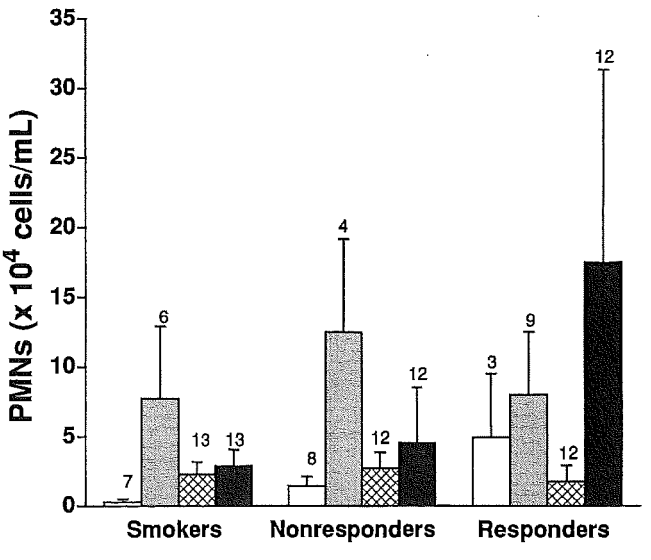

Figure 19. Recovery of PMNs in NL fluid. Open bars: air early; stippled bars: air late; cross-hatched bars: ozone early; solid bars: ozone late. Number of subjects is shown above each bar. Data are expressed as means ± SE.

smoking abstention increases ozone responsiveness by exposing nine smokers to 0.4 ppm ozone for 2 hours both before and 6 months after smoking cessation. Maximal midexpiratory flow rates increased with smoking cessation and this indicator became responsive to ozone, but no significant effect of ozone exposure on FVC was seen either before or after smoking cessation. However, the exposure incorporated only a brief period of light exercise, which may have been insufficient to provoke a change in FVC even in nonsmokers.

CELLULAR RESPONSES

Our studies also confirm previous observations (Seltzer et al. 1986; Kehrl et al. 1987; Koren et al. 1989a,b, 1991; Devlin et al. 1991) that healthy, nonsmoking subjects develop an airway inflammatory response to ozone exposure. The magnitude of PMN influx in our study at 18 hours after exposure is similar to that observed in previous studies; for example, Koren and coworkers (1989b) observed an approximately eightfold increase in PMNs 18 hours after exposure to 0.4 ppm ozone for 2 hours with exercise. However, our observations differ from previous reports in two respects. First, the observed time course of the PMN response differs in our study from that reported by investigators at the U.S. Environmental Protection Agency (Koren et al. 1991). We observed an influx of PMNs at 18 hours after exposure that was greater than the influx immediately after exposure; this observation contrasts with previous studies that reported a greater PMN influx 1 hour after exposure to 0.4 ppm ozone for 2 hours. Previous reports involved separate studies on different subjects, and the findings may have been influenced by group differences. This underscores the importance of obtaining time-course observations using the same subjects. Our observation has important implications: the inflammatory response to ozone appears to be increasing or persisting at a time when symptoms and decrements in lung function have largely resolved.

Second, in contrast with previous BAL studies, our data suggest the airway cellular response to ozone is not limited to PMNs, but involves virtually all cells recovered by BAL. We observed increases in lymphocytes, eosinophils (in nonsmokers), and mast cells associated with ozone exposure. Specific lymphocyte marker studies were not performed, so we cannot exclude the possibility that monocytes or small macrophages recruited to the alveolar space following ozone exposure were mistakenly counted as lymphocytes. However, the absence of changes in AM size by Coulter measurements and the pattern of AM surface marker expression do not support an influx of monocytes or immature macrophages. Future studies of ozone exposure should include identifying lymphocyte cell markers and determining cellular subsets.

We observed small decreases in the number of AMs recovered at the early time point following ozone exposure, which could indicate direct toxicity of ozone or induced programmed cell death. This is supported by the small decrease in the viability of cells obtained early after exposure with BAL. Alternatively, AM recovery could be reduced as a consequence of (1) altered membrane adhesive properties making cells less accessible by BAL, (2) accelerated clearance of airway cells during ozone exposure, or (3) other airway effects altering total cell recovery.

A striking finding in our study was the similarity in the pattern and intensity of the cellular inflammatory response among the three subject groups, despite differences in cell recovery following air exposure. After air exposure, smokers had nearly threefold more AMs, fewer lymphocytes, and more eosinophils than nonsmokers. As shown in Figure 6, smokers had slightly more PMNs in BAL fluid than nonsmokers after air exposure and early after ozone exposure, but the mean recovery of PMNs 18 hours after exposure was similar for all groups. Analysis of variance of the recovery of PMNs from BL and AL fluids (Figure 7) revealed no interaction effects between group and exposure, suggesting similar effects of ozone for all three groups. In addition, the similar cellular responses seen in the BL and AL fractions suggest that ozone-induced inflammation is not limited to the conducting airways. These findings indicate that individuals without symptoms or lung function changes may still experience airway inflammation following exposure to ozone. To the extent that airway inflammation is considered an adverse health effect, this observation has implications for determining who is most susceptible to the health effects of ozone exposure.

Interleukin-6 and IL-8 are involved in the recruitment, activation, and persistence of PMNs and other cells at sites of inflammation (Levine 1995). We observed increases in BAL fluid levels of both cytokines, especially IL-6, early after ozone exposure with a return toward baseline at 18 hours. This time course is consistent with PMNs having a contributing role in the response; furthermore, ANCOVA showed a significant correlation between increases in both IL-6 and IL-8 and increases in PMNs when all subjects were considered together. The approximately 10-fold increase in IL-6 early after ozone exposure particularly implicates this cytokine in the pathogenesis of the response to ozone. Bronchial epithelial cells release IL-8 and IL-6 in response to ozone exposure in vitro (Devlin et al. 1994), suggesting that epithelial cells may be the source of the changes in BAL fluid. However, the magnitude of the ozone-induced in-

crease in both cytokines was similar for BL and AL fractions, suggesting that epithelial cells lining the conducting airways may not be the only source. In our study, all subject groups showed similar patterns of cytokine responses, although smokers had smaller increases in IL-6 than nonsmokers.

ALVEOLAR MACROPHAGE RESPONSES

Previous BAL studies have shown changes in protein synthesis by AMs (Devlin and Koren 1990) and reduction in complement receptors on AMs mediating phagocytosis of *Candida albicans* (Devlin et al. 1991) following ozone exposure in vivo. We sought to determine whether influx of newly recruited macrophages could account for changes in AM function in response to ozone. Surprisingly, we found no change in AM size following ozone exposure in any subject group using Coulter measurement techniques. We also found no change in AM expression of receptors for FcRI (CD64), FcRII (CD32), and the third component of complement (CD11b). In contrast, FcRIII (CD16) increased slightly 18 hours after exposure. CD16 is the low-affinity Fc receptor, which is present on AMs but not on monocytes (Levy et al. 1991); an influx of monocytes or immature AMs in response to exposure would be expected to decrease, rather than increase, expression of CD16. Thus our observations are not consistent with an influx of less mature AMs. We cannot exclude the possibility of an influx of small monocytes that were excluded from the macrophage gates set during analysis; however, gates were set to include small as well as large AMs, and microscopic differential counts did not suggest an influx of monocytes.

PRODUCTION OF TOXIC OXYGEN SPECIES

Our findings suggest that smokers may differ from nonsmokers in the generation of TOS by cells recovered through BAL and by AMs. Stimulated O_2^- generation by the whole cell population in BAL fluid from nonsmokers showed small progressive increases following ozone exposure (Figure 14B), whereas cells from smokers showed increases in both unstimulated and stimulated O_2^- release early after ozone exposure. The small increase in nonsmokers may reflect superoxide generation by newly recruited PMNs in the BAL cell population. For smokers, AMs that are continously activated may respond directly to ozone exposure by releasing O_2^- at the early time point.

We used DCFD staining and flow cytometry to measure the generation of TOS by AMs, separate from other cells in BAL fluid. Alveolar macrophages from both nonsmokers and smokers showed constitutive production of TOS, which decreased following ozone exposure in nonsmokers but increased in smokers (Figure 15).

These patterns of changes in production of TOS suggest that, in nonsmokers, AMs may decrease generation of TOS following ozone exposure, even while other newly recruited inflammatory cells increase the overall burden of TOS. These findings are consistent with studies in animals: ozone exposure has been shown to decrease the oxidative burst of AMs obtained from mice (Ryer-Powder et al. 1988), rats (Grose et al. 1989), and rabbits (Schlesinger et al. 1992). Witz and coworkers (1987) have demonstrated in mice that reactive aldehydes, which are products of ozone lipid peroxidation, decrease AM superoxide generation.

α_2-Macroglobulin is known to suppress macrophage activation. Hoffman and coworkers (1983) demonstrated that a2M complexed with protease suppressed PMA-stimulated superoxide generation by activated murine peritoneal macrophages. It is unknown whether a2M suppresses the oxidative burst of human AMs; however, the time course of the increase in a2M in BAL fluid was similar to the time course of the decrease in AM production of TOS in our studies.

We hypothesize that, in nonsmokers, AM production of TOS may be suppressed following ozone exposure by a2M or other components of the epithelial lining fluid, thus protecting the respiratory epithelium from further injury. In smokers, AM production of TOS is not suppressed, and may increase following ozone exposure, possibly as a result of continuous activation. This raises the question of whether prolonged or repeated exposures to ozone could aggravate the epithelial injury and airway dysfunction caused by smoking.

Albumin concentration in BAL fluid increased following exposure to ozone in all groups, consistent with an increase in permeability of the respiratory epithelium. The greatest increase in albumin was found 18 hours after exposure, similar to the pattern of increase in PMNs. Increases in PMNs 18 hours after exposure correlated strongly with increases in albumin in smokers and nonsmoker-nonresponders, suggesting that PMN influx may play a role in epithelial injury in these groups. No such correlation could be found for nonsmoker-responders, even though increases in albumin were slightly larger than for the other two groups. This difference between groups may be a chance observance, particularly considering that a single outlier may strengthen the apparent correlation in the smoker group (Figure 17).

NASAL LAVAGE

Nasal lavage proved disappointing as a predictor of airway inflammation following ozone exposure. For some subjects, recovery of PMNs appeared more dependent on the timing of the NL than on ozone exposure (Figure 19), suggesting that exercise may have enhanced clearance of nasal inflammatory cells prior to lavage. Our study did not specifically exclude subjects with allergic or vasomotor rhinitis, which may account in part for the variability. In addition, mouth-breathing during exercise may have minimized exposure of the nasal mucosa. Our data indicate that findings from NL do not reliably predict the lower airway inflammatory response to ozone.

CONCLUSIONS

These findings may not be representative of the response to ozone in all segments of the population. Subjects studied were young, healthy, motivated, and able to sustain exercise. Although no subject declined participation because of ozone-related symptoms, we cannot exclude the possibility of selection bias. Despite these caveats, these data suggest the following:

1. Smokers experience smaller decrements in lung function and fewer symptoms than nonsmokers in response to ozone exposure.
2. The intensity of the airway inflammatory response following ozone exposure is independent of smoking status or airway responsiveness to ozone.
3. Inflammation and changes in epithelial permeability to proteins at 18 hours after ozone exposure are greater than immediately after exposure with the ozone concentration used in this study.
4. Interleukin-6 and IL-8 may be important in recruiting inflammatory cells following ozone exposure.
5. Alveolar macrophage production of TOS may be suppressed in nonsmokers following ozone exposure.
6. The burden of TOS presented to the lower respiratory epithelium following ozone exposure may be greater in smokers than nonsmokers.
7. Inflammation following ozone exposure appears to be inversely associated with age; gender, nonspecific airway responsiveness, and allergy history are not predictive of airway inflammation.
8. Findings from NL do not mirror the lower airway inflammatory response following ozone exposure.

In our study, the prospective selection of subjects based on ozone lung function responsiveness, with sampling at two time points after exposure, proved to be a useful experimental design for comparing responders and nonresponders with regard to airway inflammation and injury. The striking similarity between responders and nonresponders in virtually all response indicators is worthy of note. It is clear from these and other studies that symptoms and spirometric effects do not provide sufficient bases for assessing risks associated with ozone exposure. Smokers, and possibly other individuals with underlying airway inflammation, may be at particular risk from the effects of ozone exposure, even in the absence of symptoms or spirometric changes. Unfortunately, individual subject characteristics and noninvasive techniques such as NL are not helpful in determining who is susceptible to the lower airway inflammatory effects of ozone exposure.

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APPENDIX A. Screening Questionnaire

Height _____

Weight _____

(for office use only)

For Office Use

Card #030 (1-3)

SCOR ID _____ (4-9)

Person # _____ (9-10)

1. WHAT IS YOUR FULL NAME? _____ (11-30)

1a. Address _____

2. WHAT IS YOUR SOCIAL SECURITY NUMBER? ☐☐☐ - ☐☐ - ☐☐☐☐ (31-39)

3. WHAT SEX ARE YOU? (40)
1. Male
2. Female

4. WHAT IS YOUR ETHNIC GROUP OR ANCESTRY? (41)
1. White
2. Mexican-American
3. Black
4. Other

5. WHAT IS YOUR MARITAL STATUS? (42)
1. Married
2. Separated
3. Never married
4. Widowed
5. Divorced

6. HOW MANY YEARS OF FORMAL EDUCATION OR SCHOOLING HAVE YOU HAD?
(For example, completion of high school = 12) _____ (43-44)

7. WHAT IS YOUR BIRTHDATE? (45-50)
mo. day year

8. HOW TALL ARE YOU (without shoes)? (51-52)
ft. inches

9. HOW MUCH DO YOU WEIGH? _____ pounds (53-55)

For Office Use

10. HAVE YOU EVER HAD ANY OF THE FOLLOWING DISEASES? *(If uncertain, circle NO)*

a. Arthritis (57)
1. YES, I still have it.
2. YES, but I no longer have it.
3. NO.

b. Ulcer of the stomach or duodenum (58)
1. YES, I still have it.
2. YES, but I no longer have it.
3. NO.

c. Bowel trouble or colitis (59)
1. YES, I still have it.
2. YES, but I no longer have it.
3. NO.

d. Kidney trouble (60)
1. YES, I still have it.
2. YES, but I no longer have it.
3. NO.

e. Liver trouble (61)
1. YES, I still have it.
2. YES, but I no longer have it.
3. NO.

f. Any kind of heart trouble (62)
1. YES, I still have it.
2. YES, but I no longer have it.
3. NO.

g. High blood pressure (63)
1. YES, I still have it.
2. YES, but I no longer have it.
3. NO.

h. Diabetes (sugar in urine) (64)
1. YES, I still have it.
2. YES, but I no longer have it.
3. NO.

i. Stroke (65)
1. YES, I still have it.
2. YES, but I no longer have it.
3. NO.

j. Hardening of the arteries (66)
1. YES.
2. NO.

k. A serious skin rash in infancy (eczema) (67)
1. YES.
2. NO.

11a. DO YOU USUALLY COUGH FIRST THING IN THE MORNING IN THE BAD WEATHER? (68)
(If you usually cough in the morning regardless of the weather, circle YES)
1. YES.
2. NO.

11b. DO YOU USUALLY COUGH AT OTHER TIMES DURING THE DAY OR NIGHT IN THE BAD WEATHER? (69)
(If you usually cough regardless of the weather, circle YES)
1. YES.
2. NO.

IF YES TO EITHER 11a or 11b, ANSWER c AND d

c. DO YOU COUGH ON MOST DAYS FOR AS MUCH AS 3 MONTHS OF THE YEAR? (70)
1. YES.
2. NO.

d. FOR HOW MANY YEARS HAVE YOU HAD THIS COUGH? (71)
1. Less than 2 years.
2. 2-5 years.
3. More than 5 years.

12. HOW MUCH ARE YOU BOTHERED BY COUGH COMPARED TO MOST PEOPLE? (72)
1 2 3 4 5
much less than most — much more than most
(circle appropriate number)

For Office Use

13a. DO YOU USUALLY BRING UP PHLEGM, SPUTUM, OR MUCOUS FROM YOUR CHEST FIRST THING IN THE MORNING IN THE BAD WEATHER? (73)
(If you usually bring up phlegm from your chest in the morning regardless of the weather, circle YES)
1. Yes
2. No

13b. DO YOU USUALLY BRING UP PHLEGM, SPUTUM, OR MUCOUS FROM YOUR CHEST AT OTHER TIMES DURING THE DAY OR NIGHT IN THE BAD WEATHER? (74)
(If you usually bring up phlegm from your chest, regardless of the weather, circle YES)
1. Yes
2. No

IF YES TO EITHER 13a or 13b, ANSWER c AND d:

c. DO YOU BRING UP PHLEGM, SPUTUM, OR MUCOUS FROM YOUR CHEST ON MOST DAYS FOR AS MUCH AS 3 MONTHS OF THE YEAR? (75)
1. Yes
2. No

d. FOR HOW MANY YEARS HAVE YOU RAISED PHLEGM, SPUTUM, OR MUCOUS FROM YOUR CHEST? (76)
1. Less than 2 years.
2. 2-5 years.
3. More than 5 years.

14. HOW OFTEN DO YOU BRING UP PHLEGM FROM YOUR CHEST? (77)
1 2 3 4 5
never — very often
(circle appropriate number)

Card #042 (1-3)
ID DUP (4-10)

15a. DOES YOUR CHEST EVER SOUND WHEEZY OR WHISTLING? (11)
1. YES.
2. NO.

IF YES TO 15a:

b. DO YOU GET THIS WITH COLDS? (12)
1. YES.
2. NO.

c. DO YOU GET THIS EVEN WHEN YOU DON'T HAVE A COLD? (13)
1. YES.
2. NO.

d. DO YOU GET THIS ON MOST DAYS? (14)
1. YES.
2. NO.

