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## **Effects of Combined Ozone and Air Pollution Particle Exposure in Mice**

Lester Kobzik, Carroll-Ann W Goldsmith, Yao Yu Ning,  
Guozhong Qin, Bill Morgan, Amy Imrich, Joy Lawrence,  
GG Krishna Murthy, and Paul J Catalano



# HEI STATEMENT

Synopsis of Research Report 106

## Effects on Mice of Exposure to Ozone and Ambient Particle Pollution

### INTRODUCTION

Epidemiologic studies have indicated that small short-term increases in the concentration of particulate matter (PM), the complex and variable mixture of particles in the atmosphere, are associated with short-term increases in human morbidity and mortality. Particularly at risk are elderly people and individuals with compromised cardiac or airway function, such as those with asthma.

Plausible biologic mechanisms that would link low-level PM exposure with pathophysiologic effects or would explain why people with asthma may be more sensitive than healthy individuals to PM exposure have not yet been established. Because PM is present with other airborne pollutants, it is also important to evaluate the health effects of simultaneous exposure to multiple pollutants.

### APPROACH

Dr Lester Kobzik and colleagues at Harvard School of Public Health used a mouse model of asthma to evaluate how inhaling pollutants affects the airways. The mice were sensitized to the allergen ovalbumin and later challenged with the same aerosol to induce a lung condition in the mice similar to that found in people with asthma. This mouse model of asthma has some appropriate characteristics of human asthma (most notably, allergen-induced airway inflammation), but lacks others (eg, hypersecretion of mucus).

The investigators studied concentrated ambient particles (CAPs) of respirable size and ozone, a gaseous pollutant known to cause airway inflammation and to compromise respiratory function. Kobzik hypothesized that exposure to CAPs plus ozone would cause a synergistic (or greater-than-additive) response. To maximize possible CAPs effects, he used a new version of the Harvard Ambient Particle Concentrator to deliver particles at concentrations that were many times higher than those in ambient Boston air (varying from 12-fold to 67-fold on different days of the study). He exposed mice to ozone at 0.3 ppm, a concentration

similar to levels found in urban ambient air. Kobzik also measured daily levels of elements in the PM and CAPs so that, if he found a change in airway function, he could associate it with the elemental composition of the particles.

Kobzik and colleagues evaluated two endpoints associated with the asthmatic response: airway responsiveness and airway inflammation. They evaluated airway responsiveness by challenging the mice with various aerosol concentrations of methacholine, a bronchoconstrictor or agent that narrows the small airways. Mice were challenged with the methacholine aerosol immediately after a 5-hour exposure to one or both pollutants (CAPs and ozone). In some experiments, airway responsiveness was also measured beginning 24 hours after the exposure.

One valuable aspect of the investigators' approach was that they used whole-body plethysmography to measure airway responsiveness. This technique is noninvasive and allows a large number of animals to be tested repeatedly. They placed a mouse in an exposure chamber that was connected to a reference chamber; as the mouse breathed the methacholine entering the exposure chamber, the difference in pressure between the two chambers was measured. From these pressure readings, they derived a recently defined parameter known as enhanced (enh) pause (P), or Penh.

They also evaluated the effects of the pollutants on airway inflammation 24 or 48 hours after the exposure by assessing cells in bronchoalveolar lavage fluid to determine whether (1) the total number of cells increased, and (2) the numbers of cells associated with an inflammatory response, such as eosinophils, increased.

In addition, Kobzik and colleagues evaluated *in vitro* how concentrated particles might induce the synthesis of cellular mediators associated with inflammation. They did this by resuspending particles collected on different days of the *in vivo* exposure study, adding them to lung cells (derived from rats), and measuring

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24 hours later the levels of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and macrophage inflammatory protein-2 (MIP-2) produced by the cells.

### RESULTS AND INTERPRETATION

Kobzik and colleagues used four statistical approaches to evaluate the effects of pollutants on Penh. One approach indicated that CAPs (without ozone) slightly increased Penh immediately after exposure, but values returned to baseline by 24 hours later. This suggests that the particles' effect on Penh is small and transient, which is consistent with the effects of various air pollutant components on airway function that have been described in other studies. Ozone alone did not increase Penh after exposure. Only one of the four statistical approaches suggested a synergistic effect of CAPs plus ozone, and the investigators were appropriately cautious in interpreting this finding.

Another approach—factor analysis—suggested that different elemental components of the CAPs were associated with different effects on Penh; in particular, that Penh increased on days with high aluminum and silicate levels in the particles. Some particle components also appeared to be associated with decreased Penh. These findings support the idea that the magnitude of a specific health outcome on a particular day is a function

of the aggregate elemental composition and concentration of pollutants in ambient air on that day.

In vivo, CAPs had little or no effect on the numbers of cells in bronchoalveolar lavage fluid 24 or 48 hours after exposure; ozone slightly increased cell numbers in a pattern consistent with inflammation, which was to be expected. In vitro, resuspended CAPs induced the synthesis of high levels (frequently more than 1000-fold increases above background levels) of TNF- $\alpha$  and MIP-2 from rat lung cells. These results confirm previous findings from the investigators that suggest that components present in resuspended particles can induce the release of mediators associated with lung inflammation.

Overall, the results indicate that respirable PM, even when concentrated to levels higher than normally found in ambient air, had little effect on the airway mechanical and inflammatory parameters measured in this small-animal model of asthma. In addition, the effects of exposure to CAPs plus ozone did not achieve synergy that could be convincingly demonstrated. Because this mouse model mimics only some characteristics of human asthma, which is a complex illness, we cannot be certain to what extent these results may or may not predict the effects of ambient PM and ozone exposure on people with asthma.



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### Effects of Combined Ozone and Air Pollution Particle Exposure in Mice

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*Departments of Environmental Health in the Physiology Program, Biostatistical Science, and Biostatistics, Harvard School of Public Health, Boston, Massachusetts; Dana-Farber Cancer Institute, Boston, Massachusetts*

#### HEI STATEMENT

This Statement is a nontechnical summary of the Investigators' Report and the Health Review Committee's Commentary.

#### PREFACE

The Preface describes the general regulatory and scientific background for the HEI Research Program that produced this and other reports on related topics.

#### INVESTIGATORS' REPORT

When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three outside technical reviewers and a biostatistician. The Report and the reviewers' comments are then evaluated by members of the HEI Health Review Committee, who had no role in selecting or managing the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, if necessary, revise the report.

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#### CRITIQUE Health Review Committee

The Critique about the Investigators' Report is prepared by the HEI Health Review Committee and staff. Its purpose is to place the study into a broader scientific context, to point out its strengths and limitations, and to discuss the remaining uncertainties and the implications of the findings for public health.

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### RELATED HEI PUBLICATIONS

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## PREFACE

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In 1994, HEI initiated a research program to investigate the complex issues associated with the health effects of exposure to particulate matter (PM\*) in the air. This program was developed in response to growing concern about the potential public health significance of reported associations between daily fluctuations in levels of PM and changes in daily morbidity and mortality in time-series epidemiologic studies. These results were questioned for a variety of reasons, including the lack of support from experimental studies and the lack of a mechanism to explain how such effects would occur. To address these issues, HEI funded research projects in 1994, 1996, and 1998. In 1994, the Particle Epidemiology Evaluation Project (Samet et al 1995, 1997) evaluated six of the time-series epidemiologic studies that had reported effects of PM on mortality; under RFA 94-2, "Particulate Air Pollution and Daily Mortality: Identification of Populations at Risk and Underlying Mechanisms", epidemiologic and toxicologic studies were funded that aimed at understanding how PM might cause toxicity and what factors might affect susceptibility. In 1996, HEI issued RFA 96-1, "Mechanisms of Particle Toxicity: Fate and Bioreactivity of Particle-Associated Compounds", which sought studies that would improve our understanding of toxicologically relevant characteristics of ambient particles. In 1998, HEI issued RFA 98-1, "Characterization of Exposure to and Health Effects of Particulate Matter", which targeted a broad and ambitious set of research goals relating to both exposure assessment and health effects. In all, HEI has issued five requests for research on PM and funded 34 studies or reanalyses over the last 5 years.

This Preface provides general regulatory and scientific background information relevant to studies funded from RFA 98-1, including the study by Dr Lester Kobzik that is described in the accompanying Report and Commentary. This is one of five studies from RFA 98-1 that have been completed; eight additional studies are in progress. The "HEI Program Summary: Research on Particulate Matter" (Health Effects Institute 1999) provides information on PM studies funded since 1996.