16a. HAVE YOU EVER HAD ATTACKS OF SHORTNESS OF BREATH WITH WHEEZING? (15)
1. YES.
2. NO.

IF YES TO 16a:

b. HOW OFTEN ARE YOU BOTHERED BY SUCH ATTACKS? (16)
1 2 3 4 5
rarely — very often
(circle appropriate number)

For Office Use

17. ARE YOU MORE SHORT OF BREATH THAN MOST PEOPLE YOUR AGE? (17)
1. YES.
2. NO.

18. ARE YOU TROUBLED BY SHORTNESS OF BREATH WHEN HURRYING ON LEVEL GROUND OR WALKING UP A SLIGHT HILL? (18)
1. YES.
2. NO.

19. DO YOU GET SHORT OF BREATH WALKING WITH OTHER PEOPLE OF YOUR OWN AGE ON LEVEL GROUND? (19)
1. YES.
2. NO.

20. DO YOU HAVE TO STOP FOR BREATH WHILE WALKING AT YOUR OWN PACE ON LEVEL GROUND? (20)
1. YES.
2. NO.

21a. HAVE YOU EVER HAD ANY KIND OF CHEST TROUBLE? (21)
1. YES.
2. NO.

IF YES TO 21a:

b. WHAT SORT OF TROUBLE? _____ (22-57)

c. HAVE YOU HAD THIS DURING THE PAST YEAR? (58)
1. YES.
2. NO.

22. DID YOU HAVE ANY RESPIRATORY TROUBLE BEFORE AGE 16? (59)
1. YES.
2. NO.

23. DURING THE PAST THREE YEARS, HOW MUCH TROUBLE HAVE YOU HAD WITH ILLNESSES SUCH AS CHEST COLDS, BRONCHITIS, OR PNEUMONIA? (60)
(Does not refer to head colds)
1 2 3 4 5
none — a great deal
(circle appropriate number)

24. DURING THE PAST THREE YEARS, HOW OFTEN WERE YOU UNABLE TO DO YOUR USUAL ACTIVITIES BECAUSE OF ILLNESSES SUCH AS CHEST COLDS, BRONCHITIS, OR PNEUMONIA? (61)
(Does not refer to head colds)
1. Never.
2. During one such illness.
3. During 2-5 illnesses.
4. During 6 illnesses or more.

25. DURING THE PAST YEAR, FOR HOW MANY DAYS HAVE YOU BEEN UNABLE TO DO YOUR USUAL ACTIVITIES BECAUSE OF SUCH ILLNESSES? _____ days. (62-63)

26. *BEFORE* THREE YEARS AGO, HOW MUCH TROUBLE DID YOU HAVE WITH ILLNESSES SUCH AS CHEST COLDS, BRONCHITIS, OR PNEUMONIA? (64)
(Includes such illnesses during childhood)
1 2 3 4 5
none — a great deal
(circle appropriate number)

27. DO YOU THINK YOU HAVE EVER HAD ANY OF THESE CHEST DISORDERS—ASTHMA, ANY KIND OF BRONCHIAL TROUBLE, OR EMPHYSEMA? (65)
1. YES.
2. NO.

28. HAS A DOCTOR EVER TOLD YOU THAT YOU HAD ASTHMA, SOME KIND OF BRONCHIAL TROUBLE, OR EMPHYSEMA? (66)
1. YES.
2. NO.

For Office Use

IN THIS SECTION, PLEASE INDICATE WHAT SPECIFIC LUNG DISEASES YOU HAVE HAD.

29. HAVE YOU HAD ANY OF THE FOLLOWING? *(If uncertain, circle NO)*

a. Emphysema — 1. YES, I still have it. 2. YES, but I no longer have it. 3. NO. (27)

b. Chronic Bronchitis — 1. YES, I still have it. 2. YES, but I no longer have it. 3. NO. (28)

c. Bronchiectasis — 1. YES, I still have it. 2. YES, but I no longer have it. 3. NO. (29)

IF YES TO EMPHYSEMA, CHRONIC BRONCHITIS, OR BRONCHIECTASIS:

d. DID YOU SEE A DOCTOR ABOUT THE CONDITION(S)? — 1. YES. 2. NO. (70)

e. HAVE YOU HAD MEDICATION OR TREATMENT FOR THE CONDITION(S)? — 1. YES. 2. NO. (71)

f. HOW OLD WERE YOU WHEN YOU FIRST DEVELOPED SYMPTOMS FROM EMPHYSEMA, CHRONIC BRONCHITIS, OR BRONCHIECTASIS? — _____ (age) (72-73)

Card #030 (1-3) ID DUP (4-10)

30a. HAVE YOU EVER HAD ASTHMA? — 1. YES, I still have it. 2. YES, but I no longer have it. 3. NO. (11)

IF YES TO 30a:

b. IN THE PAST YEAR, HOW MANY ASTHMA ATTACKS DID YOU HAVE? — 1. No attacks. 2. A few (1-3) attacks. 3. Several (4-12) attacks. 4. Many (13 or more) attacks. 5. Attacks almost every day. (12)

c. CIRCLE THE MONTHS IN WHICH YOUR ATTACKS HAVE BEEN MOST FREQUENT. OR: ☐ Check here if no relation to time of year.

1 2 3 4 5 6 7 8 9 10 11 12
Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec (13-24)

d. HAVE YOU EVER SEEN A DOCTOR ABOUT YOUR ASTHMA? — 1. YES. 2. NO. (25)

e. ARE YOU PRESENTLY TAKING MEDICATION OR TREATMENT FOR YOUR ASTHMA? — 1. YES. 2. NO. (27)

f. HOW OLD WERE YOU WHEN YOU HAD YOUR FIRST ASTHMA ATTACK? — _____ (age) (28-29)

31. HAVE YOU HAD ANY OF THE FOLLOWING? *(If uncertain, circle NO)*

a. Tuberculosis — 1. YES, 2. NO. (30)

b. Valley Fever (coccidiodomycosis) — 1. YES, 2. NO. (31)

c. Histo (histoplasmosis) — 1. YES, 2. NO. (32)

d. Pneumonia or Bronchopneumonia — 1. YES, 2. NO. (33)

For Office Use

32a. HAVE YOU EVER HAD HAY FEVER OR ANY OTHER ALLERGY THAT MAKES YOUR NOSE RUNNY OR STUFFY, APART FROM COLDS? — 1. YES, I still have it. 2. YES, but I no longer have it. 3. NO. (34)

IF YES TO 32a:

b. DURING THE PAST YEAR, HOW MUCH HAVE YOU BEEN BOTHERED BY IT? — 1 2 3 4 5 (very little … very much) (circle appropriate number) (35)

c. CIRCLE THE MONTHS IN WHICH YOUR EPISODES HAVE BEEN MOST FREQUENT. OR: ☐ Check here if no relation to time of year. (36-47)

1 2 3 4 5 6 7 8 9 10 11 12
Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec

33. DO YOU THINK YOU HAVE EVER HAD SINUS TROUBLE? — 1. YES. 2. NO. (48)

34. HAS A DOCTOR EVER TOLD YOU THAT YOU HAD SINUS TROUBLE? — 1. YES. 2. NO. (49)

35. HAVE YOU EVER BEEN ALLERGIC TO ANY FOOD OR MEDICINE? — 1. YES. 2. NO. (50)

36. HOW MUCH EXERCISE DO YOU GET (work or recreation)? — 1. None. 2. Little. 3. Moderate amount. 4. A great deal. (51)

37. DO YOU USE ANY ESTROGEN OR PROGESTERONE (HORMONE) MEDICATIONS, SUCH AS BIRTH CONTROL PILLS? — 1. YES. 2. NO. (52)

38. FOR WOMEN ONLY: ARE YOU NOW PREGNANT? — 1. YES. 2. NO or Uncertain. (53)

39. DO YOU USUALLY USE ANY OF THE FOLLOWING AEROSOLS (pressurized spray cans) 3 OR MORE TIMES PER WEEK?

a. SPRAY ROOM FRESHENERS? — 1. YES. 2. NO. (54)

b. HAIR SPRAY? — 1. YES. 2. NO. (55)

c. UNDERARM DEODORANT SPRAY? — 1. YES. 2. NO. (56)

d. OTHER AEROSOL SPRAYS? — 1. YES. 2. NO. (57)

For Office Use

40a. IN THE PAST TWO YEARS, HAVE YOU HAD A CHEST X-RAY? — 1. YES. 2. NO. (58)

IF YES TO 40a:

WHERE WAS IT DONE? _____

41. HAVE YOU EVER BEEN TOLD YOU HAD AN ABNORMAL CHEST X-RAY? — 1. YES. 2. NO. (59)

42. IN THE PAST YEAR, HAVE YOU BEEN HOSPITALIZED FOR ANY CHEST PROBLEM? — 1. YES. 2. NO. (60)

43. HAVE YOU EVER HAD ANY CHEST OR LUNG SURGERY? (Do not include breast surgery) — 1. YES. 2. NO. (61)

44a. DO YOU DRINK ANY ALCOHOLIC BEVERAGES? — 1. YES. 2. NO. (62)

IF YES TO 44a:

b. HOW MANY GLASSES OF BEER PER WEEK? (on the average) — _____ glasses (63-65)

c. HOW MANY GLASSES OF WINE PER WEEK? (on the average) — _____ glasses (66-67)

d. HOW MUCH HARD LIQUOR PER WEEK? (on the average) — _____ shots (68-69) OR _____ pints (70-71)

45. DID YOU DRINK MORE HEAVILY IN THE PAST THAN YOU DO NOW? — 1. YES. 2. NO. (72)

46. HAVE YOU EVER HAD A PROBLEM WITH YOUR DRINKING? — 1. YES. 2. NO. (73)

(74)

47. IN WHAT TYPE OF AREA HAVE YOU SPENT MOST OF YOUR LIFE? — 1. a very large city *(million or more)* 2. a large city *(100,000-million)* 3. a suburb in a metropolitan area 4. a small city *(5000-100,000)* 5. a town *(under 5000)* 6. a rural area (75)

_____ years (76-7...

For Office Use

Card # 040 (1-3) ID DUP (4-10)

SMOKING:

48a. DO YOU NOW SMOKE CIGARETTES REGULARLY, OCCASIONALLY, OR NEVER? — 1. Regularly. 2. Occasionally *(usually less than 1 each day)*. 3. Never. (11)

IF YOU SMOKE REGULARLY NOW:
(If you do not usually smoke at least one cigarette each day, GO TO #49)

b. DO YOU INHALE? — 1. YES. 2. NO. (12)

c. DO YOU SMOKE CIGARETTES WITH FILTERS OR WITHOUT FILTERS? — 1. With filters. 2. Without filters. 3. Both with and without filters. (13)

d. HOW MANY CIGARETTES DO YOU USUALLY SMOKE EACH DAY AT THE PRESENT TIME? (Please give best estimate: One pack contains 20 cigarettes.) — _____ number per day (14-15)

e. HOW OLD WERE YOU WHEN YOU BEGAN TO SMOKE CIGARETTES? — _____ (age) (16-17)

f. WHAT IS THE USUAL NUMBER OF CIGARETTES YOU HAVE SMOKED PER DAY SINCE YOU BEGAN TO SMOKE? (Please give best estimate: One pack contains 20 cigarettes.) — _____ number per day (18-19)

(If you have completed this section, SKIP question 49 and GO TO #50)

49a. IF YOU DO NOT SMOKE CIGARETTES NOW, DID YOU EVER SMOKE THEM REGULARLY OR OCCASIONALLY? — 1. Regularly. 2. Occasionally *(usually less than 1 each day)*. 3. Never smoked cigarettes. (20)

IF YOU DO NOT SMOKE CIGARETTES REGULARLY NOW BUT USED TO SMOKE THEM:
(If you have never smoked one cigarette or more each day, GO TO #50)

b. WHAT WAS THE USUAL NUMBER OF CIGARETTES YOU SMOKED PER DAY? (Please give best estimate: One pack contains 20 cigarettes.) — _____ number per day (21-22)

c. DID YOU INHALE? — 1. YES. 2. NO. (23)

d. HOW OLD WERE YOU WHEN YOU BEGAN TO SMOKE CIGARETTES? — _____ (age) (24-25)

e. HOW OLD WERE YOU WHEN YOU STOPPED SMOKING CIGARETTES REGULARLY? — _____ (age) (26-27)

f. WERE YOU INFLUENCED TO STOP BECAUSE YOU HAD A COUGH, WHEEZING, OR SHORTNESS OF BREATH? — 1. YES. 2. NO. (28)

For Office Use

50a. DO YOU NOW SMOKE PIPES OR CIGARS REGULARLY, OCCASIONALLY, OR NEVER? (25)
1. Regularly.
2. Occasionally *(usually less than 1 each day).*
3. Never.

IF YOU SMOKE PIPES OR CIGARS REGULARLY NOW:
(If you do not usually smoke at least one cigar or pipeful each day, GO TO #51)

b. HOW MANY PIPEFULS OR CIGARS DO YOU USUALLY SMOKE EACH DAY? _____ number each day (30-31)

c. HOW OLD WERE YOU WHEN YOU FIRST SMOKED? _____ (age) (32-33)

d. DO YOU USUALLY INHALE WHEN YOU SMOKE EITHER PIPES OR CIGARS? (34)
1. YES.
2. NO.

(If you completed this section, SKIP question 51 and GO TO #52)

51a. IF YOU DO NOT SMOKE CIGARS OR PIPES NOW, DID YOU EVER SMOKE THEM REGULARLY OR OCCASIONALLY? (35)
1. Regularly.
2. Occasionally *(usually less than 1 each day).*
3. Never.

IF YOU DO NOT SMOKE PIPES OR CIGARS REGULARLY NOW BUT USED TO SMOKE THEM:
(If you have never smoked at least one cigar or pipeful each day, GO TO #52)

b. HOW MANY PIPEFULS OR CIGARS DID YOU USUALLY SMOKE EACH DAY? _____ number each day (36-37)

c. HOW OLD WERE YOU WHEN YOU FIRST SMOKED PIPES OR CIGARS? _____ (age) (38-39)

d. HOW OLD WERE YOU WHEN YOU STOPPED SMOKING PIPES OR CIGARS? _____ (age) (40-41)

e. DID YOU USUALLY INHALE WHEN YOU SMOKED EITHER PIPES OR CIGARS? (42)
1. YES.
2. NO.

For Office Use

Card # 1272 (1-2)
ID DUP (4-10)

52. MAY WE INFORM YOUR DOCTOR OF THE RESULTS OF THIS STUDY? (11)
1. YES.
2. NO.

53. MAY WE OBTAIN INFORMATION FROM YOUR DOCTOR REGARDING YOUR HEALTH? (12)
1. YES.
2. NO.

54. WHAT IS YOUR DOCTOR'S NAME AND LOCATION? _____ (13-41)

_____ (42-72)

FOR OFFICE USE:

55. |_____| PMD CODE (74-77)

56. R-FORM (78)
1 2 3 4

57. ADMIN. (79)
I R

58. NI ___ (80)

2/15/72

(55) Does anyone in your immediate family (parents, brothers, sisters, or children) have asthma now or a history of asthma in the past.

(1) Yes
(2) No

(56) In the past six weeks have you had any symptoms of a cold, "flu", or any other respiratory infection.

(1) Yes
(2) No

(57) What is your occupation?

(1) Student
(2) Other - Please describe briefly

(58) Are you routinely exposed to any fumes or dusts that make you cough, wheeze, or short of breath?

(1) Yes
(2) No

(59) Are you presently taking any medications?

(1) Yes
(2) No

(60) If answer Yes to number (59), list medications and dosage.

(61) If you have asthma and are not currently taking medication, what medication have you taken in the past 5 years?

(62) If you have asthma, when did your last attack occur?

(63) Do you take any Vitamin C or Vitamin E (including what might be in a multivitamin)?

(1) Yes
(2) No

(64) If the answer to number (63) is "Yes" please write the doses of Vitamin C and Vitamin E you take and how often you take them. If you take a multivitamin, give the brand name and the dose of Vitamin C and Vitamin E in it. (If you do not know them off-hand please find them out and get back to us.)

(65) Are there any gas burning appliances in use in your home on a regular basis or that have been in use in the past few weeks? (Stoves, unvented heaters, etc.)

(1) Yes
(2) No

(66) Has there been a kerosene heater in use in your home in the past few weeks?

(1) Yes, frequently used
(2) Yes, occasionally used

(3) If yes, how long ago was it used (Days or Weeks)

(4) No

(67) Are you getting desensitization injection?

(1) Yes
(2) No

Have you ever had shots?

(1) Yes
(2) No
(3) If yes, when?

(68) Have you ever had formal skin testing?

(1) Yes
(2) No
(3) If yes, when?

(69) Do you now smoke marijuana regularly, occasionally, or never?

(1) Regularly
(2) Occasionally (usually less than once each week)
(3) Never

(70) If you do not smoke marijuana now, did you ever smoke it regularly or occasionally?

(1) Regularly
(2) Occasionally (usually less than once each week)
(3) Never smoked marijuana

(71) Do you presently reside with anyone who smokes cigarettes regularly?

(1) Yes
(2) No

APPENDIX B. Exposure Protocol

Protocol for HEI Ozone Lavage Study

Screening Day: Questionnaire and Informed Consent
History and Physical Examination
Baseline PFT's
- FRC
- Sgaw
- Spirometry
- FV Loop

Methacholine Challenge
Repeat PFT's
Exercise for 20 min with VE (25L/MIN/M^2BSA)
Repeat Spirometry

Criteria: 18 -40 years, Responsive to Ozone (FEV1 >15%)
Nonresponsive to Ozone (FEV1 < 5%)
Nonsmokers: no Tobacco use x 3 yrs, lifetime < 1 pkyr
Smokers: Now >1 pk/day, lifetime > 3 pkyr

Exposure 1: Chamber: 0.22 ppm Ozone x 4 hours

8:00 - 9:00	Baseline PFT's, Resting VE & O_2 Sat Chamber brought up to concentration Patient 1	Patient 2
9:00 - 0:05	Enter and Cal VE	
0:05 - 0:25	Exer 1 with VE	
0:25 - 0:35	Rest	9:30 - 0:35 Enter and Cal VE
0:35 - 0:55	Exer 2	Exer 1 with VE
0:55 - 1:05	Rest	Rest
1:05 - 1:25	Exer 3 with VE	Exer 2
1:25 - 1:35	Rest	Rest
1:35 - 1:55	Exer 4	Exer 3 with VE
1:55 - 2:05	PFT's	Rest
2:05 - 2:25	Exer 5	Exer 4
2:25 - 2:35	Rest	PFT's
2:35 - 2:55	Exer 6 with VE	Exer 5
2:55 - 3:05	Rest	Rest
3:05 - 3:25	Exer 7	Exer 6 with VE
3:25 - 3:35	Rest	Rest
3:35 - 3:55	Exer 8 with VE	Exer 7
3:55 - 4:00	PFT's, questionnaire	Rest
4:05 - 4:25	Home	Exer 8 with VE
4:25 - 4:30		PFT's questionnaire Home

Exposure 2: Chamber: 0.22 ppm Ozone x 4 hours - 3 weeks later

7:00 - 8:00 Baseline PFT's, Resting VE
Chamber brought up to concentration
Patient schedule same as Exposure 1
except : start at 8:00am.

Patient 1 - BAL & Nasal Lavage immediately
Patient 2 - BAL & Nasal Lavage 18 Hour after exposure

Exposure 3: Same as Exposure 2 except:
Patient 1 - BAL & Nasal Lavage 18 hour after exposure
Patient 2 - BAL & Nasal Lavage immediately

Exposure 4: Air x 4 hours (Randomized with Ozone)
Patient 1- BAL & Nasal Lavage 18 hour after exposure
Patient 2 - BAL & Nasal Lavage 18 Hour after exposure

Order of Exposures 2, 3, and 4 will be randomized for each subject pair. Each Exposure will be separated by at least 3 weeks.

APPENDIX C. Symptom Questionnaire

CODE:_____

DATE:_____

During the exposure period, did you develop any of the following symptoms:

a)	Cough	Ø 1 2 3 4 5
b)	Sputum production	Ø 1 2 3 4 5
c)	Shortness of breath	Ø 1 2 3 4 5
d)	Chest pains on deep inspiration	Ø 1 2 3 4 5
e)	Throat irritation	Ø 1 2 3 4 5
f)	Nasal conjestion or irritation	Ø 1 2 3 4 5
g)	Headache	Ø 1 2 3 4 5
h)	Fatigue (beyond that attributable to exercise)	Ø 1 2 3 4 5
i)	Nausea	Ø 1 2 3 4 5
j)	Wheeze	Ø 1 2 3 4 5
k)	Chest tightness	Ø 1 2 3 4 5
l)	Eye irritation	Ø 1 2 3 4 5
m)	Anxiety	Ø 1 2 3 4 5

Ø = Not Present

1 = Minimal -- not noticeable unless specifically asked about

2 = Mild -- noticeable but not annoying

3 = Moderate -- noticeable and annoying

4 = Severe -- limits activity or performance

5 = Incapacitating -- causes ongoing activity to be discontinued

Could you smell or taste anything unusual about the air you were breathing?

Yes No

Do you think you were exposed to ozone today? Yes No

GENERAL COMMENTS:

APPENDIX D. Research Team

PERSONNEL

MW Frampton, Associate Professor of Medicine and Environmental Medicine, Principal Investigator

PE Morrow, Emeritus Professor of Environmental Medicine, Coinvestigator

KZ Voter, Associate Professor of Pediatrics, Coinvestigator

JC Whitin, Senior Research Scientist, Stanford University Medical School, Coinvestigator

A Torres, Instructor and Fellow, Coinvestigator

PC Levy, Associate Professor of Medicine, Coinvestigator

MJ Utell, Professor of Medicine and Environmental Medicine, Coinvestigator

G Cox, Associate Professor of Biostatistics, Consultant in Statistics

DM Speers, Senior Technical Associate in Medicine

Y Tsai, Associate in Medicine

FR Gibb, Associate in Environmental Medicine

D Chalupa, Laboratory Technician IV

L Frasier, Laboratory Technician IV

HE Beiter, Quality Assurance Officer

DESIGNATED RESPONSIBILITIES

Exposure, Analytical, and Physical Plant Team: Morrow, Gibb, Chalupa

Clinical Evaluations, Physiologic Assessments,and Bronchoscopy: Frampton, Torres, Levy, and Speers

Cell Biology Laboratory: Frampton, Voter, Whitin, Torres, Tsai and Frasier

Statistical Analyses: Cox and Speers

Quality Assurance: Beiter and Morrow

APPENDIX E. Quality Assurance Report

The conduct of this study has been subjected to periodic audits by the Quality Assurance Officers at the University of Rochester, Ms. Ellen Miles and Ms. Harriet Beiter. The audits included observations of study activities and audits of the data (see Table E.1 on the next page). The results of the audits were reported to the Director of Research of the Health Effects Institute and to the Principal Investigators. The activities of the university's Quality Assurance Officers were overseen by HEI's Quality Assurance Officer, Ms. Denise Hayes of BioDevelopment Laboratories.

Observations made during these visits indicate that the study is well documented, and that the report describes the methods used and reflects the raw data. The effect of deviations from the protocol and standard operating procedures on the results of the study have been considered and addressed, as appropriate, in the data or final report.

Denise Hayes, M.S.
Quality Assurance Officer
BioDevelopment Laboratories

APPENDICES AVAILABLE ON REQUEST

The following appendices may be obtained by writing the Health Effects Institute, 955 Massachusetts Avenue, Cambridge, MA 02139 or sending your request via e-mail to pubs@healtheffects.org. Please provide the first author's name, the Investigators' Report title, and the titles of the appendices you would like.

- Appendix F. Consent Form
- Appendix G. Raw Data (Group Means)

ABOUT THE AUTHORS

Mark W. Frampton is Associate Professor of Medicine and Environmental Medicine at the University of Rochester Medical Center. He received his M.D. degree from New York University School of Medicine in 1973. His research interests have focused on the health effects of exposure to atmospheric pollutants.

Paul E. Morrow received his B.S. and M.S. degrees in chemistry from the University of Georgia and his Ph.D. in pharmacology from the University of Rochester in 1951. He received postdoctoral training at the University of Göttingen (1959) and the University of Zurich (1960) and spent sabbatical leaves with the MRC Toxicology Unit, Carshalton, England, and the Comitato Nacionale Energie Nucleare (Casaccia), Rome, Italy, in 1968 and 1969. He served on the University of Rochester faculty from 1952 to 1985, at which time he retired as Emeritus Professor of Toxicology and Biophysics. Dr. Morrow's primary research interest is the pulmonary toxicology of inhaled substances.

Alfonso Torres is Senior Instructor in Medicine at the University of Rochester Medical Center. He received his M.D. degree from Cornell University Medical College in 1990. His research interests focus on alveolar macrophage biology in individuals with respiratory disease.

Karen Z. Voter is Associate Professor of Pediatrics at the University of Rochester Medical Center. She received her M.D. degree from the University of Virginia School of Medicine in 1981. She is Director of the Pediatric Pulmonary Function Laboratory and the Pediatric Pulmonary Bronchoscopy Service at Strong Memorial Hospital, University of Rochester. Dr. Voter's research interests focus on the production of toxic oxygen species by human alveolar macrophages.

John C. Whitin is currently Associate Professor of Pediatrics at Stanford University. He received his Ph.D. degree in biochemistry from Boston University in 1982. He received postdoctoral training at the University of Rochester and joined the faculty as an Assistant Professor of Pediatrics in 1985. He left the University of Rochester for Stanford University in 1994. Dr. Whitin's research interests have focused on mechanisms for the control of superoxide production by polymorphonuclear leukocytes.

Christopher Cox is Associate Professor of Biostatistics and Environmental Medicine. He also holds an appointment in the University of Rochester Center for Biomedical Ultrasound. He received his Ph.D. degree in mathematics from the University of Illinois in 1972. He collaborates actively with a number of research groups at the University of Rochester Medical Center. His research interests are in the area of exponential family regression models.

Donna M. Speers is a Senior Research Associate in the Pulmonary and Critical Care Division at the University of Rochester Medical Center. She received her B.S. degree in

Table E.1. Audits by Quality Assurance Officers

Date	Procedure or Data Reviewed	QA Officer
November 21, 1991	Initial QA oversight visit	Denise Hayes
January 24, 1992	Review procedures for collecting and processing lavage fluids	Ellen Miles
	Audit data including lavage cell counts and ELISA protein assays, superoxide anion generation, and flow cytometry	
	Audit files for subjects who had completed the study	
June 1, 1992	Audit data including lavage cell counts, superoxide anion generation, and flow cytometry	Ellen Miles
February 4, 1993	Audit exposure chamber logs	Harriet Beiter
February 16, 1993	Audit flow cytometry data	Harriet Beiter
February 26, 1993	Review lavage sample and pulmonary function test data	Harriet Beiter
March 9, 1993	Audit superoxide anion generation assay data	Harriet Beiter
January 27, 1994	Review pulmonary function test, protein assay, and lavage cell characterization data	Harriet Beiter
February 2, 1994	Review exposure, ELISA, and α_2-macroglobulin data	Harriet Beiter
February 11, 1994	Review superoxide anion generation and flow cytometry data	Harriet Beiter
May 18, 1994	Review exposure data	Harriet Beiter
June 16, 1994	Final review of exposure data	Harriet Beiter
June 21, 1994	Final review of pulmonary function test data	Harriet Beiter
	Review lavage data	
October 31, 1994	Review lavage data	Harriet Beiter
	Final review of protein, albumin ELISA, α_2-macroglobulin, and superoxide anion generation data	
December 9, 1994	Review interleukin assay logbook	Harriet Beiter
June 20-22, 1995	Final audit of interleukin data	Harriet Beiter

chemistry from Valparaiso University and her M.S. degree in statistics from the Rochester Institute of Technology. She recruits and screens volunteers for clinical studies, administers pulmonary function testing, assists with bronchoscopy, and performs data analysis.

Ying Tsai was an Associate in Medicine at the University of Rochester Medical Center until her departure in 1996. She received her B.S. and M.S. degrees in agricultural chemistry from National Taiwan University. She directed the Cell Biology Laboratory in the Pulmonary and Critical Care Division at the University of Rochester.

Mark J. Utell is Professor of Medicine and Environmental Medicine and Director of the Pulmonary/Critical Care and Occupational/Environmental Medicine Divisions at the University of Rochester Medical Center. He is also Associate Chairman of the Department of Environmental Medicine. He received his M.D. from Tufts University School of Medicine in 1972. His research interests have centered on the effects of environmental pollutants on the human respiratory tract.

PUBLICATIONS RESULTING FROM THIS RESEARCH

Frampton MW, Morrow PE, Gibb FR, Speers DM, Gavras JB, Utell MJ. 1993. Airways responsiveness to ozone in smokers and nonsmokers (Abstract). Am Rev Respir Dis 147:A636.

Voter KZ, Frampton MW, Whitin JC, Cox C, Tsai Y, Morrow PE, Utell MJ. 1993. Immediate and delayed effects of ozone exposure on bronchoalveolar lavage cells in smokers and nonsmokers (Abstract). Am Rev Respir Dis 147:A483.

Frampton MW, Malek F, Fogarty JP, Looney RJ, Morrow PE, Utell MJ. 1994. Ozone exposure, exercise, and the systemic acute phase response in humans (Abstract). Physiologist 37:45.

Frampton MW, Voter KZ, Fogarty JP, Morrow PE, Gibb FR, Speers DM, Tsai Y, Utell MJ. 1994. Airway inflammation and functional responsiveness to ozone in humans (Abstract). Am J Respir Crit Care Med 149:A150.

Frampton MW, Morrow PE, Gibb FR, Speers DM, Cox C, Utell MJ. 1995. Predictors of lung function responsiveness to ozone exposure in humans (Abstract). Am J Respir Crit Care Med 151:A500.

Malek FY, Utell MJ, Looney RJ, Morrow PE, Levy PC, Tsai Y, Barth R, Frampton MW. 1995. Does exposure to ozone induce a systemic acute phase response in humans? (Abstract). Am J Respir Crit Care Med 151:A27.

Torres A, Utell MJ, Morrow PE, Voter KZ, Tsai Y, Barth R, Frampton MW. 1995. Airway inflammatory responses to ozone: Relationship to lung function changes in smokers and nonsmokers (Abstract). Am J Respir Crit Care Med 151:A27.

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Avissar N, Finkelstein J, Horowitz S, Willey JC, Coy E, Frampton MW, Watkins RH, Khullar P, Xu Y-L, Cohen HJ. 1996. Extracellular glutathione peroxidase in human lung epithelial lining fluid and in lung cells. Am J Physiol: Lung Cell Mol Physiol 270:L173–L182.

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

Torres A, Voter KZ, Utell MJ, Whitin JC, Morrow PE, Frampton MW. 1996. Production of reactive oxygen intermediates following exposure to ozone: The relative contribution of alveolar macrophages. Chest 109:8S.

ABBREVIATIONS

AL	alveolar lavage
AM	alveolar macrophage
a2M	α_2-macroglobulin
ANCOVA	analysis of covariance
ANOVA	analysis of variance
BAL	bronchoalveolar lavage
BL	bronchial fraction of lavage
DCFD	2′,7′-dichlorofluorescin diacetate
ELISA	enzyme-linked immunosorbent assay
FEF_{25-75}	forced expiratory flow between 25% and 75% of FVC
FEV_1	forced expiratory volume in 1 second
FVC	forced vital capacity
IL-6	interleukin 6

IL-8	interleukin 8
MESF	molecules of equivalent soluble fluorochrome
NAAQS	National Ambient Air Quality Standards
NL	nasal lavage
O_2^-	superoxide anion
PBS	phosphate-buffered saline
PD_{50}	concentration of methacholine that produces a 50% decrease in sGaw
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocyte
ppm	parts per million
sGaw	specific airway conductance
SOD	superoxide dismutase
TOS	toxic oxygen species
$\dot{V}_E$	volume of expired air per minute

Effects of Ozone on Normal and Potentially Sensitive Human Subjects

Part III: Mediators of Inflammation in Bronchoalveolar Lavage Fluid from Nonsmokers, Smokers, and Asthmatic Subjects Exposed to Ozone: A Collaborative Study

Mark W. Frampton, John R. Balmes, Christopher Cox, Peter M. Krein, Donna M. Speers, Ying Tsai, and Mark J. Utell

ABSTRACT

To provide bases of comparison between the studies described in Parts I and II of this Research Report, concentrations of interleukin 6 (IL-6)*, interleukin 8 (IL-8), and α_2-macroglobulin (a2M) were measured in airway lavage fluids obtained in the Balmes study (Part I) and compared with the same measurements in the Frampton study (Part II). For healthy subjects in the Balmes study, IL-6 and a2M, but not IL-8, increased in association with ozone exposure. Statistical analyses suggested that effects of ozone on IL-8 levels observed in the first exposure and bronchoscopy may have carried over to the second exposure and bronchoscopy, which may have obscured an effect of ozone on IL-8 after the second exposure. For asthmatic subjects in the Balmes study, IL-6 and IL-8 increased in both bronchial and alveolar lavage fluid, but not in proximal airway lavage fluid. The mean interval between exposures was longer for asthmatic subjects than for healthy subjects, and no carryover effects were seen. When the Balmes and Frampton data were analyzed together, subject groups in the two studies (nonsmokers, smokers, and subjects without and with asthma) did not differ significantly in the response of cytokines to ozone exposure. The finding of possible carryover effects in one group suggests that subtle effects of ozone exposure, or bronchoscopy including proximal airway lavage and biopsy, or both, may persist for three weeks in some subjects.

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

This Investigators' Report is Part III of Health Effects Institute Research Report Number 78, which also includes *Part I: Airway Inflammation and Responsiveness to Ozone in Normal and Asthmatic Subjects*, by John R. Balmes and associates; *Part II: Airway Inflammation and Responsiveness to Ozone in Nonsmokers and Smokers*, by Mark W. Frampton and associates; a Commentary by the HEI Health Review Committee, and an HEI Statement about the research project. Correspondence concerning this Investigators' Report may be addressed to Dr. Mark W. Frampton, University of Rochester School of Medicine, 601 Elmwood Avenue, Box 692, Rochester, NY 14642-8692.

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INTRODUCTION

One problem in assessing the experimental data base on effects of exposure to atmospheric ozone and other pollutants is the difficulty in comparing findings among different laboratories. This is particularly true for human clinical studies. Differing criteria for subject selection, exposure regimens, outcome variables, sampling times, and measurement techniques often lead to results that are difficult to compare.

The two projects presented in this Research Report provided an opportunity to compare effects of similar exposure regimens in different laboratories. The two studies examined the effects of ozone exposure in healthy nonsmokers, but also in different subgroups with potential susceptibility to ozone exposure: smokers in the study by Frampton and coworkers (see Part II of this Research Report), and people with asthma in the study by Balmes and associates (see Part I of this Report). The studies used similar ozone exposure levels (0.22 ppm in the Frampton study, and 0.20 ppm in the Balmes study), duration (4 hours), and exercise intensity (minute ventilation of 25 L/min/m^2), with a minimum of three weeks between exposures. Both used bronchoalveolar lavage (BAL) techniques in sampling. The Balmes study involved sampling at 18 hours after exposure, whereas the Frampton study design included sampling immediately after exposure and 18 hours after exposure. The similarities in the protocols allowed the results from the sampling at 18 hours after exposure to be directly compared.