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## BACKGROUND

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*Particulate matter* is the term used to define a complex mixture of anthropogenic and naturally occurring airborne

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\* A list of abbreviations and other terms appears after the Investigators' Report.

particles. In urban environments, these particles derive mainly from combustion, including mobile sources such as motor vehicles and stationary sources such as power plants. The size, chemical composition, and other physical and biologic properties of PM depend on the sources of the particles and the changes the particles undergo in the atmosphere. The most commonly used descriptor of particles is size measured as *aerodynamic diameter*. On the basis of this parameter, ambient particles tend to fall into three size classes (often defined as modes): ultrafine or nuclei mode (particles less than 0.1  $\mu\text{m}$  in diameter); fine or accumulation mode (particles between 0.1 and 2.5  $\mu\text{m}$  in diameter), and coarse (particles larger than 2.5  $\mu\text{m}$  in diameter). Fine and ultrafine particles are found primarily in emissions from combustion processes, whereas coarse particles are mostly generated by mechanical processes from a variety of noncombustion sources. Generally, the ultrafine and fine fractions are composed of carbonaceous material, metals, sulfate, nitrate, and ammonium ions. The coarse fraction consists of insoluble minerals and biologic aerosols, with smaller contributions from primary and secondary aerosols and sea salts (US Environmental Protection Agency [US EPA] 1996).

A number of early epidemiologic studies indicated that human exposure to high concentrations of PM, such as London fog, had deleterious effects (such as an increased number of deaths) particularly in children, the elderly, and those with cardiopulmonary conditions (Firket 1931; Logan 1953; Ciocco and Thompson 1961; Gore and Shaddick 1968). Because of this apparent relation to increased mortality, the EPA has regulated the levels of ambient PM since 1971, when the Clean Air Act was first promulgated. This act authorized the EPA to set National Ambient Air Quality Standards (NAAQs) for a number of potentially harmful air pollutants (including PM) in order to protect the health of the population, particularly those people thought to be sensitive to the effects of pollution.

The first NAAQs for PM were based on controlling total suspended PM or particles up to 40  $\mu\text{m}$  in diameter. In 1978, the standards were revised to regulate inhalable particles, or particles that can deposit in the respiratory tract and therefore have greater potential for causing adverse health effects. These particles measure 10  $\mu\text{m}$  or smaller in aerodynamic diameter ( $\text{PM}_{10}$ ). More recent epidemiologic studies, published in the early 1990s, indicated a relatively consistent association between short-term small increases in PM levels and increases in both morbidity and mortality from respiratory and cardiovascular diseases (reviewed by the

**Preface Table 1.** Current National Ambient Air Quality Standards for Particulate Matter (Set in 1997)

Time Period	PM <sub>10</sub>	PM <sub>2.5</sub>
Daily	150 µg/m <sup>3</sup>	65 µg/m <sup>3</sup>
Annual	50 µg/m <sup>3</sup>	15 µg/m <sup>3</sup>

Committee of the Environmental and Occupational Health Assembly, American Thoracic Society [Bascom et al 1996]).

Some studies also suggested that long-term exposure to low levels of PM is associated with adverse effects (Dockery et al 1993; Pope et al 1995). These latter studies also pointed to a possible role of fine particles (less than 2.5 µm in aerodynamic diameter [PM<sub>2.5</sub>]). In 1997, the EPA decided that the evidence for the effects of fine particles was sufficient to promulgate a PM<sub>2.5</sub> standard while retaining the PM<sub>10</sub> standard (US EPA 1997) (see Preface Table 1). The next review of the PM NAAQS is scheduled to be completed by 2002.

#### HEI's PARTICULATE MATTER RESEARCH PROGRAM

The wealth of epidemiologic data published in the early 1990s suggested an association between PM and health effects, but aspects of these findings were not well understood. Problems involved uncertainties in the exposure estimates, confounding by weather or other factors, the role of copollutants, and the mechanisms by which particles may cause effects. Moreover, although the epidemiologic findings were consistent across different communities exposed to distinct mixtures and levels of pollutants, they were not well supported by either human exposure chamber studies or animal inhalation studies aimed at delineating the pathologic changes that might result in death. Failure of the experimental studies to provide support for the epidemiologic findings was attributed to insufficient statistical power, use of particles not representative of ambient particles, or use of animals not representative of the individuals susceptible to increased mortality.

By the mid 1990s, it became apparent that the research to advance our understanding of the association between exposure to particles and daily mortality found in the epidemiologic studies needed to focus on identifying (1) susceptible populations, (2) mechanisms by which particles may lead to increased mortality, and (3) characteristics of the particles responsible for the effects. It was recognized

that both epidemiologic and experimental studies would be required. The HEI program from RFA-94 was aimed at addressing these research needs. In 1994, HEI also initiated the Particle Epidemiology Evaluation Project to address the validity and replicability of key epidemiologic studies (Samet et al 1995, 1997). Out of that project evolved the National Morbidity, Mortality, and Air Pollution Study to continue the epidemiologic evaluation in a large number of cities across the US with varying levels of PM and other air pollutants (Samet et al 2000a,b). Subsequently, HEI funded studies under RFA 96-1 that would use fine and ultrafine particles to test specific hypotheses related to the role of particle constituents in PM toxicity.

With increased financial support from the EPA and industry, in January 1998 HEI requested applications targeting both exposure assessments and health effects. HEI held a workshop at the Offices of the National Research Council that brought together scientists and representatives of the EPA and the motor vehicle and oil industries to discuss research needs. Out of this discussion, RFA 98-1, "Characterization of Exposure to and Health Effects of Particulate Matter", was developed and issued. The exposure objectives included (1) characterizing personal exposure to particles in different indoor and outdoor microenvironments and in geographic locations that differ in the types and sources of particles, topography, and climate; and (2) improving particle characterization to increase the accuracy of exposure estimates in epidemiologic studies. The health effects objectives included (1) characterizing potential pathophysiologic effects caused by PM in sensitive subjects; (2) defining the relation between particle characteristics and dose, distribution, and persistence of particles in the respiratory tract; (3) identifying the kinds of particles or particle attributes that may cause toxicity; (4) investigating the diseases or conditions that affect sensitivity; and (5) delineating how copollutants affect or contribute to the physiologic response to particles. From this RFA, HEI funded a comprehensive set of exposure assessment and health effects studies.

Three exposure assessment studies are investigating personal exposure to PM in potentially sensitive population subgroups in several US and European cities with diverse climatic and geographic features. These studies are (1) characterizing indoor concentrations of and personal exposure to PM<sub>2.5</sub> for subjects in two European cities (Bert Brunekreef of Wageningen/Utrecht University); (2) characterizing exposure to PM<sub>2.5</sub>, ozone, nitrogen dioxide, carbon monoxide, and sulfur dioxide in children, healthy seniors, and subjects with chronic obstructive pulmonary disease (Petros Koutrakis of Harvard School of Public Health); and (3) assessing personal exposure to PM<sub>2.5</sub> and

characterizing PM in terms of mass, functional groups, trace metals, polynuclear aromatic hydrocarbons, and elemental and organic carbon (Barbara Turpin of Environmental and Occupational Health Sciences Institute). A fourth study validated a newly developed method for measuring the acidic component of ultrafine particles or PM<sub>0.1</sub> (Beverly Cohen of New York University Medical Center).

Four human experimental and epidemiologic studies are investigating several potentially important endpoints that may help elucidate the mechanisms of particle toxicity. The epidemiologic studies are investigating the relation between PM levels and specific cardiac events: arrhythmias (Douglas Dockery of Harvard School of Public Health) and nonfatal myocardial infarctions (Annette Peters of GSF-Forschungszentrum für Umwelt und Gesundheit). The experimental studies are investigating cardiovascular and pulmonary effects in healthy and asthmatic subjects exposed to ultrafine carbon particles (Mark Frampton of University of Rochester) and concentrated ambient particles (CAPs) from Los Angeles air (Henry Gong of Los Amigos Research and Education Institute).

Six animal studies are addressing a number of hypotheses about susceptibility to and toxicity of particulate matter components using different health endpoints, animal models, and types of particles. These studies were designed to investigate (1) whether coexposure to CAPs from Boston air and ozone causes a synergistic amplification of asthmatic airway inflammation and hyperresponsiveness in juvenile mice with hypersensitive airways (Lester Kobzik of Harvard School of Public Health); (2) the genetic determinants of susceptibility to morbidity and mortality from nickel particles in inbred mouse strains (George Leikauf of University of Cincinnati); (3) whether exposing healthy rats to CAPs from New York City air causes changes in blood coagulation parameters that may be involved in thrombotic effects (Christine Nadziejko of New York University Medical Center); (4) whether exposure to resuspended particles from Ottawa air samples causes changes in heart function and vascular parameters in adult rats (Renaud Vincent of Health Canada); (5) the effects of particle size and composition on the lung inflammatory and histopathologic responses in old rats and rats with preexisting inflammation (Fletcher Hahn of Lovelace Respiratory Research Institute); and (6) the effects of CAPs from Detroit air on the airway epithelium in rats with preexisting hypersecretory airway disease (Jack Harkema of Michigan State University).