In the Frampton study, healthy nonsmokers and smokers developed airway inflammation following ozone exposure. Increases in the proinflammatory cytokines IL-6 and IL-8 were observed in BAL fluid as part of this response. Interleukin-6 is a multifunctional cytokine that induces T lymphocyte proliferation and differentiation of cytotoxic T cells, macrophages, and polymorphonuclear leukocytes (PMNs) (Levine 1995). Interleukin-8 mediates PMN chemotaxis, but may also induce eosinophil and T lymphocyte chemotaxis. These cytokines therefore combine to cause airway inflammatory cell recruitment, proliferation, and differentiation.

In the Frampton study of nonsmokers and smokers, peak increases in both IL-6 and IL-8 preceded the peak increases in PMNs and lymphocytes, consistent with these cytokines having a role in recruiting inflammatory cells following ozone exposure. Both cytokines can be released by epithelial cells in vitro (Levine 1995); however, the predominant site or sites of release within the airways have not been determined.

α_2-Macroglobulin is a large molecule that is abundantly present in serum, but is also present in BAL fluid (Frampton et al. 1989). It may play an important role in protecting the epithelium from the damaging effects of inflammation. For example, a2M serves as a local antiprotease by binding protease molecules (Travis and Salvesen 1983). It may bind proinflammatory cytokines in a similar manner, modulating their effects (Bonner et al. 1989; Matsuda et al. 1989). Murine peritoneal macrophages possess membrane receptors for a2M complexed with protease; engaging these receptors appears to suppress the oxidative burst (Hoffman et al. 1983).

Ozone exposure was associated with a two- to threefold increase in a2M; the time course of the response paralleled the increase in PMNs. In addition, production of toxic oxygen species by alveolar macrophages (AMs) from nonsmokers decreased as a2M levels increased. We hypothesized that a2M may serve to down-regulate AM production of toxic oxygen species following ozone exposure, perhaps to protect the respiratory epithelium from oxidative injury. The cellular source and airway location of a2M production following ozone exposure is unknown.

In this collaborative project, we measured protein concentrations of these mediators in BAL samples from the Balmes study with three goals in mind. First, determining levels of these mediators in samples from both studies using identical techniques provides a point of comparison between the two studies, and extends the findings to a larger group of healthy, nonsmoking subjects. We hypothesized that changes in cytokines and a2M would be similar for the two studies. To the extent the findings agree between the two studies, the results could be extrapolated to the general population of young, healthy nonsmokers with greater confidence.

Second, the lavage protocol for the Balmes study provided an opportunity to determine the source within the lung for these mediators. The protocol included proximal airway lavage (PAL), which avoids contamination from epithelial lining fluid and cells from the lower airways by performing the lavage between occluding balloons in a mainstem bronchus. We hypothesized that, if airway epithelial cells are the primary source for the increase in these proteins following ozone exposure, the increases would be greater in PAL fluid than in the initial return from standard BAL (bronchial lavage fraction or BL) or in the pooled subsequent return from BAL (alveolar lavage fraction or AL). (In this collaborative report [Part III], two terms from Part I [the Balmes study] have been changed to correspond with terms in Part II [the Frampton study]: "bronchial fraction" in Part I is now "bronchial lavage [BL]", and "bronchoalveolar lavage" in Part I is now "alveolar lavage [AL]".

Third, in order to determine whether asthmatic subjects have increased susceptibility to ozone exposure, we sought to compare changes in the production of these mediators following ozone exposure across four subject groups from the two studies: nonsmokers and smokers from the Frampton study, and subjects without and with asthma from the Balmes study.

METHODS

Protocols and methods for subject recruitment, ozone exposure, and BAL in these studies are found in the individual accompanying reports. In brief, the Balmes study examined healthy nonsmokers and subjects with mild asthma. Subjects were exposed to air or 0.20 ppm ozone for 4 hours with exercise for 50 of each 60 minutes at a target minute ventilation of 25 L/min/m^2. Bronchoscopy with PAL, BL, AL, and endobronchial biopsy was performed 18 hours after exposure. Exposures were separated by at least 3 weeks. The Frampton study examined healthy nonsmokers and smokers who had been selected based on responsiveness or nonresponsiveness to a previous ozone exposure. Each subject underwent a total of four exposures (including the initial classification exposure) and three bronchoscopies. Subjects were exposed to air or 0.22 ppm ozone for 4 hours, with exercise to achieve a minute ventilation of 25 L/min/m^2 for 20 of each 30 minutes. Bronchoscopy with BL and AL was performed immediately after one ozone exposure, 18 hours after the other ozone exposure, and either immediately or 18 hours after air exposure. Exposures were separated by at least 3 weeks. Only the results from bronchoscopy 18 hours after exposure are presented for comparison with the Balmes study.

Materials and methods used in the measurement of IL-6, IL-8, and a2M are provided in Part II of this report by Frampton and coworkers. Methods had been previously validated for use with BAL fluid, as described. Aliquots of supernatant fluid from PAL, BL, and AL from the Balmes

study were stored at –70°C and shipped on dry ice to the University of Rochester by overnight mail. Analyses were performed in duplicate using techniques identical to those used in the Frampton study. Investigators were blinded to the identity of the exposures until analyses were complete. Lavage fluids from both exposure days for each subject were analyzed on the same day.

Statistical analysis was performed with two objectives. First, we wanted to compare the levels of the markers of inflammation (IL-6, IL-8, and a2M) after ozone exposure with the levels after air exposure in the PAL, BL, and AL samples from the Balmes study. We did so by using the paired crossover *t* test (Brown Jr. 1980), which allowed for possible period effects and interaction effects to be recognized. A significant period effect would indicate that responses to exposure varied with time (time of day, time between exposure and lavage) or season (of year) independent of the treatment (i.e., ozone or air). A significant interaction effect would indicate that one exposure may have altered the results of the next exposure (also referred to as "carry-over effect").

Second, we wanted to determine whether differences in the effects of ozone exposure could be found among the four groups of subjects in the two studies: nonsmokers and smokers in the Frampton study, and nonasthmatic and asthmatic subjects in the Balmes study. Analysis of variance with the Tukey method of multiple comparisons was used to compare the four groups; the absolute differences between air and ozone exposure for each subject 18 hours after exposure were compared, recognizing that significant differences could be due either to actual differences between groups of subjects, or to procedural or subject selection differences between the studies. Percentage of change was not used because values measured after air exposure included significant numbers of zeros.

A p value of less than 0.05 was required for significance; the overall error rate for the Tukey method was 5%.

RESULTS

Lavage samples were received for 19 healthy subjects and 18 subjects with asthma. Because of occasional technical difficulties, not all lavage samples were obtained on every subject. The numbers of samples analyzed and the results of statistical comparisons are shown in the figures. Individual and mean data are provided in Appendix A, which is available on request from the Health Effects Institute.

NORMAL SUBJECTS

Figures 1A through 3A show the changes in IL-6, IL-8, and a2M, respectively, for nonasthmatic subjects. Interleukin-6 increased following ozone exposure in PAL, BL, and AL. Increases in IL-6 ranged from nearly fourfold in PAL to more than twofold in AL. Using the crossover *t* test, a significant interaction effect was seen for IL-6 in PAL (Figure 1A, $p < 0.05$), suggesting the possibility of carry-over effects from the first exposure to the second.

For IL-8, no significant ozone effect was observed (Figure 2A). However, significant interaction effects were seen for IL-8 in BL and AL. In order to determine whether carry-over effects from the first bronchoscopy may have obscured an ozone effect on IL-8, two analyses were performed. First, we compared IL-8 levels in BL following air exposure between subjects receiving air as the first exposure and subjects receiving air as the second exposure. Interleukin-8

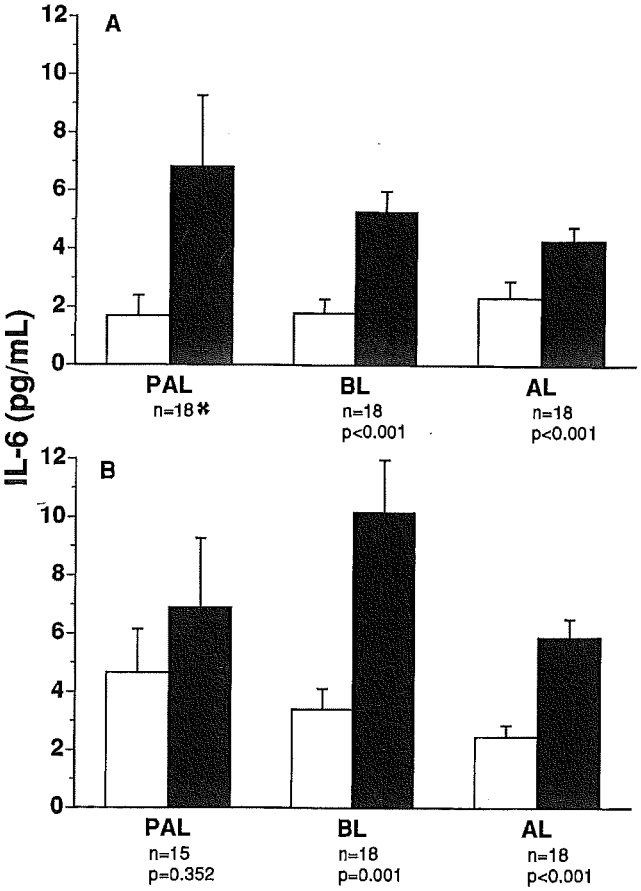

Figure 1. Interleukin 6 in nonasthmatic (A) and asthmatic (B) subjects. Concentration of IL-6 in PAL, BL, and AL fluids. Open bars: air exposure; solid bars: ozone exposure. * = Significant interaction effect on crossover *t* test ($p < 0.05$ for PAL). Data are expressed as means ± SE.

levels after air exposure were higher in the group exposed to air second (mean ± SE, 289.3 ± 156.4 pg/mL; n = 9) compared with the group exposed to air first (62.3 ± 7.9 pg/mL; n = 9). Second, we compared IL-8 levels in BL after air exposure in those subjects exposed to air first (62.3 ± 7.9 pg/mL; n = 9) with levels after ozone exposure in those exposed to ozone first (240.1 ± 42.6 pg/mL; n = 9); the difference was significant using an unpaired t test (p = 0.003). This suggested that ozone exposure caused an increase in IL-8 in BL fluid, but that carry-over effects from the first exposure and bronchoscopy may have obscured that effect for the study as a whole.

For IL-8 in PAL, no significant interaction effect was found, and the observed difference between air and ozone exposure (treatment effect) was not significant.

α_2-Macroglobulin increased following ozone exposure in all samples (Figure 3A). However, analysis of data from PAL showed a significant period effect (p = 0.007), indicating that the timing of the exposures, independent of treatment (air or ozone), may have influenced the measurement of a2M levels. No significant interaction (carry-over) effect was noted. The increases in a2M in BL and AL were highly significant, without period or interaction effects.

SUBJECTS WITH ASTHMA

Figures 1B through 3B provide the findings for subjects with asthma. Interleukin-6 increased significantly in BL and AL, but the increase in PAL was not significant (Figure 1B). Interleukin-8 also showed no significant change in PAL, but increased more than two-fold in BL (Figure 2B).

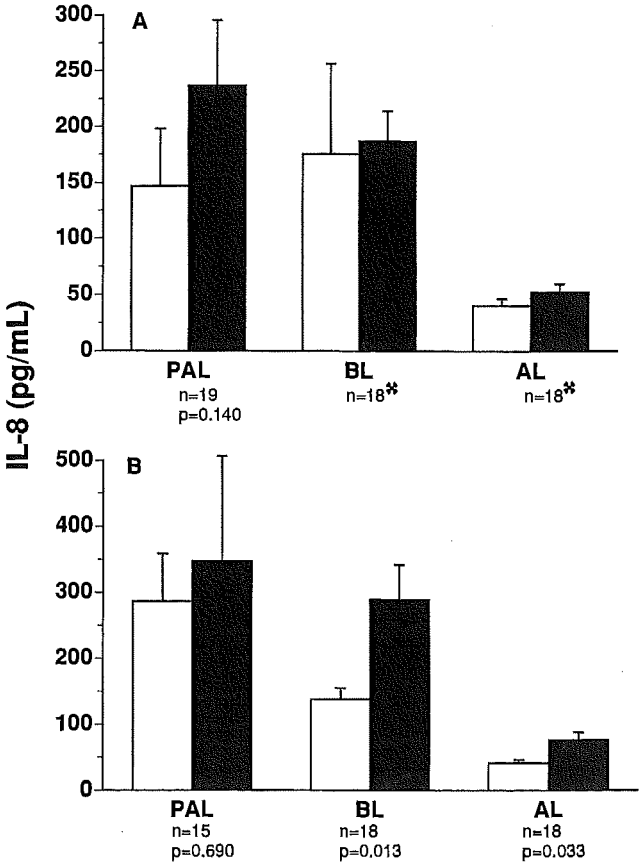

Figure 2. Interleukin 8 in nonasthmatic (A) and asthmatic (B) subjects. Concentration of IL-8 in PAL, BL, and AL fluids. Open bars: air exposure; solid bars: ozone exposure. ✖ = Significant interaction effects on crossover t test ($p < 0.050$ for BL, $p = 0.035$ for AL). Data are expressed as means ± SE.

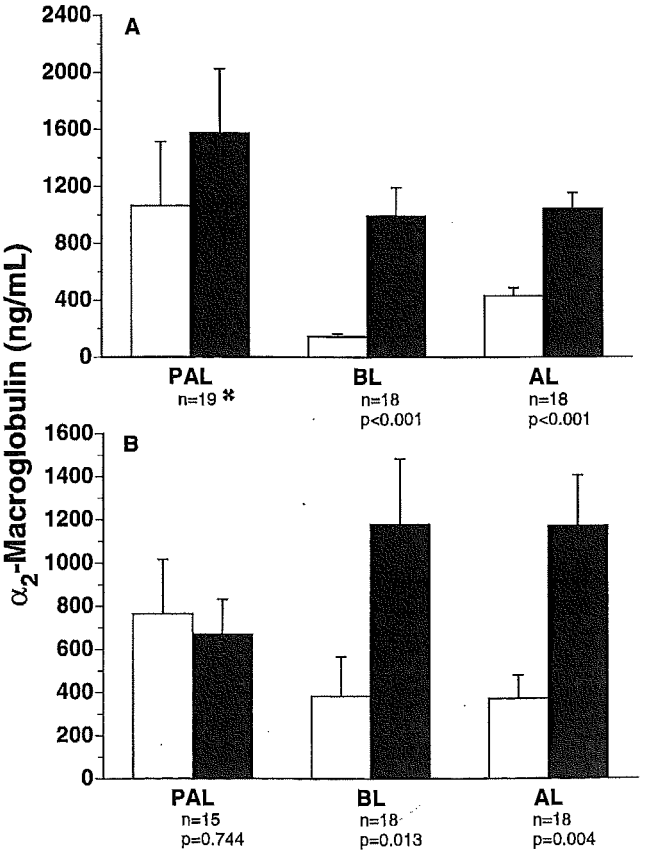

Figure 3. α_2-Macroglobulin in nonasthmatic (A) and asthmatic (B) subjects. Concentration of a2M in PAL, BL, and AL fluids. Open bars: air exposure; solid bars: ozone exposure. ✖ = Significant period effect on crossover t test (p = 0.007 for PAL). Data are expressed as means ± SE.

Levels were lower in AL, but also increased significantly. α_2-Macroglobulin was unchanged in PAL, with substantial increases in BL and AL, similar to nonasthmatics (Figure 3B). After air exposure, levels of a2M were generally higher in PAL than in BL or AL, for both nonasthmatic and asthmatic subjects.

In contrast to the data for nonasthmatic subjects, no significant period or interaction effects were observed for any variable. The mean (± SE) interval between air and ozone exposures for asthmatic subjects was 85 ± 18 days, compared with 50 ± 7 days for nonasthmatic subjects.

SUBJECT GROUP COMPARISONS

Figures 4 through 8 show IL-6, IL-8, and a2M data for BL fluid and AL fluid for all four subject groups: nonasthmatic and asthmatic groups (from the Balmes study) and nonsmoker and smoker groups (from the Frampton study). Concentrations of IL-6 in BL and AL fluid (Figures 4 and 5) from subjects in the Balmes study were lower after air exposure than for the subjects from the Frampton study (nonasthmatic subjects compared with nonsmokers, $p < 0.001$); however, IL-8 (Figures 6 and 7) and a2M (Figure 8) levels after air exposure were similar between the two studies.

Analysis of variance with multiple comparisons was performed, using the absolute change in the levels of these mediators following ozone exposure as the variable of interest. The increase from air to ozone exposure did not differ among subject groups for IL-6, IL-8, or a2M from BL or AL fluids. Only for IL-6 in BL fluid did the differences among groups approach significance ($p = 0.061$); smokers had the largest increase in levels of IL-6 in BL fluid.

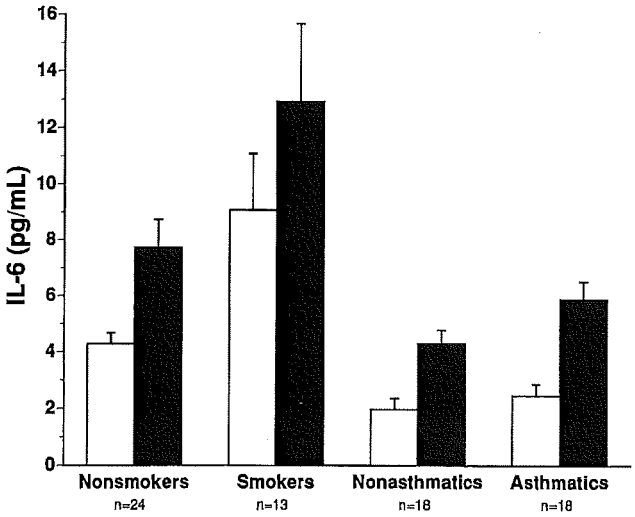

Figure 5. Concentration of IL-6 in alveolar lavage fluid: Comparison of four subject groups. Data from the Frampton study are presented for nonsmokers and smokers; data from the Balmes study are presented for nonasthmatic and asthmatic subjects. Open bars: air exposure; solid bars: ozone exposure. Data are expressed as means ± SE.

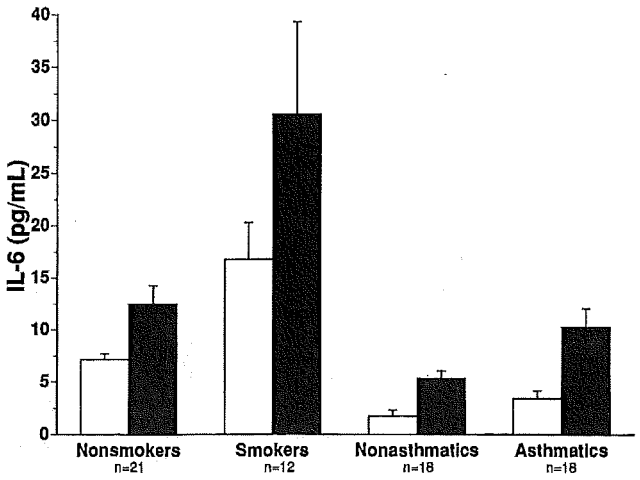

Figure 4. Concentration of IL-6 in bronchial lavage fluid: Comparison of four subject groups. Data from the Frampton study are presented for nonsmokers and smokers; data from the Balmes study are presented for nonasthmatic and asthmatic subjects. Numbers of smokers and nonsmokers (Frampton study) differ from Figure 5 because bronchial lavage was not performed separately on all subjects. Open bars: air exposure; solid bars: ozone exposure. Data are expressed as means ± SE.

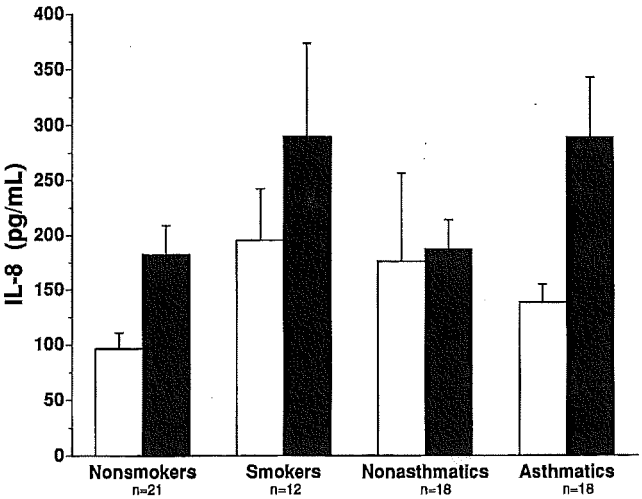

Figure 6. Concentration of IL-8 in bronchial lavage fluid: Comparison of four subject groups. Data from the Frampton study are presented for nonsmokers and smokers; data from the Balmes study are presented for nonasthmatic and asthmatic subjects. Numbers of smokers and nonsmokers (Frampton study) differ from Figure 7 because bronchial lavage was not performed separately on all subjects. Open bars: air exposure; solid bars: ozone exposure. Data are expressed as means ± SE.

DISCUSSION

Interleukin-6, IL-8, and a2M were measurable in PAL after air exposure, suggesting that epithelial cells produce these mediators constitutively or in response to bronchoscopic manipulation. Interleukin-6 increased substantially in all lavage components 18 hours after exposure, except in PAL in asthmatic subjects. In nonasthmatic subjects, the largest increase was in PAL fluid, and in asthmatic subjects the largest increase was in BL fluid. However, IL-6 levels are known to be much higher immediately after exposure than 18 hours later; therefore, these measurements at 18 hours may not reflect the peak concentrations. The time course for the increase in IL-6 in PAL is unknown, but is likely to be similar to that for BL fluid.

Mean levels of a2M were actually higher in PAL than in BL or AL fluids after air exposure, for both nonasthmatic and asthmatic subjects. Nevertheless, the largest ozone-related increases in a2M were in BL and AL fluids for both healthy and asthmatic subjects, with little or no increase in PAL fluid. This suggests that the source of the increased synthesis of a2M in response to ozone exposure may be in the distal airways or alveolar space.

Interleukin-8 also tended to remain increased 18 hours after exposure in asthmatic subjects (Figure 5). For nonasthmatic subjects, analysis suggested unexpected carry-over effects in the measurement of IL-8 levels in BL and AL fluids. Levels of IL-8 were higher when air exposure was performed as the second exposure rather than the first, which suggests that persistent effects on IL-8 release by airway epithelial cells may have resulted from the previous bronchoscopy or ozone exposure. The occasional significant carry-over or interaction effects could be by chance, considering that a total of 18 comparisons were performed in the analysis. However, interaction effects occurred exclusively in nonasthmatic subjects, and the interval between exposures was shorter for this group than for the asthmatic subjects.

Bronchoscopy with BAL has been shown to induce an influx of PMNs, but this resolves within 72 hours after the procedure (Von Essen et al. 1991). It seems unlikely that changes in IL-8 production following bronchoscopy could persist beyond three weeks. However, we cannot exclude the possibility that some component of the procedure, which included PAL and endobronchial biopsies, might have sensitized the epithelium to respond by releasing more IL-8 following the second procedure in some subjects. In this study, carry-over effects would tend to obscure the effects of ozone. Indeed, significant ozone-induced increases in IL-8 in BL fluid were seen only when data from first exposures were used.

We conclude that ozone exposure causes increases in lavage fluid levels of IL-6, IL-8, and a2M that persist to 18 hours after exposure. The effects of ozone were similar in two different laboratories, suggesting that they are representative of the general population. Increases in IL-6 and

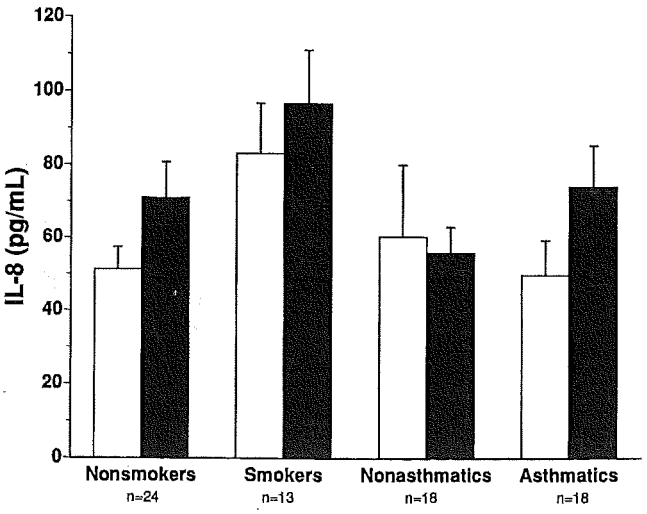

Figure 7. Concentration of IL-8 in alveolar lavage fluid: Comparison of four subject groups. Data from the Frampton study are presented for nonsmokers and smokers; data from the Balmes study are presented for nonasthmatic and asthmatic subjects. Open bars: air exposure; solid bars: ozone exposure. Data are expressed as means ± SE.

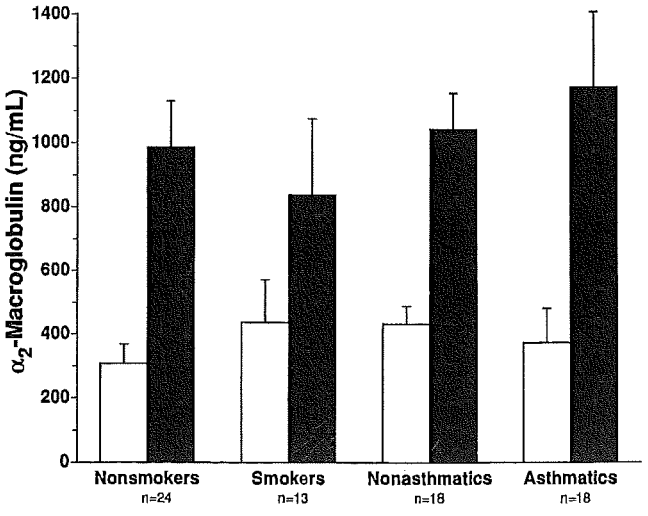

Figure 8. Concentration of a2M in alveolar lavage fluid: Comparison of four subject groups. Data from the Frampton study are presented for nonsmokers and smokers; data from the Balmes study are presented for nonasthmatic and asthmatic subjects. Open bars: air exposure; solid bars: ozone exposure. Data are expressed as means ± SE.

IL-8 occurred in all lavage fractions, indicating that the source for the increased production of these cytokines is not limited to the proximal conducting airways. The increase in a2M was greater in BL and AL fluids than in PAL fluids, suggesting a source in the more distal airways. No significant differences were noted between healthy nonsmokers, smokers, and asthmatic subjects in the effects of ozone on these mediators. The finding of possible carryover effects on lavage fluid levels of IL-8 has implications for all studies involving repeated exposures and bronchoscopic procedures, and suggests that subtle effects of ozone exposure, bronchoscopy (including PAL and biopsy), or both may persist for three weeks in some subjects.

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APPENDIX AVAILABLE ON REQUEST

The following appendix may be obtained by writing the Health Effects Institute, 955 Massachusetts Avenue, Cambridge, MA 02139 or sending your request via e-mail to pubs@healtheffects.org. Please provide the first author's name, the Investigator's Report title, and the title of the appendix you would like.

- Appendix A. Collaborative Study Raw Data

ABBREVIATIONS

AL	alveolar fraction of lavage
AM	alveolar macrophage
a2M	α_2-macroglobulin
BAL	bronchoalveolar lavage
BL	bronchial fraction of lavage
IL-6	interleukin 6
IL-8	interleukin 8
PAL	proximal airway lavage
PMN	polymorphonuclear leukocyte

INTRODUCTION

Since its formation, the Health Effects Institute has supported studies on oxidants, primarily nitrogen dioxide and ozone. A variety of experimental approaches have been used, including in vitro, animal, controlled human exposure, and epidemiologic studies. One goal of HEI's ozone research program has been to provide scientific information to aid the U.S. Environmental Protection Agency's (EPA)* deliberations of whether the National Ambient Air Quality Standard (NAAQS) for ozone adequately protects human health.

The current primary NAAQS for ozone is 0.12 parts per million (ppm), a level not to be exceeded for more than one hour, once per year. The ozone standard is being reevaluated and the EPA has proposed changing the level and the form of the standard to 0.08 ppm based on an eight-hour, rather than a one-hour, average (U.S. Environmental Protection Agency 1996b). The EPA's proposal for an eight-hour standard is based on evidence (discussed in the Scientific Background section) that ozone causes adverse effects on the respiratory system after multihour exposures. The adverse effects following short-term exposure to ozone include shortness of breath, coughing, reversible changes in pulmonary function, and airway inflammation (reviewed in U.S. Environmental Protection Agency 1996a). Little is known about the impact of repeated or prolonged episodes of airway inflammation due to ozone on lung structure and function. This information is crucial to understanding the role that exposure to this pollutant may have in the etiology of chronic lung disease. In particular, it is important to determine whether subpopulations with preexisting respiratory disease, including people with underlying airway inflammation, are more susceptible to the effects of ozone than people with healthy lungs.

In order to address this particular issue, HEI issued a Request for Applications (RFA) in 1990 entitled "Clinical Studies of Sensitivity to Ozone." This RFA had two objectives: (1) to determine the range of responsiveness to ozone in the general population; and (2) to identify subgroups within the general population who are particularly sensitive to ozone. In response to RFA 90-2, Drs. John Balmes of the University of California at San Francisco and Mark Frampton of the University of Rochester Medical Center submitted separate proposals for studies examining normal volunteers and volunteers expected to have underlying airway inflammation that might influence their response to ozone. Common goals of both studies were to determine if lung function responses to ozone exposure correlated with the appearance of markers of inflammation, and if potentially sensitive individuals, such as smokers and people with asthma, had greater adverse responses to ozone than normal subjects. The reports for the Balmes and Frampton studies form Part I and Part II of this Research Report, respectively. Because each investigator used similar ozone exposure protocols, it was possible to compare selected endpoints for both population groups in a collaborative study (Part III)[†]. The common goals and similar ozone exposure protocols provided the HEI Health Review Committee with the opportunity to evaluate and compare the interpretations and conclusions of all studies in a single Commentary.

SCIENTIFIC BACKGROUND

OZONE'S EFFECTS ON PULMONARY FUNCTION

In some individuals, ozone causes acute changes in pulmonary function. These changes are primarily of the restrictive type (i.e., decreased lung volume as a result of impaired ability to take a deep breath), although some bronchoconstriction (i.e., narrowing of the conducting airways) may occur as well. Common pulmonary function tests used to measure ozone's effects are the forced expira-

* A list of abbreviations appears at the end of this Commentary for your reference.

This document has not been reviewed by public or private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views of these parties, and no endorsements by them should be inferred.

[†] Dr. Balmes' study, *Airway Inflammation and Responsiveness to Ozone in Normal and Asthmatic Subjects*, began in March 1991 and total expenditures were $932,712. The Investigators' Report from Dr. Balmes and colleagues was received for review in September 1995. A revised report was accepted for publication by the Health Review Committee in March 1996. Dr. Frampton's study, *Airway Inflammation and Responsiveness to Ozone in Nonsmokers and Smokers*, began in July 1991 and total expenditures were $816,197. The Investigators' Report from Dr. Frampton and colleagues was received in October 1995. A revised report was accepted for publication by the Health Review Committee in October 1996. The report on the collaboration, *Mediators of Inflammation in Bronchoalveolar Lavage Fluid from Nonsmokers, Smokers, and Asthmatic Subjects Exposed to Ozone: A Collaborative Study*, was received in October 1995. A revised report was accepted for publication by the Health Review Committee in October 1996. During the review process, the HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Reports and in the Review Committee's Commentary. The following Commentary is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the studies and by placing the Investigators' Reports into scientific and regulatory perspective.

tory volume in one second (FEV_1), which is the volume exhaled in the first second following maximal inspiration; the forced vital capacity (FVC), which is the maximum exhaled volume following maximal inspiration; and their ratio (FEV_1/FVC), a measure of airflow during exhalation. With lung restriction, the absolute values of FEV_1 and FVC decrease, but the FEV_1/FVC ratio remains relatively unchanged. With bronchoconstriction, the absolute value of FEV_1 and the FEV_1/FVC ratio decrease, whereas FVC tends not to change. There are age-, gender-, and height-specific normal values for FEV_1, FVC, and FEV_1/FVC from reference populations that can be compared to subject values.

Bronchoconstriction (which occurs when the bronchi and bronchioles constrict) is associated with increases in airway resistance (defined as the driving pressure of air through the airways divided by the air flow rate) or decreases in the reciprocal function, airway conductance. Because airway resistance and airway conductance vary with factors such as lung volume, resistance and conductance values are divided by the lung volume at which they are measured and expressed as specific airway resistance (sRaw) and specific airway conductance (sGaw). Both measurements change according to airway smooth muscle tone. For example, resistance decreases when smooth muscle relaxes and increases when smooth muscle tightens. Inflammation of the airways can lead to irritation and constriction of the bronchi and small bronchioles, which increases airway resistance and decreases airway conductance.

A large body of evidence indicates that decreases in FEV_1 and increases in sRaw occur in some people who are exposed to the maximal allowable ambient or near-ambient levels of ozone while exercising. These changes become worse with longer exposure (Folinsbee et al. 1988), higher ozone concentration, and deeper breathing (Gong et al. 1986; Hazucha 1987). The FEV_1 response to ozone is reproducible within an individual (McDonnell et al. 1985), and is attenuated by multiple exposures on consecutive days (discussed by Tepper et al. 1989). Individuals who are exposed to 0.08 ppm ozone and higher over 4- to 6-hour periods experience transient changes in FEV_1, ranging from very slight increases to 40% decreases from baseline. Many laboratory studies involve healthy adults from 18 to 50 years of age; this carefully selected group of normal subjects may not provide adequate information to assess the health effects of ozone in the general population and in susceptible populations. For example, the effects of ozone on individuals with asthma, who may be more sensitive to the acute effects of ozone than their healthy counterparts, are not clear. Some studies suggest that the magnitude of ozone-induced decrements in pulmonary function in asthmatic subjects is no greater than those in normal subjects (Linn et al. 1978; Silverman 1979; Koenig et al. 1985, 1987, 1988), whereas another study indicates that ozone causes greater decrements in people with asthma (Kreit et al. 1989).

OZONE'S EFFECTS ON AIRWAY INFLAMMATION

When inhaled, ozone reacts with surface mucus and the airway epithelium. The consequence of such exposure is the appearance of inflammatory cells in the airways (reviewed by Bates 1995). These recruited cells are mainly polymorphonuclear (PMN) leukocytes (a subgroup of white blood cells). Inflammatory cell recruitment also involves activating immune cells and airway epithelial cells. When activated, these cells produce and release specific proteins, known as cytokines, that further stimulate the growth and activation of inflammatory cells (Mantovani et al. 1992). Cytokines upregulate the expression of adhesion molecules on the surfaces of vascular endothelial and inflammatory cells, and act as chemoattractants (called chemokines) to attract more inflammatory cells. The presence of adhesion molecules facilitates movement of PMNs from the blood into the tissue. Inflammatory cells also can adhere to the tissue's epithelial layer and produce highly reactive oxygen species that damage epithelial cells.