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## CONTINUING RESEARCH

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Many of the key questions identified in the early 1990s are still relevant and many research projects continue to address them. The research strategies have evolved, however, as results from completed studies have provided insights into which animal models and which endpoints may be the most helpful to evaluate. In addition, advances in exposure assessment and statistical methods have pointed to new approaches for conducting epidemiologic studies. In the past year HEI has published ten reports from its PM research program (Checkoway et al 2000; Gerde et al 2001; Godleski et al 2000; Goldberg et al 2000; Gordon et al 2000; Krewski et al 2000a,b; Lippmann et al 2000; Oberdörster et al 2000; Samet et al 2000a,b; Wichmann et al 2000). Many additional PM studies that are currently under review will be published in 2001 and 2002.

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### ABSTRACT

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Epidemiologic studies indicate that ozone (O<sub>3</sub>\*) and air pollution particles can exacerbate asthma symptoms. We investigated whether coexposure to inhaled particles and O<sub>3</sub> causes a synergistic effect on airway responsiveness and allergic inflammation in a murine (BALB/c) model of ovalbumin (OVA)-induced asthma.

Half of the mice were sensitized by intraperitoneal injection of OVA and then exposed to OVA aerosol on 3 successive days to create the asthmatic phenotype; the other half were sensitized to OVA and exposed to phosphate-buffered saline (PBS) to create the nonasthmatic control group. On the same 3 days that the OVA or PBS challenge was administered, mice were further divided into groups that were exposed for 5 hours to concentrated ambient particles (CAPs; mass values ranging from 63 to 1,569 µg/m<sup>3</sup> for 1 day's exposure), 0.3 ppm O<sub>3</sub>, both, or neither ( $n \geq 61$  total mice per exposure group for all 12 experiments). Whole-body plethysmography was used to measure airway responsiveness after challenge with aerosolized methacholine (MCh). Enhanced pause (Penh), an index that closely correlates with pulmonary resistance (Hamelmann et al 1997), was measured daily in each mouse immediately after pollutant exposure and, for 7 of

the 12 experiments ( $n \geq 36$ /exposure group), beginning 24 hours after the final OVA or PBS challenge.

Using several complementary statistical models, we found that exposure to CAPs alone caused a small but significant increase in Penh in both normal and asthmatic mice immediately after exposure (an increase of ~1% per 100-µg/m<sup>3</sup> increase in CAPs). No increase in Penh was found in animals exposed to O<sub>3</sub> alone or to filtered air. Compared with control animals, no combination of exposure atmosphere plus asthma produced a synergistic effect on Penh. By 24 hours after the last OVA or PBS challenge, any enhanced response induced by pollutant exposure had declined to control levels. The pollutant exposures did not significantly increase airway inflammation (assessed by bronchoalveolar lavage [BAL] fluid analysis beginning 24 or 48 hours after the final OVA or PBS challenge).

Because CAPs are a heterogeneous mixture of particles, elemental analysis was conducted and associations between specific elemental groupings (present in daily samples) and airway responsiveness were analyzed. This analysis showed that increased Penh in asthmatic mice exposed to CAPs plus O<sub>3</sub> was associated with the AlSi fraction of CAPs. No such association was found in control mice or in asthmatic mice not exposed to O<sub>3</sub>.

We conclude that CAPs exposure causes an immediate, short-lived (< 24-hour), small increase in airway responsiveness in mice and that changes in airway physiology are correlated with specific elements found within the particle mixture.

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\* A list of abbreviations and other terms appears at the end of the Investigators' Report.

This Investigators' Report is one part of Health Effects Institute Research Report 106, which also includes a Preface, a Critique by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr Lester Kobzik, Harvard School of Public Health, Department of Environmental Health, 665 Huntington Avenue, Room 221, Boston MA 02115-6021.

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### INTRODUCTION

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Epidemiologic studies show increases in morbidity and mortality associated with air pollution particles of respirable size (Bates et al 1990; Dockery et al 1993; Schwartz 1994). People with asthma are particularly affected (Dockery et al 1989; Pope et al 1991; Forsberg et al 1993; Yang and Yang 1994), but the mechanisms underlying their increased sensitivity are not well understood.

Patients with asthma show exacerbation of their disease (eg, increased use of bronchodilatory agents, increased school absenteeism, increased hospitalization) during episodes of increased particulate air pollution (Dockery et al 1989; Pope et al 1991; Pope and Dockery 1992; Forsberg et al 1993; Yang and Yang 1994).

At least two mechanisms have been identified by which particles might exacerbate asthma. One is that particles act indirectly by amplifying the allergic airway inflammation central to asthma. The other is that particles directly increase airway responsiveness to triggers of bronchospasm. These mechanisms are not mutually exclusive.

Although no mechanism has yet been established, clues have been found in studies using surrogate particles such as diesel exhaust or residual oil fly ash (particles collected in the environmental controls of oil-burning power plants). Some studies have shown that diesel exhaust and residual oil fly ash can act as adjuvants or enhancers of allergic inflammation and airway hyperresponsiveness in humans and other species (Yang and Yang 1994; Shima and Adachi 1996; Diaz-Sanchez et al 1997; Fujimaki et al 1997; Ichinose et al 1997, 1998; Takano et al 1997, 1998a,b; Miyabara et al 1998a,b,c,d; Goldsmith et al 1999; Hamada et al 1999). Other studies have shown that particles can alter the profile of immunoglobulins, proteins, and cytokines toward an allergic profile in humans and other species (Shima and Adachi 1996; Diaz-Sanchez et al 1997; Fujimaki et al 1997; Ichinose et al 1997; Takano et al 1997; Bayram et al 1998a,b; Miyabara et al 1998a,b,c,d; Devalia et al 1999). Furthermore, normal human bronchial epithelial cells exposed to diesel exhaust have shown physical and biochemical changes. These cells showed no changes in permeability, but did have increased ciliary beat frequency and increased production of interleukin-8, granulocyte-macrophage colony-stimulating factor, and soluble intercellular adhesion molecule-1 (Bayram et al 1998a,b). When bronchial epithelial cells from asthmatic subjects were studied, lower particle concentrations were required to cause cellular release of the aforementioned mediators than the concentrations needed for responses in these cells from control subjects (Devalia et al 1999). These studies show that particles may increase the symptoms of asthma by affecting the pulmonary environment, by creating an allergic profile of cytokines and immunoglobulins, and by directly affecting the cells of the lung.

Air pollution is a complex mixture of substances; particles are not the only component known to affect pulmonary function. Many studies have shown declines in pulmonary function in subjects exposed to O<sub>3</sub> (Silverman et al 1976; Dimeo et al 1981; Liroy et al 1985; McDonnell et al 1985; Hazucha et al 1989; Kinney et al 1989; Castillejos et al 1992; Cody et al 1992). These respiratory changes

were seen in normal and asthmatic subjects, in both young and old individuals, and at O<sub>3</sub> levels both above and below the National Ambient Air Quality Standard of 120 ppb.

As with particles, clues point to the mechanisms that may be involved in the increased susceptibility that asthmatic subjects demonstrate in the respiratory effects of O<sub>3</sub> exposure. Koenig and colleagues (1990) showed that exposure to 120 ppb O<sub>3</sub> before exposure to 100 ppb SO<sub>2</sub> caused significant decreases in pulmonary function (FEV<sub>1</sub>, total respiratory resistance, and maximal flow) that were not seen with SO<sub>2</sub> exposure alone. Other researchers have shown that exposing asthmatic subjects to O<sub>3</sub> before allergen challenge resulted in decreases in pulmonary function (Molfino et al 1991) and increases in upper and lower respiratory symptoms, including increases in inflammatory cells, such as neutrophils and eosinophils, and in protein concentrations in lavage fluid, which indicate tissue damage (Bascom et al 1990).

Previously, we found that exposing asthmatic and normal mice to air pollution particles alone was not sufficient to exacerbate airway inflammation and airway hyperresponsiveness in the asthmatic mice, nor to increase airway responsiveness or airway inflammation in normal animals 24 to 48 hours after pollutant exposure (Goldsmith et al 1999). Based upon such experimental and epidemiologic findings, we postulated that a combination of pollutants might be necessary to elicit physiologic responses. We hypothesized that exposing asthmatic mice to a combination of CAPs plus O<sub>3</sub> would exacerbate the airway inflammation and airway responsiveness associated with the asthma model. To test this postulate, we studied the *in vivo* effects of CAPs plus O<sub>3</sub> exposure in a mouse model of asthma induced by OVA sensitization and challenge. This model of allergen-induced airway inflammation and hyperresponsiveness is similar but not identical to human asthma (Drazen et al 1996; Herz et al 1996a,b; Krinzman et al 1996; Galli 1997; Corry et al 1998). We also considered that pollutant effects might be relatively short-lived and elude detection if assayed solely at 24 hours after exposure. Hence, we analyzed physiologic effects immediately after exposure in these experiments.