Analysis of bronchoalveolar lavage (BAL) fluid obtained by fiberoptic bronchoscopy has made it possible to characterize the inflammatory response in the lungs of patients with respiratory disease (reviewed by Russi and Crystal 1997) and in the lungs of normal volunteers exposed to air pollutants. Many components of the airway inflammation and tissue repair processes can be identified in lavage fluid. (Table 1 lists the components examined in the Balmes and Frampton studies.) For example, it has been established that BAL fluids obtained 1 to 24 hours after subjects were exposed to ozone contain higher than normal levels of a broad range of markers of inflammation, including inflammatory cells, cytokines, chemokines, fibronectin (a protein involved in tissue repair), lactate dehydrogenase (LDH, a marker of cell injury), and markers of epithelial layer permeability such as total protein, albumin, and immunoglobulin G (Koren et al. 1989; Devlin et al. 1991; Schelegle et al. 1991).

Researchers also have studied the time course for appearance of several markers of inflammation and cell damage in BAL fluid. For example, levels of interleukin 6 (IL-6) and prostaglandin E_2 (PGE_2) were reported to be higher one hour after exposure to ozone than 18 hours later; the reverse is true for fibronectin and tissue-plasminogen activator (Koren et al. 1991; Devlin et al. 1996). The early appearance

Table 1. Markers of Inflammation Analyzed in the Balmes and Frampton Studies

Marker	Function	Role in Inflammation
Intercellular adhesion molecule-1 (ICAM-1)	Adhesion molecule	Involved in inflammatory cell migration; facilitates attachment and spread of inflammatory cells on vascular epithelium; facilitates movement through endothelium
Vascular cell adhesion molecule (VCAM)	Adhesion molecule	Involved in inflammatory cell migration; facilitates attachment and spread of inflammatory cells on vascular epithelium; facilitates movement through endothelium
Interleukin 6 (IL-6)	Cytokine	A multifunctional cytokine that may contribute to inflammatory cell chemotaxis and persistence at sites of inflammation
Interleukin 8 (IL-8)	Cytokine	Stimulates growth and activation of inflammatory cells; upregulates expression of adhesion molecules on vascular endothelial and inflammatory cell surfaces; has potent neutrophil chemoattractant properties
Polymorphonuclear (PMN) leukocyte		
Neutrophil	Inflammatory cell	Granulocytic cell with phagocytic properties: destroys invading substances including bacteria; produces toxic oxygen species
Eosinophil	Inflammatory cell	Modulates allergic inflammatory response
Alveolar macrophage (AM)	Cell derived from blood monocyte	Phagocytic properties: destroys invading substances; produces toxic oxygen species
T Lymphocyte	Cell derived from lymphoid cell line	Kills virus-infected cells; regulates other white blood cells
Mast cell	Inflammatory cell	Key cell in allergic response
α_2-Macroglobulin	Protein found in serum	Antiprotease activity; interacts with certain cytokines and inflammatory cells
Transforming growth factor $\beta 2$ (TGF-β_2)	Growth factor found in serum	A protein produced in response to tissue injury; regulates cellular growth and differentiation
Fibronectin	Extracellular protein secreted by fibroblasts	Provisional matrix protein that participates in tissue repair and may lead to fibrosis
Lactate dehydrogenase (LDH)	Enzyme found in muscle tissue and serum	Marker of tissue damage; a measure of ozone-induced cell injury
Granulocyte–macrophage colony–stimulating factor (GM-CSF)	Cytokine	Activates PMNs
Integrins	Proteins that bind cells to extracellular matrix	Involved in inflammatory cell migration
Myeloperoxidase	Enzyme produced by neutrophils	Degrades peroxidase
Immunoglobulin M (IgM)	An immunoglobulin	One measure of exposure to an antigen

of IL-6 and PGE_2 in BAL fluid is significant because they are believed to recruit inflammatory cells into the airways. Because fibronectin can participate in tissue repair, its late elevation suggests a tissue response to injury. The inflammatory response to ozone appears to attenuate after continued exposure, as do lung function and symptom responses. Devlin and coworkers (1997) reported that the increases in several markers of inflammation seen in the BAL fluid of humans one hour after a two-hour exposure to 0.4 ppm ozone attenuated after five consecutive days of exposure. However, the BAL fluid levels of compounds indicative of cell damage (LDH, epithelial cells) or leakage of plasma components into lung air spaces (total protein, α_1-antitrypsin) remained elevated. The bulk of the evidence indicates that inflammation is an early response to ozone and that it persists for several hours after exposure ceases (Koren et al. 1991; Devlin et al. 1996). The impact of repeated episodes of ozone-induced inflammation on subsequent airway disease is an unanswered question.

CONDITIONS THAT MAY INFLUENCE THE AIRWAY'S RESPONSE TO OZONE

Preexisting Airway Inflammation

Chronic airway inflammation is characteristic of smoking and several respiratory illnesses, including asthma, chronic obstructive pulmonary disease, and cystic fibrosis. These are common disorders; for example, in the United States, asthma affects 14 to 15 million people; it is the most common chronic disease of childhood, affecting an estimated 4.8 million children (National Heart, Lung and Blood Institute 1997). Smoking is also associated with pulmonary inflammation (Mullen et al. 1986), and approximately 48 million Americans smoke cigarettes (Fiore et al. 1994). Because inflammation under these conditions is longstanding, there is concern that people who smoke or have lung diseases may respond differently to ozone exposure than healthy people; furthermore, repeated exposure to ozone could lead to permanent structural damage in the airways and more severe chronic lung disease.

Inflammatory cells infiltrate the bronchial mucosa and airways of people with asthma; eosinophils especially accumulate in the airways and are believed to play a major role in airway inflammation. During an allergic episode, other cells, especially mast cells and lymphocytes, also accumulate, and may play a role in the pathogenesis of the airway response (Lukacs et al. 1995). When activated, inflammatory cells release an array of cytokines that perpetuate inflammation. Continued inflammation, in turn, affects the reactivity of the bronchial smooth muscle cells.

Smokers differ from nonsmokers in the numbers of inflammatory cells obtained from BAL fluid and in the ability of those cells to produce oxidants damaging to the lungs. For example, when compared with healthy nonsmokers, alveolar macrophages (AMs) are more plentiful in the lavage fluid (Martin et al. 1985); more neutrophils are found in the alveolar walls, and AMs are more dense in the lung parenchyma (Finkelstein et al. 1995); PMNs and AMs release more reactive oxygen intermediates, including superoxide anion (Hubbard et al. 1987); and blood neutrophils release more superoxide anion (Rahman et al. 1996). These findings suggest that inflammatory cells are present and sequestered in the pulmonary microvasculature of smokers, and that they are primed to release oxidant species and may be triggered by inhaling reactive pollutants such as ozone.

Airway Hyperresponsiveness

Airway hyperresponsiveness is defined as a heightened tendency of the airways to constrict in response to irritant stimuli, viral infections, or chemical injuries. One way to test airway responsiveness is to expose subjects to an aerosol containing increasing concentrations of bronchoconstrictive agents such as methacholine. Individuals with hyperresponsive airways require significantly less methacholine aerosol to increase sRaw than individuals with normally responsive airways. By measuring the airway constriction in response to methacholine before and after ozone exposure, some studies have demonstrated that people and animals exposed to ozone also show increased hyperresponsiveness to the methacholine challenge (Seltzer et al. 1986; Tan and Bethel 1992; McFadden 1997).

The causes and contributing factors of airway hyperresponsiveness are not fully understood; however, airway inflammation is thought to play a role. Eosinophils release proteins that damage the airway epithelium, making it more responsive to stimulation and less responsive to a relaxing factor derived from the epithelium itself (Flavahan et al. 1988; Ohashi et al. 1992; Gleich and Adolphson 1993; Bousquet et al. 1994). Because underlying airway inflammation may play a role in hyperresponsiveness, one concern is that people with asthma and smokers (who are known to have preexisting airway inflammation) may also experience increased hyperresponsiveness after exposure to ozone.

JUSTIFICATION FOR THE STUDIES

The HEI Health Research Committee thought that the proposals from Drs. Balmes and Frampton were highly responsive to the goals of RFA 90-2. Both investigators proposed (1) to study populations who were responsive and

nonresponsive to ozone on the basis of changes in pulmonary function as measured by spirometry; (2) to study normal subjects from the general population and potentially sensitive subpopulations: people with asthma in the Balmes study and smokers in the Frampton study; and (3) to investigate the degree of the inflammatory response in normal and potentially sensitive populations. Changes in spirometry are a common measure of ozone sensitivity and only a few investigators (Koren et al. 1989; Schelegle et al. 1991) have attempted to correlate changes in FEV_1 and FVC with the degree of airway inflammation. The HEI Research Committee thought it was important to replicate the Koren and Schelegle studies in normal subjects and to extend the study population to include individuals with underlying airway inflammation. Both studies were expected to provide new information on ozone-induced inflammatory responses. The Balmes technique of separating proximal and distal airway lavage samples would evaluate whether ozone caused inflammation throughout the airways or in a specific region. The Frampton study was expected to provide new information about the effect of ozone on the production of toxic oxygen species. The collaborative study would compare markers of inflammation from smokers, people with asthma, and normal subjects; this comparison would provide an internal check of the results and allow the results from both studies to be extrapolated to a broader general population.

TECHNICAL EVALUATION OF PART I: AIRWAY INFLAMMATION AND RESPONSIVENESS TO OZONE IN NORMAL AND ASTHMATIC SUBJECTS

SPECIFIC AIMS AND ATTAINMENT OF STUDY OBJECTIVES

The specific aims of Dr. Balmes' study were to determine whether:

1. Airway responsiveness to any of a variety of chemical stimuli (e.g. methacholine) is correlated with sensitivity to ozone as measured by pulmonary function tests;
2. Short-term exposure to ozone induces inflammation in the proximal airways and distal regions of the lungs in subjects classified as sensitive or insensitive to ozone on the basis of their ozone-induced FEV_1 responses; and
3. Ozone-induced decrements in lung function predict the degree of inflammation in normal and asthmatic subjects.

The investigators attained their study objectives and obtained new information about the relation between ozone sensitivity and the degree of the inflammatory response in normal subjects and about differences in the ozone-induced inflammatory response between normal and asthmatic subjects.

STUDY DESIGN AND METHODS

This was a well-designed, rigorously controlled study that was divided into three phases. Phase I tested whether the degree of nonspecific airway responsiveness predicted the degree of ozone sensitivity in normal, nonsmoking men and women. In initial experiments, the investigators measured each subject's nonspecific airway responsiveness by administering increasing concentrations of a methacholine aerosol until a specific level of change in responsiveness was achieved. On later days, subjects were exposed in random order to either 0.2 ppm ozone or clean air for four hours while exercising moderately. The investigators separated the air and ozone exposures by at least three weeks to minimize the possible effects of one exposure on the other. They measured FEV_1, FVC, and sRaw before exposure (baseline), at hourly intervals during exposure, and at the end of each exposure. Balmes and coworkers analyzed their data to look for correlations between methacholine responsiveness prior to ozone exposure and ozone-induced FEV_1, FVC, and sRaw responses. They then determined each subject's ozone sensitivity on the basis of the degree of ozone-induced decreases in FEV_1. Based on the results of this test, they divided the subjects into tertiles and analyzed data from subjects in the upper (most-sensitive) and lower (least-sensitive) tertiles. Data from subjects in the middle tertile were not analyzed because the middle tertile of data often introduces random variations or "noise" into statistical analyses, and removing those data is a useful way to compare the extreme levels of responsiveness.

Phase II followed the same exposure protocol and division of subjects into tertiles as Phase I. It was designed (1) to determine if ozone-induced changes in lung function correlated with the appearance of markers of inflammation in lung lavage fluids or bronchial mucosal biopsy specimens obtained 18 hours after exposure, and (2) to compare the inflammatory responses in the airways of subjects classified as most-sensitive or least-sensitive to ozone. The investigators chose the 18-hour time point because previously published studies had demonstrated that subjects exposed to ozone showed an inflammatory response at this time (Koren et al.1989; Aris et al. 1993a,b). However, the time separation between measuring physiologic responses (during exposure) and levels of markers of inflammation (18 hours after exposure) makes it difficult to correlate these

two types of responses, and provides a single snapshot of responsiveness rather than information on the time course of the appearance or disappearance of each inflammatory marker.

The investigators measured markers of airway inflammation in lavage fluids obtained from the proximal airways, bronchus, and bronchoalveolar regions of the lungs. Proximal airway lavage (PAL) fluid was collected from the left main bronchus and the bronchial fraction lavage (BL) fluid and BAL fluid from the right middle lobe. They analyzed lavage fluids for levels of total protein, fibronectin, the potent neutrophil chemokine interleukin 8 (IL-8), and granulocyte–macrophage colony–stimulating factor (GM-CSF), a general activator of PMNs.

A unique aspect of this study was the evaluation of bronchial mucosal biopsy specimens to determine the effects of ozone on lung tissue (as opposed to the more common measurements of the constituents of lung fluids). The biopsy specimens were analyzed for integrins and cell adhesion molecules by immunohistochemistry, for neutrophils and eosinophils by morphometry, and for damage to the bronchial epithelium by electron microscopy. Initial technical difficulties limited the number of biopsy specimens available for analysis. Because the investigators could not always compare paired tissues from both air and ozone exposures for the same subject, the statistical power of this aspect of the study was limited. Also, because the investigators took tissues from only one site, sampling errors may have compromised their analyses of inflammatory cells. Another limitation was that the investigators performed immunohistochemical staining for cell surface molecules (e.g., integrins, adhesion molecules) using primary antibodies at only one dilution and without positive staining control cells for the antibodies. The absence of data from staining performed at lower dilutions and on positive control cells allows only limited interpretation of the immunohistochemistry data.

In Phase III, the investigators tested the hypothesis that exposure to ozone produces a greater inflammatory response in subjects with asthma than in normal control subjects. They compared the effects of ozone on lung function responses for 18 subjects with mild asthma with those obtained previously from the normal subjects who participated in Phases I and II. They also compared the levels of markers of inflammation in lavage fluids and bronchial mucosal biopsy specimens with those in normal subjects. The lavage fluid analyses included additional markers: myeloperoxidase (a product of neutrophils), transforming growth factor β2 (a protein involved in the repair of cell injury), and LDH (a measure of cell injury).

For statistical analyses, the investigators used parametric tests, such as two-tailed *t* tests, to compare the differences in physiologic responses (sRaw, FEV_1, and FVC) between the most-sensitive and least-sensitive subjects. They also appropriately used nonparametric methods, such as the Wilcoxon signed rank test (used to compare two groups via paired samples), and the Wilcoxon rank sum test (used to compare two groups from independent samples). For example, they used the signed rank test to compare (1) markers of inflammation in lavage fluids from each subject after air and ozone exposures, (2) symptom scores between normal and asthmatic subjects after exposure to air or ozone, (3) the physiologic responses in asthmatic subjects after exposure to air and ozone, and (4) the staining intensity of adhesion molecules, neutrophils, and eosinophils. They used the Wilcoxon rank sum test to compare (1) symptom responses between the most-sensitive and least-sensitive groups, (2) lavage fluid markers of inflammation between the most-sensitive and least-sensitive groups and between normal and asthmatic groups, and (3) the physiologic responses between normal and asthmatic subjects.

RESULTS AND INTERPRETATIONS

Phase I. Relationship of Nonspecific Airway Responsiveness to Ozone Sensitivity

As expected, the FEV_1 and FVC values for normal subjects decreased progressively from baseline during ozone exposure. However, the degree of responsiveness to methacholine challenge was not associated with the degree of ozone-induced decline in FEV_1 or FVC. Although the authors suggest that such a relationship may not have been detectable because there were too few subjects to encompass the full range of methacholine responsiveness, as discussed below, the lack of association agrees with Frampton's results. Balmes and coworkers did report a weak association between the degree of nonspecific airway responsiveness and ozone-induced increases in sRaw; however, the magnitude of the increase in sRaw was small and did not differ between groups of subjects defined as most-sensitive or least-sensitive to ozone. An association between nonspecific airway responsiveness and increased sRaw would suggest that people who are hyperresponsive to certain stimuli may experience a greater degree of airway constriction after exposure to ozone than normal people; however, it requires confirmation.

Subjects defined as most-sensitive to ozone on the basis of their FEV_1 response reported significantly more coughing and chest discomfort after ozone exposure than the least-sensitive group; both groups reported similar levels of wheezing. Because separate regression analyses indicated

that the association between symptoms and pulmonary function responses was weak, the authors proposed that exposure to ozone can produce symptoms without affecting lung function responses, or conversely, can cause decreases in lung function in the absence of symptoms.

Phase II. Effects of Ozone Exposure on Proximal Airways and Distal Lung in Normal Subjects

Lavage Fluid Analyses For each source of lavage fluids (PAL, BAL, and BL), the cellular and biochemical characteristics did not differ between the groups defined as most-sensitive and least-sensitive to ozone on the basis of the subjects' FEV_1 responses. Therefore, the data from both groups were combined to compare the results of air and ozone exposures. The results of these analyses are shown in Table 2. The investigators observed ozone-induced increases in several markers of inflammation compared with the levels after exposure to air. No statistically significant correlations were found among the ozone-induced increases in markers of inflammation and declines in FEV_1 and FVC; however, increased sRaw correlated significantly with ozone-induced increases in the percentage of neutrophils and amount of total protein in the BAL fluid. The increased number of neutrophils in the PAL fluid after ozone exposure supports the concept that the inflammatory response to ozone involves the proximal airways as well as the distal lung.

The lack of correlation between FEV_1 or FVC and inflammatory markers reported by Balmes may lie in the choice of markers for study, or in the fact that the measurements of these endpoints were widely separated in time. However, these findings agree with the conclusions of other researchers (Koren et al. 1989; Schelegle et al. 1991; Devlin et al. 1996). Bates (1995) suggested that the decrements in FEV_1 that occur immediately after ozone exposure involve a neurally mediated reduction in lung volume. This mechanism differs from that causing a cascade of events leading to inflammation. Therefore, it is not surprising that the investigators found no correlation between physiologic and inflammatory responses to ozone.