Finally, we analyzed CAPs *in vitro* using samples collected onto filters during the animal exposures. We postulated that the phlogistic potency of CAPs samples *in vitro* would correlate with the bioactivity *in vivo* (ie, enhanced airway responsiveness). We measured cytokine production (TNF- $\alpha$  and MIP-2) by normal or lipopolysaccharide (LPS)-primed rat alveolar macrophages in response to *in vitro* incubation with particle suspensions, as recently described (Imrich et al 1999).

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## SPECIFIC AIM

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The specific aim of this study was to measure the effects of exposure to CAPs, or O<sub>3</sub>, or both together on the asthmatic phenotype in a mouse model of airway hypersensitivity.

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## MATERIALS AND METHODS

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### IN VIVO ANIMAL STUDIES

#### Animals

BALB/c mice (3 days old) were purchased from Harlan (Indianapolis IN) as litters with their mothers. Each mother and litter were housed separately in a virus- and antibody-free animal facility that was maintained at 22–24°C and had 12-hour dark-light cycles. Mice were fed commercial mouse food pellets and given water ad libitum. All protocols used in these experiments were approved by Harvard University's Animal Care and Use Committee and conformed to the National Institutes of Health standards defined by the US Department of Agriculture Animal Welfare Acts.

#### Experimental Design

Experiments followed the scheme outlined in Figure 1. On days 7 and 14 of life, all animals were sensitized to OVA via intraperitoneal injection (see Animal Sensitization and Allergen Challenge section below). On days 21, 22, and 23 of life, mice were challenged with an aerosol of 3% OVA in PBS; control animals were challenged with PBS aerosol only. These challenges occurred for 10 minutes/day in individual compartments of a pie-shaped chamber.

In some experiments (3 through 7 out of 12), both OVA-challenged and control mice were then further exposed to one of six pollutant atmospheres for 5 hours each day: CAPs at high or low concentration, 0.3 ppm O<sub>3</sub>, CAPs at high or low concentration plus 0.3 ppm O<sub>3</sub>, or neither. This resulted in 12 exposure groups (with 5 or 6 mice per group) in each experiment conducted (total sample size per experiment was ≥ 61 mice). Further details as to concentrations and exposure conditions are provided below. Subsequent analysis revealed (1) an absence of clear effects of the CAPs exposure at the low concentration, and (2) the low concentrations of CAPs were occasionally below ambient (see Table 1) and indicative of technical problems. Hence, the exposures to low-concentration CAPs without and with O<sub>3</sub> were excluded from the final analyses.

In the remaining seven experiments (1, 2, and 8 through 12), both OVA-challenged and PBS-challenged mice were

further exposed to one of four pollutant atmospheres for 5 hours each day: the highest concentration of CAPs possible on that day, 0.3 ppm O<sub>3</sub>, CAPs plus 0.3 ppm O<sub>3</sub>, or neither. This resulted in eight exposure groups in each experiment conducted. Further details as to the concentrations and exposure conditions are provided below.

For all 12 experiments, pulmonary function was assessed in each mouse each day immediately after exposure; testing started 15 minutes after the pollutant exposure ended and required 2 hours to complete. For experiments 1 through 7, pulmonary function tests were also done beginning 24 hours after the last OVA or PBS challenge (day 24 of life), and lavage was performed starting 24 hours after the pulmonary function tests (day 25 of life) (Figure 1). For experiments 8 through 12, the animals were killed for lavage starting 24 hours after the last OVA or PBS challenge and no pulmonary function tests were conducted.

#### Animal Sensitization and Allergen Challenge

All animals were sensitized on day 7 of life by intraperitoneal injection of 10 µg OVA (Grade III, Sigma Chemical, St Louis MO) with 1 mg aluminum hydroxide [Al(OH)<sub>3</sub>; JT Baker Chemical, Phillipburg NJ] in 0.2 mL PBS, pH 7.4. A boosting injection using identical reagents was done on day 14 of life.

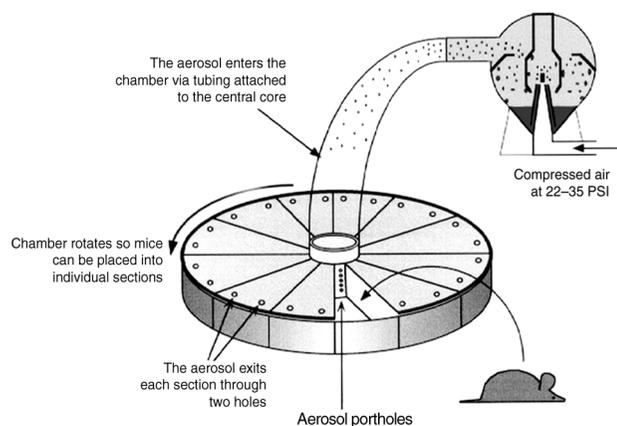
On days 21, 22, and 23 of life, mice were challenged with a nebulized aerosol of 3% OVA in PBS (w/v) or with PBS alone (control animals) for 10 minutes/day in individual compartments of a pie-shaped chamber (Figure 2, Braintree Scientific, Braintree MA) (Rudmann et al 2000). Aerosols were generated using a Pari IS2 nebulizer (Sun Medical Supply, Kansas City KS) connected to an air compressor (PulmoAID, DeVilbiss, Somerset PA). As reported by Cippola and coworkers, among the systems they tested, this one resulted in a median particle droplet size of ~2.75 µm, which was optimal for delivery into the lungs of small animals (mice) (Cippola et al 1996).

#### Exposure to Concentrated Ambient Particles and Ozone

Challenge with aerosolized OVA or PBS on days 21, 22, and 23 was followed by 5 hours of exposure each day to CAPs, O<sub>3</sub>, CAPs + O<sub>3</sub>, or filtered air. The mice were contained within the same type of pie-shaped chambers (Figure 2) placed within a Plexiglas sealed container with an inlet port (for pollutant or air) and an outlet port. O<sub>3</sub> was added through a T joint in the inlet port just before it enters the chamber. Mice were exposed to CAPs in the Harvard Ambient Particle Concentrator (Sioutas et al 1995a,b), which was developed to concentrate respirable particles from ambient air. One of the major advantages of this system is that particles remain airborne throughout the

Experiment Number	Day of Mouse Life						
	7	14	21 and 22 and 23		24	25	
All ( <i>n</i> = 40–72 mice per experiment; administered twice: All mice total)  1 2 3 4 5 6 7 8 9 10 11 12 Total mice	OVA sensitization administered (10 min) at start of each day		Exposure to Pollutant Atmosphere (5 hours/day)		Whole-body plethysmography at end of each day		
			OVA-Challenged Mice	PBS-Challenged Mice			
			O <sub>3</sub> ↑ CAPs + O <sub>3</sub>	Air ↑ CAPs + O <sub>3</sub>	Air		
			@ <i>n</i> ≥ 40 animals per experiment, <i>n</i> = 5 or 6 animals per exposure group; for these two experiments combined, <i>n</i> = 10 to 12 mice per exposure group.			0 mg (baseline) and 12 mg/mL MCh	
			O <sub>3</sub> ↑ CAPs + O <sub>3</sub>	Air ↓ CAPs* + O <sub>3</sub> *	Air ↓ CAPs* + O <sub>3</sub> *		
			@ <i>n</i> ≥ 61 animals per experiment, <i>n</i> = 5 or 6 animals per exposure group; for these five experiments combined, <i>n</i> = 25 to 30 mice per exposure group.			Whole-body plethysmography starting 24 hours after last OVA or PBS challenge; baseline, PBS, 6, 12, 25, 50, and 100 mg/mL MCh	
			O <sub>3</sub> ↑ CAPs + O <sub>3</sub>	Air ↓ CAPs* + O <sub>3</sub> *	Air ↓ CAPs* + O <sub>3</sub> *		
			@ <i>n</i> ≥ 40 animals per experiment, <i>n</i> = 5 or 6 animals per exposure group; for these five experiments combined, <i>n</i> = 25 to 30 mice per exposure group.			Killed starting 48 hours after last OVA or PBS challenge and BAL performed	
			O <sub>3</sub> ↑ CAPs + O <sub>3</sub>	Air ↑ CAPs + O <sub>3</sub>	Air		
			@ <i>n</i> ≥ 40 animals per experiment, <i>n</i> = 5 or 6 animals per exposure group; for these five experiments combined, <i>n</i> = 25 to 30 mice per exposure group.			0 mg (baseline) and 12 and 50 mg/mL MCh	
			60–72 per exposure group	60–72 per exposure group	60–72 per exposure group		

**Figure 1. Experimental design.** ↑ = High-level; ↓ = low-level. In experiments 3 through 7, an \* indicates that because the low levels of CAPs were erratic, we discontinued these exposures and eliminated the data from the statistical analyses. † In experiments 1 through 7 on days 24 and 25, the *n* values reflect the exclusion of animals exposed to low-level CAPs and low-level CAPs + O<sub>3</sub>.



**Figure 2. Diagram of the pie-shaped chamber used to challenge mice with PBS or OVA.** (Used by courtesy of T Sweeney.)

concentration process, allowing delivery directly to an inhalation exposure chamber (Sioutas et al 1995a,b, 1997). An undiluted stream of CAPs was used for the high exposure concentrations. The lower concentrations used in some experiments were achieved by diluting the CAPs stream with filtered air.

CAPs concentrations differed from day to day and from one set of mice to another (each set of mice, assigned to 1 of 12 experiments, was exposed during a different week from May through December of 1999). Daily CAPs concentrations ranged from  $\sim 63 \mu\text{g}/\text{m}^3$  to  $1,569 \mu\text{g}/\text{m}^3$  (Table 1).

Chemical and physical characterization of ambient and concentrated particles for each exposure included fine particle mass, sulfate ion concentration, nitrate ion concentration, trace metal concentration, and black carbon (a surrogate for elemental carbon). Methods for characterizing the chemical and physical properties of particles are described in subsequent sections.

Ozone was generated by passing filtered dry 100% oxygen through a high-voltage (7,000-V) discharge device that produced ultraviolet light; the resulting gas was mixed with a diluting flow of filtered room air and pumped into the exposure chamber. The air entering and leaving the exposure chamber was continually sampled via two ports to analyze  $\text{O}_3$  concentrations, which were measured with a chemiluminescent analyzer (Model 49, Thermoelectron, Hopkinton MA). The  $\text{O}_3$  analyzer was calibrated by reference to an ultraviolet photometer (Model 1003, PC S/N 3419, Daisibi, Glendale CA) that serves as an  $\text{O}_3$  primary standard as defined by the US Environmental Protection Agency. Control mice were

placed in identical chambers but were exposed to clean filtered room air for the same duration. Typical ambient  $\text{O}_3$  levels in the room outside the chamber were  $< 10$  ppb.

### Measurement of Airway Responsiveness

Immediately after exposure on each of the 3 days, Penh was measured using whole-body plethysmography (Buxco, Troy NY). Mice were placed in a plethysmograph and continuous measurements of box-pressure/time wave were recorded via a connected transducer and computer data acquisition system. Penh, as an indicator of airway responsiveness, was calculated from the waveform (Hamelmann et al 1997). Penh is a unitless parameter described by the following equation:

$$\text{Penh} = \frac{\text{PEP}}{\text{PIP}} \times \text{Pause}$$

where PEP is peak expiratory pressure, PIP is peak inspiratory pressure, and Pause is a timing comparison between early and late expiration. A more detailed explanation and description of both the Buxco equipment and the use of Penh to determine airway responsiveness is provided by Hamelmann and associates (1997).

For data acquired immediately after pollutant exposures, Penh was measured at baseline (resting measurements in the plethysmograph) and after challenge with 12 mg/mL aerosolized MCh in experiments 1 through 7; for experiments 8 through 12, mice were also challenged with 50 mg/mL MCh. Aerosols of MCh were generated for 2 minutes and entered the plethysmograph via a port at the top of each chamber. Data were acquired on each animal for 9 minutes after the 2 minutes of aerosol challenge. Penh values for the first 5 minutes after MCh challenge were averaged and results were compared across individual mice and pollutant exposure groups.

For experiments 1 through 7, starting 24 hours after the last OVA or PBS challenge, mice were placed in the whole-body plethysmograph and airway responsiveness to increasing concentrations of aerosolized MCh was measured. Baseline Penh measurements were recorded; then each animal was challenged with aerosols of PBS (for control animals) or increasing concentrations of MCh (6, 12, 25, 50, and 100 mg/mL). Aerosols were generated for 2 minutes and data were acquired on each animal for 9 minutes after each aerosol challenge. Penh values for the first 5 minutes after each MCh challenge were averaged and results were compared across individual mice and pollutant exposure groups.

To conduct these experiments on a large number of animals required that we use two different Buxco systems, each of which contained six plethysmograph chambers.

**Table 1.** Levels of Mass for Various Particle Components During Exposures<sup>a</sup>

Date	Ambient ( $\mu\text{g}/\text{m}^3$ )	High-Level CAPs ( $\mu\text{g}/\text{m}^3$ )	High-Level CAPs $\div$ Ambient	Low-Level CAPs ( $\mu\text{g}/\text{m}^3$ )	Black Carbon ( $\text{ng}/\text{m}^3$ )	Sulfate ( $\mu\text{g}/\text{m}^3$ )	Nitrate ( $\mu\text{g}/\text{m}^3$ )
5/10	9.2	109.9	11.9	ND	1,583	29	5.7
5/11	4.7	109.3	23.2	ND	2,720	19	5.6
5/12	4.8	140.3	29.2	ND	NA	29.6	5.9
6/14	7.6	232.3	30.6	ND	2,829	106.9	7.3
6/15	5.2	161.8	31.3	ND	2,543	44.3	5.5
6/16	5.4	156.5	29.2	ND	2,642	13.4	9.1
7/19	18.5	660.1	35.7	133.1	12,655	243.3	7.9
7/20	9.0	226.5	25.1	47.6	6,777	26	9.1
7/21	9.8	244.8	24.9	24.1	5,802	16.3	12.1
7/26	31.7	1,568.6	49.5	8.8	20,836	885.4	14.6
7/27	7.8	331.8	42.4	48.8	5,805	108.8	7
7/28	7.7	308.6	40.2	28.3	5,568	36.9	9.5
8/23	9.9	425.9	43.0	42.6	9,337	82.1	9.6
8/24	16.3	875.2	53.8	73.5	19,605	148.1	16.1
8/25	22.6	1,518.2	67.2	125.5	23,634	557.7	15.9
8/30	5.1	174.7	34.0	4.1	3,548	8.9	10.8
8/31	5.7	254.5	44.8	13.2	3,830	13.3	12.4
9/1	9.3	373.6	40.0	NA	10,367	22.8	16
10/4	6.6	144.8	21.9	1.6	3,515	25	NA
10/5	4.3	149.0	34.5	3.9	3,470	42.8	10.6
10/6	7.0	239.1	34.0	20.7	3,861	81	8.1
10/18	5.6	109.9	19.5	ND	1,578	38.6	4.5
10/19	6.9	156.7	22.7	ND	2,567	27.3	13
10/20	7.5	127.5	17.1	ND	5,124	26.7	10.3
10/25	4.9	108.9	22.4	ND	1,523	29.9	7.9
10/26	9.3	253.4	27.2	ND	4,029	83.4	14
10/27	7.3	176.5	24.1	ND	3,461	34.1	8.7
11/15	2.1	63.3	30.8	ND	796	5.8	5.2
11/16	2.7	79.7	29.4	ND	2,080	9.9	4.4
11/17	2.3	91.1	39.4	ND	1,174	11.5	6.4
11/29	4.3	142.9	33.0	ND	4,084	30.6	5.8
11/30	4.9	135.0	27.5	ND	2,450	17.4	14
12/1	6.4	135.9	21.3	ND	1,963	12.3	8
12/6	13.3	519.1	39.1	ND	10,876	123.4	80.8
12/7	5.5	216.7	39.1	ND	4,167	85	8.3
12/8	5.3	159.4	30.2	ND	3,901	42.1	4.9

<sup>a</sup> All dates are in 1999. ND = not done; NA = not available.

Although we made the systems as comparable as possible, using two monitoring systems could have introduced variability in our experimental results. For this reason, we carefully tracked which animals were analyzed in which system and accounted for this in our statistical analyses. In addition, for every pulmonary function test conducted, half of each exposure group was analyzed on one system and the other half on the other system.

#### **Assessment of Cells in Bronchoalveolar Lavage Fluid**

Beginning either 24 hours (experiments 8 through 12) or 48 hours (experiments 1 through 7; see Figure 1) after the last OVA or PBS challenge, mice were killed with an overdose of sodium pentobarbital (Veterinary Laboratories, Lenexa KS). The chest wall was opened and the animals were exsanguinated by cardiac puncture. The trachea was cannulated and BAL performed. PBS (0.5 mL) was instilled into the lung six times and the lavage fluid was collected after each instillation. Recovery of the instillate was greater than 90%. These samples were kept on ice and then centrifuged for 10 minutes at 1,200 rpm ( $300 \times g$ ). The cell pellet was resuspended in 0.5 mL PBS and cell counts and viability were determined using a hemocytometer and trypan blue dye exclusion. For differential cell counts, 50,000 to 100,000 cells/lavage sample were centrifuged (Cytospin 2, Shandon, Pittsburgh PA) for 5 minutes at 800 rpm and pelleted onto a slide. After fixing in 95% ethanol and staining with a modified Wright-Giemsa stain, 200 cells/lavage sample were differentially identified (Diff-Quick, VWR, Boston MA). Alveolar macrophages, polymorphonuclear cells, eosinophils, and lymphocytes were counted. After lavage, the lungs were removed and fixed in formalin; tissues were later stained with hematoxylin and eosin for histopathologic assessment.