Table 2. Balmes Study: Lavage Fluid Analyses of Healthy and Asthmatic Subjects 18 Hours After Ozone Exposure[a]

Marker of Inflammation or Tissue Damage	Healthy Subjects (Phase II)	Asthmatic Subjects (Phase III)
PMNs	↑ BAL, BL, PAL	↑↑ BAL, ↑ BL, PAL
Total protein	↑ BAL, BL	↑↑ BAL, ↑ BL
IL-8	↑ BL	↑ BAL, BL
GM-CSF	↑ BAL	↑ BAL
Fibronectin	↑ BAL	↑ BAL
Myeloperoxidase	Not done	↑ BAL

[a] Compared with air exposure. A single arrow indicates a statistically significant change. Double arrows indicate a greater response than a single arrow.

Analyses of Bronchial Biopsy Specimens Table 3 lists the results of the analyses of bronchial tissues. Because the investigators initially experienced technical problems in obtaining bronchial biopsy specimens, only a limited number of samples from normal subjects were available for analysis. Electron microscopy of four of the six biopsy specimens from subjects exposed to ozone showed evidence of epithelial injury. Balmes found no effects of ozone exposure on the expression of vascular cell adhesion molecules (VCAM), E-selectin, and the integrins. Analyses of unpaired biopsy specimens (five from individuals exposed to air and four from subjects exposed to ozone) revealed a nonsignificant trend toward an elevation in the expression of intercellular adhesion molecule-1 (ICAM-1) after ozone exposure. Because the ICAM-1 data from the unpaired biopsy samples agree with the statistically significant elevation in ICAM-1 from three (of six) sets of paired biopsy specimens, the investigators concluded that ICAM-1 levels were elevated in endothelial tissue from the bronchial mucosa after ozone exposure. However, the degree of ICAM-1 upregulation and the consistency of this response are uncertain because increases were found in only half of the paired biopsy tissues and were not observed in the asthmatic subjects.

The investigators concluded that there were weak, nonsignificant trends toward increased numbers of neutrophils and eosinophils in the bronchial mucosa specimens isolated from normal subjects exposed to ozone. Although this result is consistent with their observation of increased levels of neutrophils in the lavage fluids and the apparent upregulation of ICAM-1 (which may recruit peripheral blood neutrophils to the airways), it should be interpreted cautiously. Examination of Figure 12 in the Investigators' Report indicates that the apparent increase in the mean numbers of neutrophils and eosinophils in the limited number of bronchial biopsy specimens was driven by outlier data points.

Summary of Phase II The overall results of Phase II indicate that sensitivity to ozone (as measured by spirometry) does not correlate with the magnitude of the inflammatory response; thus, individuals exposed to ozone who do not have changes in measurements of pulmonary function may still be at risk of respiratory tract inflammation.

Phase III. Effects of Ozone on Proximal Airways and Distal Lung in Subjects with Asthma

Lavage Fluid Analyses and Physiologic Responses The key results of analyzing lavage fluid from asthmatic subjects 18 hours after exposure to 0.2 ppm ozone are compared with those from normal subjects in Table 2. The magnitude of ozone-induced changes in FEV_1, FVC, and sRaw in subjects with asthma was similar to that seen in normal subjects. In addition, the ozone-induced increases in several markers of inflammation (IL-8, GM-CSF, and fibronectin) in lavage fluids were similar to those in normal subjects exposed to ozone. However, the percentage of neutrophils and the total protein content in the BAL fluid of asthmatic subjects both were elevated to a greater extent than in normal subjects, and these differences were statistically significant.

While the Balmes study was underway, Basha and coworkers (1994) reported increases in neutrophils, IL-6, and IL-8 in BAL fluid from asthmatic subjects 18 hours after exposure to 0.2 ppm ozone. These ozone-induced increases in inflammatory markers in asthmatic subjects were greater than those seen in normal subjects, but the effect of ozone on the pulmonary function responses (FEV_1, FVC, FEV_1:FVC, sRaw) did not differ between normal and asthmatic subjects. Based on analyses of lung lavage fluid, these two studies suggest that people with asthma may be at risk of developing a greater inflammatory response following exposure to ozone than normal people.

Analyses of Bronchial Biopsy Specimens In Phase III, the investigators resolved their technical problems with the biopsy procedure and obtained 15 paired biopsy specimens from asthmatic subjects. The results of the morphometric and immunohistochemistry studies are shown in Table 3. No effect of ozone was found on the levels of integrins or cell adhesion molecules. A larger number of eosinophils was found in biopsy specimens from asthmatic subjects (Figure 20 in the Investigators' Report) compared with normal subjects (Figure 12 in the Investigators' Report). This finding would be expected based on the role eosinophils play in asthma and increases confidence in the biopsy procedures. The investigators suggest that nonsignificant trends were seen toward increased levels of inflammatory cells in the bronchial mucosa of subjects exposed to ozone. However, as discussed above for the normal subjects, any

Table 3. Balmes Study: Analyses of Bronchial Biopsy Specimens from Subjects Exposed to Ozone[a]

Analysis	Result	Comment[b]
Normal Subjects		
Pathologic changes	Intercellular spaces expanded in bronchial epithelium	Changes observed in 4 of 6 paired specimens
Expression of cell adhesion molecules	ICAM-1 increased in blood vessel endothelium	Statistically significant changes in ICAM-1 observed in 3 of 6 paired specimens; nonsignificant trend in 4 unpaired specimens
	No changes in VCAM and E-selectin	
Expression of integrins	No changes	
Inflammatory cells	No changes	Of 11 unpaired specimens, 1 had elevated levels of PMNs and 3 had elevated levels of eosinophils
Asthmatic Subjects		
Pathologic changes	Not reported	
Expression of cell adhesion molecules	No changes in ICAM-1, VCAM, and E-selectin	
Expression of integrins	No changes	
Inflammatory cells	No changes	Of 17 unpaired specimens, 2 had elevated levels of PMNs and eosinophils

[a] Compared with air exposure. Only changes that were statistically significant are noted.

[b] Paired specimens are samples from the same subject after air exposure and after ozone exposure.

ozone-induced increase in the levels of neutrophils and eosinophils appears to be due to outlier data points (Figure 20 in the Investigators' Report). Because the investigators obtained biopsy specimens from the larger airways, the lack of an increase in inflammatory cells in these tissues following exposure to ozone does not necessarily reflect conditions existing at peripheral sites that may be more critical targets for ozone's effect, such as the bronchoalveolar junction.

TECHNICAL EVALUATION OF PART II: AIRWAY INFLAMMATION AND RESPONSIVENESS TO OZONE IN NONSMOKERS AND SMOKERS

SPECIFIC AIMS AND ATTAINMENT OF STUDY OBJECTIVES

The overall objective of Dr. Frampton's study was to determine whether individuals who differ in their sensitivity to ozone (as evaluated by their lung function responses) also differ in their susceptibility to ozone-induced airway inflammation. The investigators' original specific aims were to:

1. Examine the relationship between ozone-induced changes in pulmonary function and the appearance of markers of inflammation in lung lavage fluids from smokers and nonsmokers who were designated as "responsive" or "nonresponsive" to ozone based on the degree of their decrement in FEV_1 after ozone exposure.
2. Define how AMs respond to ozone, and characterize ozone's effect on the in vitro generation of toxic oxygen species (TOS) by alveolar cells isolated from smokers and nonsmokers who are responsive or nonresponsive to ozone.
3. Determine if exposing subjects (smokers and nonsmokers) repeatedly to the same ozone concentration produces the same symptom responses and decrements in FEV_1.
4. Determine whether the inflammatory markers found in BAL fluid are similar to those found in nasal lavage (NL) fluid from subjects exposed to ozone.

The investigators attained most of their original objectives. However, only 4 of 34 smokers tested were responsive to ozone and only two of these four completed the BAL portion of the protocol. Because of the difficulty in finding smoker-responders, the investigators, with HEI's approval, revised their original objective of analyzing data from smoker responders and smoker nonresponders separately (objective 1) and combined the two smoker responders with the smoker nonresponders.

STUDY DESIGN AND METHODS

The investigators recruited 74 nonsmokers and 72 smokers (men and women 18 to 40 years old) and measured their airway responsiveness to methacholine challenge. Subjects were classified as "responders" to ozone if exposure to 0.22 ppm ozone for four hours (combined with intermittent exercise) decreased their FEV_1 by 15% or more from baseline (measured just before ozone exposure began), or as "nonresponders" if their FEV_1 decreased by 5% or less. Subjects with intermediate decreases in FEV_1 were not included in the study. This method of selecting subjects, differed from that of Balmes in that it was prospective rather than retrospective. Frampton and colleagues determined the cutoff for FEV_1 at the outset, and then screened enough subjects to provide the predetermined number in each group.

The final study group consisted of 13 nonsmoker nonresponders, 12 nonsmoker responders, and 13 smokers, of whom 11 were nonresponders. The preponderance of nonsmokers was accounted for by the fact that more smokers than nonsmokers were noncompliant. No smokers dropped out because of their sensitivity to ozone or their respiratory symptoms; therefore, the possibility of significant selection bias seems unlikely. Table 1 of the Investigators' Report compares recruitment and participation rates for smokers and nonsmokers.

Following the classification exposure, subjects were exposed twice to ozone and once to clean air, with each exposure accompanied by exercise. Although classification as responders or nonresponders to ozone was based on decline in FEV_1, the investigators also measured FVC and forced expiratory flow between 25% and 75% of FVC (FEF_{25-75}) immediately before exposure, and after two and four hours of exposure. One group of ozone responders was tested for pulmonary function responses at 18 hours after ozone exposure. Pulmonary function data were analyzed by a repeated measures analysis of variance.

Nasal lavage and BAL were performed immediately after one ozone exposure and 18 hours after the other. As in the Balmes study, the investigators modified their BAL procedure to separate the components of the proximal and distal airways into the bronchial lavage fraction (BL), which is thought to reflect the proximal airways primarily (Rennard et al. 1990) and which would correspond closely to Balmes' bronchial fraction, and the alveolar lavage (AL) fraction, which reflects the distal airways and would correspond with the BAL fraction in the Balmes study. The investigators analyzed lavage fluids for inflammatory cells, cytokines IL-6 and IL-8, total protein, albumin, immunoglobulin M (IgM), and α_2-macroglobulin (a2M, which is thought to suppress

macrophage activation). They tested the total cell population in the BAL fluid for its ability to produce superoxide anion (a toxic form of oxygen that can damage cell membranes and decrease the barrier function of vascular membranes), and AMs specifically for their production of the total population of TOS.

Overall, the study was well designed and measurements were done carefully with state-of-the-art facilities and procedures. An important feature of the study is that it is one of few studies that report sequential pulmonary function and lavage data on the same subjects following exposure to ozone. The investigators considered that a three-week interval between ozone exposures would be enough time for the airways to revert to their original condition.

RESULTS AND INTERPRETATION

Physiologic and Inflammatory Responses

The key physiologic, cellular, and biochemical responses following exposure of smokers and nonsmokers to ozone are listed in Table 4. Dr. Frampton and associates reported that the lung function responses to ozone were reproducible within each subject and that the degree of nonspecific airway responsiveness (before ozone exposure) was not predictive of changes in FEV_1. Measurements of lung function in subjects who were responsive to ozone returned to preexposure levels by 18 hours after exposure. Frampton's finding that smokers showed smaller decrements in lung function than nonsmokers immediately after ozone exposure is similar to the results of Kerr and colleagues (1975), but different from those of Hazucha and coworkers (1973), who reported greater decrements in FEV_1 in smokers than in nonsmokers. In the Frampton study, smokers reported fewer ozone-induced respiratory symptoms (cough, sputum production, dyspnea, wheeze, chest tightness, chest pain, and throat irritation) than nonsmokers. The Frampton results confirm and extend other reports of airway inflammation in normal subjects exposed to ozone (the Balmes Investigators' Report, Part I of this Research Report; see also Seltzer et al. 1986; Koren et al. 1989, 1991; Schelegle et al. 1991; Devlin et al. 1991). New findings were that the degree of the ozone-induced airway inflammatory response in the lower respiratory tract was independent of both smoking status and airway responsiveness as measured by spirometry, and that lymphocytes, eosinophils (in nonsmokers), and mast cells, in addition to PMNs, were elevated after ozone exposure.

The time course of the appearance of cytokines in lavage fluids of subjects exposed to ozone differed from that of the inflammatory cells (Table 4). The levels of IL-6 and IL-8 increased immediately after ozone exposure, whereas the levels of PMNs were highest 18 hours later, thus supporting

Table 4. Frampton Study: Physiologic, Cellular, and Biochemical Responses to Ozone in Smokers and Nonsmokers[a]

Analysis	Nonsmokers		Smokers	
	Immediately After Exposure	18 Hours After Exposure	Immediately After Exposure	18 Hours After Exposure
Lung Function Tests				
FEV_1, FVC, FEF_{25-75}	↓↓	NSD[b]	↓	NSD[b]
Lavage Fluid Analyses				
PMNs	NSD	↑↑	NSD	↑↑
Lymphocytes	NSD	↑	NSD	↑
Eosinophils	NSD	↑	NSD	NSD
Mast cells	↑	NSD	↑	NSD
AMs	↓	NSD	↓	NSD
IL-6	↑↑	↑	↑↑	↑
Total protein, albumin, IgM	NSD	↑	NSD	↑
a2M	NSD	↑	NSD	↑

[a] Compared with air exposure. Arrows indicate statistically significant changes. Double arrows indicate a greater response. NSD indicates no significant difference from air exposure.

[b] Lung function tests at this time point were performed only in ozone responders.

the theory that these cytokines are involved in the recruitment and activation of PMNs at sites of inflammation. Total protein, albumin, and IgM levels in the BAL fluid were higher 18 hours after ozone exposure than one hour after exposure, indicating that ozone exposure progressively increased vascular permeability. The fact that the decrements in FEV_1 and FVC observed immediately after ozone exposure returned toward normal values 18 hours later (when PMNs and the plasma proteins in the BAL fluid were at their highest levels) indicates that these measures of pulmonary function do not correlate with the inflammatory response in the lower respiratory tract.

Production of Toxic Oxygen Species

Production of superoxide anion by cells in the BAL fluid from nonsmokers (both responders and nonresponders) 18 hours after ozone exposure was higher than after air exposure. Overall, superoxide anion production was greater in smokers than in nonsmokers immediately and 18 hours after exposure to ozone. The immediate production of superoxide anion by smokers may be due to AMs in the BAL fluid that are chronically activated by exposure to cigarette smoke.

When Frampton and colleagues examined the production of the total TOS by AMs in the BAL fluid after air and ozone exposures, they found that smokers and nonsmokers responded differently. In smokers, TOS production by AMs progressively increased during the 18 hours after ozone exposure; TOS production by cells from nonsmokers decreased during the same time period. Although neither result was statistically significant, together they suggest that the levels of TOS after ozone exposure may be greater in the lungs of smokers than nonsmokers.

Nasal Lavage

The recovery of cells by nasal lavage was variable under all exposure conditions. For example, mean recovery of PMNs from smokers and nonsmokers depended on the timing of nasal lavage (immediately after or 18 hours after exposure), and was independent of ozone exposure. No statistically significant differences between smokers and nonsmokers, no significant effects of ozone exposure on PMN recovery, and no significant correlations between PMNs in nasal and BAL fluids were noted. Thus, in contrast to Graham and Koren (1990), the results of this study suggest that nasal lavage is not a useful predictor of how the lower airways respond to ozone.

TECHNICAL EVALUATION OF PART III: MEDIATORS OF INFLAMMATION IN BRONCHOALVEOLAR LAVAGE FLUID FROM NONSMOKERS, SMOKERS, AND ASTHMATIC SUBJECTS EXPOSED TO OZONE: A COLLABORATIVE STUDY

INTRODUCTION

Because Drs. Balmes and Frampton used similar ozone exposure protocols and examined lavage fluids from proximal and distal airways, they collaborated to examine further the inflammatory response of the airways after exposure to ozone. The overall goal was to analyze lavage fluids from both studies in one laboratory, thereby ensuring that all specimens would be examined by uniform methods.

SPECIFIC AIMS

The collaborative study had three objectives:

1. Compare levels of three markers of inflammation (IL-6, IL-8, and a2M) in lavage samples from normal subjects after exposure to air or ozone in both studies.
2. For lavage samples from the Balmes study, determine whether the ozone-induced increases in these three markers of inflammation were greater in the PAL fluid (which does not sample the lower airways) than in the BL fluid or in the BAL fluid from the distal airways. The investigators hypothesized that if airway epithelial cells were the primary source of the increase in markers of inflammation after ozone exposure, the increases would be greater in the PAL fluid than in the BL or BAL fluids.
3. Compare ozone-induced changes in the levels of markers of inflammation among all groups in the two studies: normal subjects, smokers, and subjects with asthma.

STUDY DESIGN AND METHODS

Because Balmes and coworkers examined lavage fluids at only one time point (18 hours) after exposure, all analyses for the Collaborative Study were performed on samples obtained 18 hours after air or ozone exposures. Dr. Balmes shipped lavage fluids on dry ice to Dr. Frampton's laboratory, where the analyses for IL-6, IL-8, and a2M were performed.

Statistical analyses were performed with two objectives: First, a crossover *t* test was used to compare the levels of the three markers of inflammation in the PAL, BL, and BAL

fluids from subjects after exposure to air with those after exposure to ozone in the Balmes study. This analysis also allowed the investigators to determine whether the analytical results were influenced by period or interaction effects. A significant period effect would indicate that responses to exposure varied with time (time of day, time between exposure and lavage), or season (of year) regardless of whether the exposure was to air or ozone. A significant interaction effect would indicate that the first exposure may have altered the results of the second exposure by the effects of the first exposure being carried over during the time interval between exposures. Second, the investigators used analyses of variance to determine whether the effects of ozone exposure on the levels of inflammatory markers differed among the four groups of subjects in the two studies: nonsmokers and smokers in the Frampton study, and normal and asthmatic subjects in the Balmes study.