#### **Physical and Chemical Characterization of Concentrated and Ambient Particles**

Several integrated and continuous sampling techniques were used to measure the concentrations and determine the composition of particles during exposure studies. Measurements were made on both ambient and concentrated particles to verify that the CAPs exposure atmospheres accurately reflected the composition of ambient particles. Levels of mass for ambient particles, CAPs, black carbon, nitrate, and sulfate are depicted in Table 1. The various measurements and methods, including sampling and analysis, are described in detail below.

To analyze ambient particles, 30 L/min of ambient air was diverted to a manifold between the 2.5- $\mu$ m size-selective inlet and the first stage of the concentrator. To analyze

concentrated particles, 12 L/min of the available 45 L/min from the concentrator output flow was diverted to a manifold for analytical measurements.

#### ***Fine Particle Mass and Sulfate and Nitrate***

**Concentrations** Both ambient and concentrated fine particle masses were sampled on Teflon filters (Teflo, 47 mm diameter, 2  $\mu$ m pore size, Gelman Sciences, Ann Arbor MI) contained in a plastic filter-holder assembly. Samples were collected for the duration of each exposure (5 hours) at typical sampling flow rates of 30 L/min for ambient mass and 3 L/min for concentrated mass. Precise weighing of the filters before and after sampling on an electronic microbalance (Mettler Model MT5, Mettler-Toledo, Columbus OH) yielded the net mass collected on the filter. Mass concentration was then determined from the net mass collected and the total measured volume of air sampled.

Following gravimetric analysis, the collected particles were extracted from the filters and analyzed by ion chromatography using a conductivity detector for sulfate. This analytic method is described in detail elsewhere (Koutrakis et al 1988a,b).

**Particle Size Distribution** The particle size distribution (as mass median aerodynamic diameter and geometric standard deviation) of ambient fine particle mass was characterized using a multiple-orifice impactor (Marple et al 1991). Particles were collected on 37-mm Teflon filter substrates mounted on the collection surface of each stage of the impactor. Particles were sampled for 5 hours at a flow rate of 30 L/min on each of the 3 days during a typical mouse exposure.

**Trace Metal Concentrations** Particle samples to analyze for trace metal concentrations by x-ray fluorescence (Dzubay and Stevens 1975; Jaklevic et al 1977) were collected on a 47-mm Teflon filter mounted in parallel with the filter assembly used for particle mass and sulfate analysis of concentrated fine particles. X-ray fluorescence analysis was performed by Chester LabNet (Tigard OR).

#### ***Black Carbon As a Surrogate for Elemental***

**Carbon** An aethalometer (model AE-14, Magee Scientific, Berkeley CA) was used to measure black carbon, a surrogate for elemental carbon, in real time. The method is based on the optical attenuation of light by elemental carbon particles collected on a quartz-fiber filter tape at a sample flow rate of 0.75 L/min. The light source was an incandescent bulb with an effective center wavelength of 820 nm. The overall decrease in optical attenuation was calculated by subtracting the transmission level at the end of the measurement cycle when the filter was full from the

transmission level at the beginning of the measurement cycle when the filter was clean. The optical transmission of an unexposed portion of the filter was measured at the end of each cycle to control for drift in instrument parameters. The change in light attenuation from one measurement cycle to the next, which decreases exponentially with filter loading, was reported as a linearized and scaled "attenuation unit". The mean concentration of black carbon during the measurement cycle was then determined from the attenuation units and sample volume data using an internal, empirically derived conversion factor (19.2 m<sup>2</sup>/g). Black carbon data from this instrument has agreed well with elemental carbon data in previous comparisons (Hansen and McMurray 1990; Allen et al 1999). The principle of the method is described in detail elsewhere (Hansen et al 1984).

## IN VITRO ANALYSIS OF PARTICLE EFFECTS

### Preparation of Particle Suspensions

Concentrated ambient particles were collected from Boston air using the Harvard concentrator (Sioutas et al 1995a,b). Particles (0.1 to 2.5 µm in aerodynamic diameter) were collected onto Teflon filters (Teflo, 47 mm diameter, 2 µm pore size, Gelman Sciences, Ann Arbor MI) during each sampling day; only the filters from the days during experiments 8 through 12 were used. To prepare suspensions of CAPs for in vitro experiments, filters were cut into pieces, suspended in 0.5 to 1 mL water and probe-sonicated for 30 seconds (Model W-p200, Ultrasonics, Plainview NY, setting 4). We purchased the urban air particle (UAP) sample SRM 1649, collected in Washington DC, from the National Bureau of Standards (Washington DC) to use as a positive control for CAPs in the in vitro experiments. Suspensions of UAP were prepared in water at 10 mg/mL and were probe-sonicated for 1 minute before use.

### In Vitro Priming and Particle Incubation

Female CD rats (250 to 275 g, Harlan, Indianapolis IN) were killed with an overdose of sodium pentobarbital, their lungs lavaged, and the cells collected from the BAL fluid. Only cells from animals yielding ≥ 95% alveolar macrophages were used in these assays.

Incubations of cells were carried out in 6-well Ultra Low Cluster plates (Costar, Cambridge MA) at 37°C in humid 5% CO<sub>2</sub>. For priming, alveolar macrophages were suspended at 10<sup>6</sup> cells per mL RPMI + 1% fetal bovine serum (R1%; BioWhittaker, Walkersville MD) and treated with either bacterial LPS (*Escherichia coli* serotype 0127:B8, 250 ng/mL) or rat interferon-γ (IFN-γ; 250 U/mL) for 3 hours. (Control, nonprimed cells were treated identically except that neither LPS nor IFN-γ was added during the

3-hour incubation.) After this priming period, alveolar macrophages were collected, washed, and adjusted to 2.4 × 10<sup>6</sup> cells per mL R1%. Cells were then dispensed (80 µL = 192,000 per well) into 96-well Ultra Low Cluster plates (preincubated with 80 µL R1% per well). Particle suspensions (80 µL) were then added for a final concentration of 100 µg/mL in 240 µL total volume. The particle concentration was selected based on our recent work showing the dose-response analysis of UAP-mediated TNF-α release by control and primed alveolar macrophages (Imrich et al 1999).

Previous time-course experiments have identified 20 hours of particle incubation as an optimal endpoint for amplified TNF-α and MIP-2 release by primed alveolar macrophages (Imrich et al 1999; and data not shown). Supernatants were collected at this time point and stored frozen. Cells were then analyzed by flow cytometry for viability and relative particle load (as assessed by right-angle light scatter; see below). All reagents were obtained from Sigma (St Louis MO) unless otherwise specified.

### Flow Cytometric Analysis

We used a Coulter ELITE flow cytometer (Coulter Corporation, Miami FL) equipped with an air-cooled argon laser (Cyronics/Uniphase) set at 488 nm to separate samples stained with propidium iodide, a red, fluorescent, DNA-binding dye. Live cells do not stain with propidium iodide, but cells without nuclear membrane integrity stain red. The percentage of viable cells was determined as the percentage of all red-negative cells within a forward-scatter versus red-bivariate histogram. Data from 3,000 alveolar macrophages were collected using a linear scale ranging from 0 to 1,023 relative intensity units.

Measurement of right-angle light scatter associated with alveolar macrophages was used to quantify particle load (Stringer et al 1995). In flow cytometry, laser light scatters in all directions as the cell (with or without associated particles) passes through the laser beam. When cells bind particles, granularity, and therefore light scatter, increases. Light scattering properties for a given particle type are determined by particle size, shape, and composition. We make the assumption that right-angle light scatter generated by different CAPs samples can be compared as one particle type. Such an assumption permits interpreting changes in light scatter as being reflective of changes in cell-associated particle load.

### Cytokine Assays: TNF-α and MIP-2

Cell supernatants from the female CD rat alveolar macrophages were assayed for TNF-α bioactivity using the

WEHI 164 clone 13, a tumor cell line sensitive to TNF- $\alpha$ , in a previously described microplate assay (Imrich et al 1998).