RESULTS AND INTERPRETATION

Normal Subjects from the Balmes Study

Additional analyses of the levels of inflammatory mediators (IL-6, IL-8, and a2M) in lavage fluids from the normal and asthmatic subjects in the Balmes study are shown in Table 5. All three markers of inflammation were found in the PAL fluid samples isolated from subjects 18 hours after air exposure, which suggests that epithelial cells (whose products predominate in the PAL fluid) either (1) produce these components constitutively, or (2) were stimulated by irritation caused by bronchoscopic manipulation to produce these compounds. Eighteen hours after ozone exposure, the levels of IL-6 were elevated in the PAL, BL, and BAL fluids from Dr. Balmes' subjects. A crossover *t* test indicated a significant interaction effect for the measurements of IL-6 in PAL but not for the analyses of BL and BAL fluids.

In the collaborative study, no statistically significant effects of ozone exposure were found on IL-8 levels in lavage fluids from Dr. Balmes' normal subjects 18 hours after exposure to ozone. This finding differs from that reported for the bronchial fraction by Balmes in Part I of this Research Report (see Table 7 of Part I). The apparently discrepant result is probably due to the different procedures used for data analysis (paired analysis of ozone versus air in Part I and crossover *t* test in Part III). Statistical analysis of IL-8 levels in BL and BAL fluids after air or ozone exposure indicated a possible interaction effect, which prompted the investigators to further analyze their data for the BL fluid. These analyses revealed that IL-8 levels in BL fluid after air exposure were higher when it was the second exposure (following ozone as the first exposure). When the investigators compared IL-8 levels in BL fluids from subjects exposed to ozone first with those from subjects exposed to air first, they detected an ozone-induced elevation in IL-8. Although these analyses were performed on different subjects, the results suggest that ozone exposure may

Table 5. Collaborative Study: Additional Comparisons of Inflammatory Markers in Lavage Fluids Obtained 18 Hours After Ozone Exposure from Subjects in the Balmes Study[a]

Marker of Inflammation	Proximal Airway Lavage	Bronchial Fraction of Lavage	Bronchoalveolar Lavage
Normal Subjects			
IL-6	↑; Interaction effect[b]	↑	↑
IL-8	No changes	No change; interaction effect; further analysis suggested an increase	No change; interaction effect; further analysis not done
a2M	↑; Period effect[c]	↑	↑
Asthmatic Subjects			
IL-6	No changes	↑	↑
IL-8	No changes	↑	↑
a2M	No changes	↑	↑

[a] Compared with air exposure. Samples were analyzed in Dr. Frampton's laboratory. An arrow indicates a statistically significant change. All effects noted were statistically significant.

[b] A significant interaction effect indicates that the first exposure may have altered the results of the next exposure; a carry-over effect.

[c] A significant period effect indicates that responses to exposure varied with time (time of day, time between exposure and lavage), or season (of year) independent of whether the exposure was to ozone or air.

have caused an increase in IL-8 in the BL fluid and that interaction effects caused by the first bronchoscopy obscured ozone-induced increases in IL-8 when data from both bronchoscopic procedures (after air and ozone) were compared. The investigators concluded that the first bronchoscopic procedure produced a persistent, but undefined, effect on IL-8 release by airway epithelial cells that obscured the difference between ozone and air exposures. Although the investigators did not expect that changes in IL-8 production following bronchoscopy would persist for three weeks, it is possible that some component of the overall study procedure, which included multiple lavages and bronchial biopsies, sensitized the epithelium resulting in a greater release of IL-8 following the second bronchoscopic procedure.

Ozone exposure caused an increase in the levels of a2M in all lavage fluids. Statistical analysis of the data from the PAL fluid showed a significant period effect, indicating that the timing of the exposures may have influenced the measurements. The ozone-induced elevated levels of a2M in the BL and BAL fluids were not influenced by period effects.

Asthmatic Subjects from the Balmes Study

As illustrated in Table 5, IL-6, IL-8, and a2M were elevated in BL and BAL fluids from asthmatic subjects in the Balmes study after exposure to ozone. (No statistically significant differences were found between the PAL fluids after air and ozone exposures.) No significant period or interaction effects were observed for the asthmatic subjects, possibly due to the longer mean interval between air and ozone exposures for asthmatic subjects (85 days) than for normal subjects (50 days).

Comparison of Subject Groups from the Balmes and Frampton Studies

Eighteen hours after exposure, the ozone-induced increases in levels of IL-6, IL-8, and a2M were similar in AL and BL fluids in smokers and nonsmokers (Frampton study) and in the BL and BAL fluids from normal subjects and asthmatic subjects (Balmes study). Because the protocol for the Balmes study did not include a lavage procedure four hours after exposure, it was not possible to make comparisons at the time point when the cytokines were at their highest level in the Frampton study.

Conclusions

Five conclusions can be drawn from the collaborative study: (1) The increased levels of IL-6, IL-8, and a2M in lavage fluids obtained 18 hours after ozone exposure by two groups of investigators using similar exposure protocols suggests that the results are representative of populations with the same general characteristics as these subjects. (2) The source of the cytokines (IL-6 and IL-8) is not limited to the proximal conducting airways because the levels of IL-6 and IL-8 increased in the BL and BAL fluids. (3) The ozone-induced increases in a2M being greater in the BL and BAL fluids than in the PAL fluid suggests that the source of a2M may be the distal, rather than the proximal, airways. (4) No statistically significant differences were found among normal subjects, smokers, and asthmatic subjects in the increases in three markers of inflammation (IL-6, IL-8, and a2M) in lavage fluid 18 hours after exposure to ozone. (5) The effects of bronchoscopy combined with lavage and endobronchial biopsy may persist for longer than three weeks in some subjects. The possibility of carryover effects has critical implications for studies involving repeated bronchoscopic procedures.

DISCUSSION OF THE STUDIES BY BALMES AND FRAMPTON

It has been well established that, for some people, inhaling ozone can lead to short-term decrements in some pulmonary function tests such as FEV_1 and FVC and to bronchopulmonary inflammation. Drs. Balmes and Frampton and their collaborators investigated the effects of ozone on the airways of people with asthma and people who smoke and compared these results with observations of normal nonsmoker subjects. Neither investigator found an association between the degree of a subject's nonspecific airway responsiveness (measured before exposure to ozone) and his or her sensitivity to ozone as measured by decrements in FEV_1. This result agrees with that of McDonnell and coworkers (1987) but differs from the results of Hackney and colleagues (1989) and Kreit and coworkers (1989).

The results of both studies highlight the fact that people who experience little or no effect of ozone on their FEV_1 measurements (the least-sensitive subjects in the Balmes study and nonresponders in the Frampton study) can nevertheless experience airway inflammation following exposure to ozone. Balmes found no association between the degree of ozone-induced decrements in pulmonary function (as assessed by FEV_1 or FVC) measured during and immediately after ozone exposure and the levels of various markers of inflammation in lavage fluids collected 18 hours later. (Balmes did not assess pulmonary function at the same time that he collected the lavage fluid.) Frampton and associates found that early decrements in pulmonary function returned toward normal 18 hours after ozone exposure when PMNs and plasma proteins in lavage fluids were at

their highest levels. Thus, the inflammatory response to ozone persisted at a time when decrements in lung function were resolving. The results of both studies suggest that ozone-induced changes in FEV_1 or FVC do not reflect the degree of the airway inflammatory response, and confirm the conclusions of other investigators (Koren et al. 1989; Schelegle et al. 1991; Devlin et al. 1996). Balmes found that ozone-induced increases in sRaw were correlated with elevations in the levels of neutrophils and total protein in the BAL fluid 18 hours after ozone exposure. These findings agree with those of Devlin and colleagues (1996), who also reported a correlation between increases in sRaw and increased inflammatory cells in the BAL fluid of subjects after exposure to 0.4 ppm ozone. Both Balmes and Frampton separated lavage fluids into fractions representing the proximal and distal airways. These techniques provided evidence that the inflammatory response elicited by ozone appeared in both regions of the airways and was not limited to the conducting airways.

Both Balmes and Frampton compared the ozone-induced responses of potentially susceptible populations with those of normal subjects. Balmes found that exposure to ozone did not cause a greater incidence of respiratory symptoms or larger decrements in spirometric tests of lung function in subjects with asthma than in normal subjects. Frampton reported that the smoker group as a whole (both responders and nonresponders) actually experienced smaller decrements in lung function and fewer respiratory symptoms in response to ozone than the nonsmoker group. This finding must be viewed in light of the difficulty the investigators had in finding smokers who were responsive to ozone. This lack of ozone responsiveness may lie, as the authors propose, in smokers being a self-selected group that is relatively tolerant to the effects of exposure to both cigarette smoke and ozone. Alternatively, tolerance to ozone could be conferred by the protective effect of increased mucous production by smokers (Fiore et al. 1994). The ozone-induced increases in PMNs from smokers were similar to those from nonsmokers. In contrast, the ozone-induced increases in certain markers of inflammation were higher in asthmatic subjects. Eighteen hours after exposure to ozone, people with asthma had more neutrophils and protein in their BAL fluids than normal subjects. Although the latter results are intriguing, they need to be placed in the context of the other data on airway inflammation. Differences between normal and asthmatic subjects were not seen for IL-8, GM-CSF, and fibronectin in the BAL fluid, nor were the levels of any marker different in the BL or PAL fluids. Moreover, although the baseline inflammatory response was generally higher in the mucosal bronchial specimens from asthmatic subjects than normal subjects, ozone exposure affected samples from both groups to the same extent.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

These and other studies discussed in the Scientific Background section indicate that short-term (1- to 6-hour) exposures of healthy humans to ozone causes the appearance of markers of inflammation in lung fluids, and that this response is not related to the magnitude of changes in commonly used spirometric tests of lung volume such as FEV_1 and FVC. However, some researchers (Weinmann et al. 1995a,b; Künzli et al. 1997) have focused on the relationship of ozone with middle or end expiratory flow measurements, FEF_{25-75} and FEF_{75}. These measures are thought to be more sensitive than FEV_1 to changes in the small airways, which are target sites for ozone deposition and damage (reviewed in U.S. Environmental Protection Agency 1996a).

In agreement with Frampton's results, Weinmann and coworkers (1995a,b) reported a reduction in isovolumetric FEF_{25-75} suggestive of small airway dysfunction in normal subjects immediately following exposure to 0.35 ppm ozone for 130 minutes. Also, the reduction in FEF_{25-75} correlated closely with the appearance of fibrinogen (a marker of vascular permeability and tissue damage) in the BAL fluid. Additional research is warranted to determine if this test reflects ozone-induced airway inflammation better than FEV_1 or FVC.

The time course of the inflammatory response following ozone exposure also needs further evaluation. We need additional studies to determine if inflammation attenuates upon repeated exposure to ozone, as do some changes in pulmonary function, and whether inflammation and cell injury attenuate differently (Devlin et al. 1997). More information also is needed on whether structural or other changes occur in the upper and lower respiratory tracts of humans as a consequence of prolonged or repeated inflammatory events. The combined effects of ozone and other pollutants (such as particulate matter) on inflammation is another important unresolved issue because multiple pollutants usually coexist in ambient environments.

Noninvasive methods are needed to evaluate markers of inflammation in the airways of ozone-exposed subjects. Many potential volunteers are unwilling to undergo bronchoscopy, and multiple bronchoscopic procedures in the same subjects are impractical over a short time period (e.g., 18 hours). Other possible approaches include analyzing nasal lavage fluid or sputum. It is not clear whether the differences in the results for the nasal lavage fluid between Frampton's study and the study by Graham and Koren (1990) are due to the different exposure protocols of these

two studies. Many investigators have identified markers of inflammation in sputum from asthmatic subjects (Frigas et al. 1981; Gibson et al. 1989; Pin et al. 1992a,b; Virchow Jr. et al. 1992; Kurashima et al. 1996; Twadell et al. 1996; Diamant et al. 1997; Keatings and Burnes 1997). However, the effects of air pollutants on markers of inflammation in sputum have not received much attention. Because sputum contains bronchial secretions produced by the epithelium and submucosal glands in the bronchi and bronchioles (sites of inflammation in asthma), it may be a better source of inflammatory markers than the BAL fluid, which includes alveolar contents (Hansel and Walker 1992). Analyzing naturally occurring sputum, or that induced by inhaling aerosolized hypertonic saline solutions, may be a promising noninvasive method of obtaining samples for measuring lower airway inflammation. Another potential noninvasive test for inflammation is measuring nitric oxide in exhaled air. Nitric oxide is elevated in the exhaled air of children and adults with asthma. It has been monitored to study the relation between inflammation and airway disease (Lundberg et al. 1997) and may prove useful for studying the effects of air pollution on airway inflammation.

Most studies of ozone's effect on people with asthma have been carried out on subjects with mild asthma. Ideally, one would want to determine the degree of airway inflammation after inhaling ozone in patients with severe asthma or obstructive lung disease. However, such studies would be difficult to conduct and may not be approved by institutional review boards on ethical grounds.

Finally, the results of the Balmes and Frampton studies suggest that although standard spirometric tests of changes in lung volume will continue to be useful for characterizing the effects of ozone exposure, more attention needs to be paid to other measurements that are indicators of airway inflammation. Such measurements, including FEF_{25-75}, sRaw, and markers associated with the inflammatory response, should be conducted in normal subjects as well as in populations with underlying airway inflammation.

CONCLUSIONS

This report describes the results of two independent studies that were designed to evaluate the effects of short-term exposure to ozone on pulmonary function and the airway inflammatory response in populations with preexisting airway inflammation (smokers and people with asthma). A second goal was to see if pulmonary function changes correlate with the degree of inflammation and to compare ozone-induced changes in pulmonary function and airway inflammation in individuals identified as responsive or nonresponsive to ozone.

The principal investigators of the two studies reported here (Drs. Balmes and Frampton) examined pulmonary function and inflammatory markers in healthy subjects following a four-hour exposure to ozone (0.20 or 0.22 ppm respectively) combined with intermittent exercise. Both investigators classified their subjects as sensitive or insensitive to ozone based on the magnitude of their ozone-induced changes in FEV_1. In addition, Dr. Balmes investigated people with asthma and Dr. Frampton examined smokers.

The results of the Balmes and Frampton studies generally agree with the results of earlier studies, and add to the body of literature by (1) providing more extensive analyses of temporal changes in markers of airway inflammation after ozone exposure, (2) analyzing a broad array of markers of inflammation, and (3) comparing the responses of two potentially sensitive populations with underlying airway inflammation undergoing similar ozone exposure regimens.

The pulmonary function observations confirm and extend the findings of earlier clinical and field studies.

- A four-hour exposure to a level of ozone that occurs in ambient settings results in reversible decrements in FEV_1 and FVC and small increases in sRaw in some people.
- Marked interindividual differences occur in ozone-induced pulmonary function changes ranging from no response to a 40% decrease in FEV_1 or FVC.

Inflammation is a complex process involving a cascade of time-dependent changes in cytokines, inflammatory cells, and permeability. Under the conditions of the Balmes and Frampton studies:

- Ozone caused airway inflammation throughout the respiratory tract in all three subject groups (normal subjects, subjects with asthma, and smokers) tested.
- A variety of markers of inflammation increased in lung fluids after ozone exposure; early increases in specific cytokines may be important for recruiting inflammatory cells to the lungs.
- Inflammatory changes in nasal lavage fluid did not mirror changes in lung lavage fluid.
- Analysis of lung lavage fluid from subjects undergoing multiple bronchoscopies suggested the effects of the procedure can last for more than three weeks.

The degree of inflammation (whether caused by ozone or other factors) that may cause permanent changes in lung structure is not yet known; therefore, the significance of the ozone-induced inflammatory response in terms of subsequent airway disease remains to be determined.

When Balmes and Frampton examined the relation between airway inflammation and pulmonary function in

healthy subjects exposed to ozone as well as in subjects with underlying airway inflammation (smokers and people with asthma), they found:

- No correlation among ozone-induced respiratory symptoms, certain spirometric tests of lung function (FEV_1 and FVC), and markers of airway inflammation. However, some markers of inflammation found in lavage fluid 18 hours after ozone exposure were correlated with ozone-induced increases in sRaw.
- No marked differences between asthmatic and healthy subjects were found in respiratory symptoms and lung function responses to ozone. However, ozone may cause a more intense inflammatory response (as judged by the presence of inflammatory markers in lavage fluids) in the airways of people with asthma than in normal subjects.
- Smokers showed smaller decrements in lung function after ozone exposure than nonsmokers; however, the potential for damage to lung tissue by reactive oxygen species after ozone exposure may be greater in smokers than nonsmokers.

These results suggest that symptoms and spirometry may not be sufficient to evaluate the potential risks associated with ozone exposure. Despite the technical demands, more attention needs to be directed toward assessing small airway function and to developing noninvasive measurements of airway inflammation.

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ABBREVIATIONS

AL	alveolar lavage
AM	alveolar macrophage
a2M	α_2-macroglobulin
BAL	bronchoalveolar lavage
BL	bronchial fraction of lavage
FEF_{25-75}	forced expiratory flow between 25% and 75% of the vital capacity
FEV_1	forced expiratory volume in one second
FVC	forced vital capacity
GM-CSF	granulocyte–macrophage colony–stimulating factor
ICAM-1	intercellular adhesion molecule-1
IgM	immunoglobulin M
IL-6, IL-8	interleukins 6 and 8
LDH	lactate dehydrogenase
NAAQS	National Ambient Air Quality Standard
NL	nasal lavage
PAL	proximal airway lavage
PGE_2	prostaglandin E_2
PMN	polymorphonuclear (leukocytes)
sGaw	specific airway conductance
sRaw	specific airway resistance
TOS	toxic oxygen species
VCAM	vascular cell adhesion molecule

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