Rat MIP-2 was measured with a sandwich ELISA using rabbit anti-rat Gro/MIP-2 (500-P75, PeproTech, Rocky Hill NJ) as the coating antibody diluted in coating buffer at 1  $\mu\text{g}/\text{mL}$  in ELISA plates (MaxiSorp NUNC, VWR, Bridgeport NJ). After overnight incubation at room temperature, wells were blocked (PBS with 2% bovine serum albumin) and samples and standards were applied (rat MIP-2 standard, 2 to 0.03  $\text{ng}/\text{mL}$ , R&D Systems, Minneapolis MN). Goat anti-rat MIP-2 (C-19, Santa Cruz Biotechnology, Santa Cruz CA) was added at 0.4  $\mu\text{g}/\text{mL}$ . To increase sensitivity, biotinylated horse anti-goat IgG (1:400) was added and then avidin-biotin-peroxidase complex (1:400; ABCelite kit, Vector, Burlingame CA) to deposit peroxidase enzyme. Normal sera were used to block species crossreactivity of reagents. Peroxidase substrate tetramethylbenzidine (DAKO, Carpinteria CA) was added for 10 minutes and then quenched with an equal volume of 2N sulfuric acid. The optical density was measured (450  $\text{nm}$ ) and MIP-2 levels were calculated from the standard curve using Soft MAX software (Molecular Devices, Menlo Park CA).

## STATISTICAL ANALYSES

Data are expressed as means  $\pm$  SEM. For cell counts (total and differential), multiway analysis of variance (ANOVA) was used to compare both total counts and log counts. For airway responsiveness data, a general linear mixed-effects model was used to analyze the replicate measurements in each mouse over MCh concentration and day, treating "mouse" as a random effect. Multiway ANOVA models were constructed in the context of the mixed-effects model to allow for multiple experimental factors (each of 3 days,  $\text{O}_3$  exposure, MCh challenge concentration, OVA challenge, and each of two Buxco systems) and multiway interactions. Total CAPs and elemental composition scores were treated as continuous predictors.

Statistics are presented both for differences in the MCh challenge concentration-response slopes and for intercepts that compare exposure groups (ie, comparisons were made to evaluate differences in the slopes of the lines generated during MCh challenge and differences in the overall magnitude [means] of these responses in the mixed-effects model, adjusting for all the other experimental factors listed above). As a result, separate *P* values were computed for comparisons of group slopes (rate of change in Penh) and comparisons of overall means (mean Penh). For the MCh challenges performed each day at the end of exposure, log or square root transformations of response were used for modeling depending on which provided better symmetry, homoscedasticity, and linearity; the square root

transformation was used for MCh concentration in experiments 1 through 7 in which a complete set of MCh challenges was performed beginning 24 hours after the last OVA or PBS challenge. All mixed-effects model analyses were done using the SAS System (SAS Institute, Cary NC). *P* values  $< 0.05$  were considered statistically significant for total CAPs models; a Bonferroni-adjusted significance level of 0.0083 (0.05/6) was used to determine formal statistical significance for the particle elemental component models that involved analyzing elemental scores. In all models and analyses, significance testing was two-sided.

To characterize elemental composition in the airway responsiveness analyses, principal components analyses were applied to particle elemental concentration data. The factor scores and loadings were calculated by positioning the correlation matrix on the diagonal and the extracted factors were rotated using the Equamax rotation procedures (SPSS 8.0 Software, SPSS, Chicago IL). Factor rotations were also conducted using oblique rotations. Orthogonal and oblique rotations yielded similar results and the oblique rotations were employed in further analyses. Finally, the absolute scores were determined using the method described by Koutrakis and Spengler (1987) and were used as input to the elemental mixed-effects model regression analyses.

Total CAPs and elemental composition scores were analyzed both as daily exposure and as cumulative exposure (accumulating over the 3 days of exposure for each mouse) for each of the airway responsiveness models.

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## RESULTS

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### AIRWAY RESPONSIVENESS: OVERVIEW OF DATA ANALYSIS

The review of the data from these experiments is complicated. For every experiment conducted, we had two initial cohorts of mice: asthmatic mice (created by the challenge with OVA) and nonasthmatic control mice (created by the same challenge with PBS alone). These two cohorts were then exposed to different pollutant atmospheres: Some animals were exposed to homogeneous atmospheres that never differed from experiment to experiment nor from day to day (eg, filtered air or 0.3 ppm  $\text{O}_3$ ). Other animals were exposed to CAPs, a heterogeneous mixture of pollutant particles that varies both in concentration and composition from day to day and therefore from experiment to experiment. Finally, some animals were exposed to the combination of CAPs plus  $\text{O}_3$ .

To further complicate the analyses, multiple outcomes were considered at different time points, including pulmonary function and assessment of cells in BAL fluid. Pulmonary function testing resulted in data gathered (1) on each of the 3 days of exposure for all animals (at baseline and after challenge with one concentration of MCh in experiments 1 through 7 and two concentrations of MCh in experiments 8 through 12); and (2) for animals in experiments 1 through 7, beginning 24 hours after the last OVA or PBS challenge (at baseline and after challenge with increasing concentrations of MCh). To assess cells in BAL fluid, BAL was performed at death in some animals starting 24 hours and in some animals starting 48 hours after the last OVA or PBS challenge.

As a result, we have applied a number of different, albeit complementary, statistical approaches to the data obtained. In the first approach (Analysis I), we used a linear regression model and CAPs exposure was considered as a category; no consideration was given to actual concentrations during exposure. Although this approach indicated some interesting effects, a more detailed evaluation of the data was warranted.

Next we incorporated CAPs concentrations into the multivariable regression model (Analysis II), which provided a more quantitative evaluation of the interaction of the various pollutant exposures on airway responsiveness. Because CAPs is a heterogeneous mixture of particles, considering CAPs exposures on the basis of concentration alone may obscure important information; in both the first and second analyses, no consideration was given to the composition of the particles. Thus, a third analysis was undertaken in which the elemental composition of the particles for each day (Table 2) was considered (Analysis III). A final variation of this last approach (Analysis IV) was used to analyze data from experiments 8 through 12. In these experiments, a second dose was used in the MCh challenge for airway responsiveness at the end of each exposure day; this resulted in a distinct subset for statistical evaluation. These related analyses are presented sequentially to provide a comprehensive view of the data and our methods of interpreting them.

#### **Analysis I: Multivariable Regression Considering Exposure to Concentrated Ambient Particles As a Category**

For all 12 experiments, the animals were divided into two groups: asthmatic (OVA-challenged) and nonasthmatic (PBS-challenged) controls. These groups were then exposed to filtered air, CAPs, 0.3 ppm O<sub>3</sub>, or CAPs + O<sub>3</sub>. Penh, an indicator of airway responsiveness, was assessed in a whole-body plethysmograph after each day's exposure.

Using a multivariable linear regression model in which CAPs exposure was considered as a category and data from each exposure group were pooled from all 3 days, both OVA challenge alone (as expected in an asthma model;  $P = 0.0017$ ) and CAPs exposure ( $P < 0.0001$ ) increased Penh. Coexposure to CAPs + O<sub>3</sub> was also found to significantly increase Penh ( $P = 0.0038$ ). On the basis of this analytic method, no synergistic effects were found for the combined effects of OVA challenge (ie, having asthma) and any pollutant exposure. No increases in airway responsiveness in either OVA-challenged or PBS-challenged animals resulted from exposure to O<sub>3</sub> alone or to filtered air alone. Figure 3 presents the daily values that were pooled for analysis.

#### **Analysis II: Multivariable Regression Considering Concentrations of Ambient Particles**

The daily concentrations of CAPs for these experiments are shown in Table 1. These values were used to assess the effects of the different pollutant atmospheres in a regression model that included these specific values rather than treating CAPs exposure categorically. Moreover, analysis considering CAPs concentrations was conducted in a number of different ways. One approach was to consider each day independently using nonlinearized unit CAPs concentrations, and another approach was to use a log conversion of the CAPs concentrations to linearize the data. Other approaches were to consider both the nonlinear and log-linearized cumulative CAPs exposures over the course of the 3 days of any given experiment. The results of these evaluations are detailed in Table 3. The major finding was that, for both asthmatic and control animals, CAPs exposure alone caused a significant increase in Penh ( $P < 0.0001$ ), regardless of the slight variations in statistical methods used. In these analyses, the other factors found to significantly increase Penh were expected: challenge with OVA (all asthmatic animals compared with all control animals regardless of pollutant exposure); and challenge with the bronchoconstrictor MCh (the daily MCh challenge at 12 or 50 mg in all animals versus the baseline values for all animals).

The effect of CAPs alone seen in Analysis I was supported by this more rigorous analysis using specific concentrations in the regression model. We sought to estimate the magnitude of the effect that each concentration of CAPs (hereafter designated as [CAPs]) had on Penh. Estimates of the effect of increasing [CAPs] on baseline Penh could be generated from the linear equation used in the analysis and are shown in Figure 4. Although the increase in Penh is statistically significant, it is relatively small for increases in particles given the typical ambient exposures in the United States. For example, the increase in Penh for

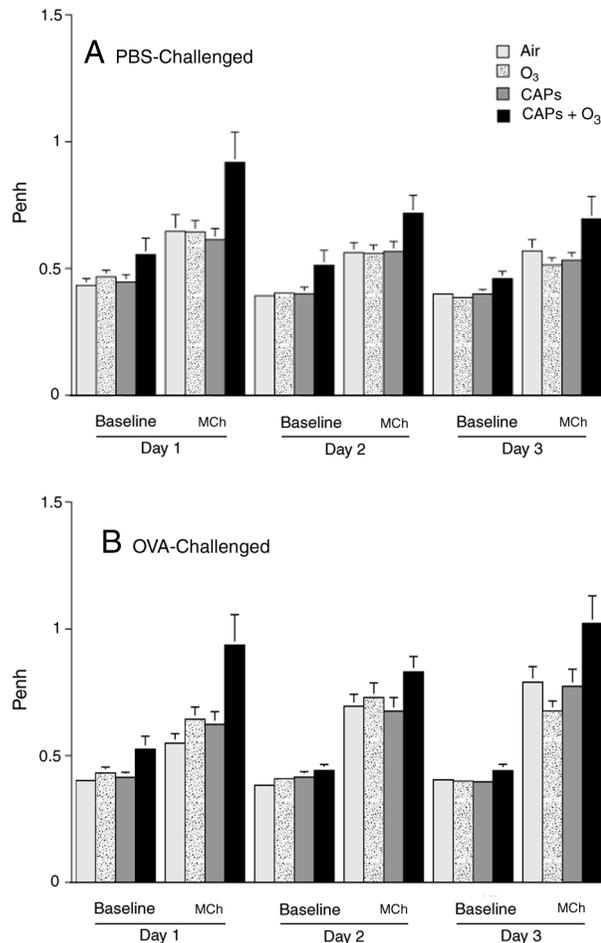
**Table 2. Elemental Concentrations During Particle Exposures<sup>a</sup>**

Date <sup>b</sup>	CAPs Mass <sup>c</sup>	Al	Si	S	Cl	K	Ca	Ti	V	Cr	Mn	Fe	Ni	Cu	Zn	As	Se	Br	Cd	Ba	Pb
5/10	119.2	0.6929	2.5205	6.4018	ND	0.5947	0.8585	ND	ND	0.0348	2.3868	0.0000	0.1159	0.1748	ND	ND	ND	ND	0.4143	0.2260	
5/11																					
5/12	141.3	2.3907	5.8979	6.6788	ND	1.3100	2.1057	0.1983	ND	0.0002	0.0742	3.8230	ND	0.0765	0.2063	ND	ND	0.0417	ND	0.4662	0.0981
6/14	247.3	ND	2.0093	23.6024	ND	0.6720	0.6084	0.0739	0.0064	ND	0.0076	1.4648	0.0216	0.0178	0.0761	ND	0.0102	ND	ND	ND	0.0456
6/15	169.9	0.8671	3.1328	10.2342	ND	0.8527	1.5758	0.1395	ND	ND	0.0343	2.8764	ND	0.0446	0.0571	ND	ND	0.0147	ND	ND	0.0012
6/16	182.9	6.4524	13.0942	3.4868	0.5818	2.3371	4.7422	0.6602	ND	ND	0.1193	6.7729	0.0035	0.0614	0.1989	ND	ND	ND	ND	0.2307	0.0330
7/19	685.0	4.4417	14.4501	51.6430	ND	2.6323	4.7013	0.9123	0.0869	0.0071	0.1780	8.7462	0.0702	0.0827	0.3831	ND	0.0138	0.0827	ND	ND	0.0647
7/20	239.5	4.4805	9.8889	6.1414	ND	2.0141	3.2495	0.6378	ND	0.0119	0.1284	6.2351	ND	0.0726	0.2426	ND	ND	0.0221	ND	0.1607	0.0363
7/21	254.5	11.8963	23.7773	4.1627	ND	4.0266	7.1613	1.1759	0.0133	0.0365	0.2467	12.6620	0.0278	0.1261	0.3414	ND	ND	0.0133	ND	0.3152	0.0816
7/26	1591.9	ND	4.1709	187.4672	ND	1.1081	1.4490	0.1877	0.2591	ND	0.1047	4.0953	0.0979	0.0566	0.4932	ND	0.0039	0.0992	ND	ND	0.1143
7/27	314.0	0.3615	3.1662	24.0616	ND	1.0419	1.1989	0.3627	ND	0.0155	0.0496	2.9505	0.0089	0.0572	0.3273	ND	ND	0.0330	ND	ND	0.0370
7/28	276.5	3.2716	9.2889	9.2988	ND	1.9526	3.6233	0.3200	ND	ND	0.1141	6.8983	0.0012	0.1153	0.2336	ND	ND	0.0259	ND	0.1595	0.0493
8/23	413.7	3.7167	11.0856	20.6074	ND	2.6880	4.6036	0.6169	0.1051	0.0016	0.1992	10.0819	0.0609	0.1704	0.7181	ND	ND	0.0580	ND	0.2379	0.1162
8/24	853.7	11.3408	28.6964	34.5675	ND	5.2548	10.5923	1.9077	0.1912	0.0518	0.4295	21.8550	0.1483	0.4295	1.1622	0.0312	ND	0.1056	ND	0.6528	0.3423
8/25	1516.2	2.9948	21.0921	124.4099	ND	4.4885	10.1375	1.2477	0.1946	0.0420	0.3035	18.3691	0.1580	0.3296	0.9764	ND	0.0558	0.2424	ND	0.6987	0.2519
8/30	261.5	11.8439	24.3953	3.7294	2.4123	3.8833	10.6084	0.9941	0.0625	0.0234	0.2887	14.4444	0.0327	0.1819	0.8874	ND	ND	ND	ND	0.1053	0.1743
8/31	230.0	10.1214	21.6344	3.4509	5.1065	3.7271	8.0904	0.7353	ND	0.0124	0.4339	13.1372	0.0043	0.1384	1.0027	ND	ND	ND	ND	0.3527	0.1083
9/1	394.8	17.1870	35.1968	6.2197	2.0541	5.6554	12.4785	1.4216	0.1173	0.0181	0.3886	21.7601	0.0554	0.2723	1.0526	ND	ND	0.0594	ND	0.0659	0.1599
10/4	167.3	0.0153	1.4773	7.8642	7.8788	0.8978	2.2817	0.2153	0.0890	0.0091	0.0916	2.6992	0.0573	0.0573	0.9760	ND	ND	0.0229	ND	0.0930	0.0599
10/5	169.5	0.4909	2.9075	10.3993	ND	0.9104	1.9183	0.1866	0.0501	ND	0.1266	3.4766	0.0468	0.0681	0.5267	ND	ND	ND	ND	ND	0.0370
10/6	237.6	0.3550	3.6171	19.0366	ND	1.0371	1.8454	0.2532	0.0025	0.0002	0.0819	4.3154	0.0225	0.1017	0.3090	ND	ND	0.0298	ND	0.0658	0.1228
10/18	108.3	0.0483	1.0966	9.0135	ND	0.3948	0.7552	0.0710	ND	ND	0.0306	1.8031	0.0066	0.0428	0.1694	ND	ND	0.0144	ND	0.0371	0.0109
10/19	137.8	2.2879	5.7176	6.3899	0.1013	1.3661	2.2101	0.2138	0.0047	ND	0.1446	4.7886	0.0471	0.0680	0.4810	ND	ND	0.0310	ND	0.3812	0.0506
10/20	135.1	0.0686	0.9318	6.1333	1.3968	0.5463	0.9538	0.2141	ND	0.0025	0.0463	2.0955	0.0036	0.0392	0.4685	ND	ND	0.0331	ND	ND	0.0248
10/25	108.6	0.4765	2.4860	7.0473	ND	0.8460	1.9652	0.1061	ND	ND	0.0352	2.3391	0.0170	0.0689	0.1084	ND	ND	0.0092	ND	0.0846	0.0655
10/26	254.0	1.2870	5.5563	18.7122	ND	1.3642	1.6022	0.2546	0.0143	0.0122	0.0566	3.9833	0.0378	0.0599	0.3321	ND	0.0220	0.0612	ND	ND	0.0232
10/27	182.4	2.7129	7.8908	8.3810	ND	2.1157	2.7496	0.2927	ND	ND	0.1000	5.2919	0.0098	0.1098	0.3258	ND	ND	0.0220	ND	0.0902	0.0439
11/15																					
11/16																					
11/17																					
11/29	135.3	0.5636	3.0505	7.7024	ND	0.7022	1.5627	0.0909	ND	0.0036	0.0477	4.6017	0.0068	0.1159	0.1920	ND	ND	0.0093	ND	0.4206	0.0273
11/30	126.6	1.8597	5.1546	4.3959	4.4382	1.0500	2.8272	0.2557	0.1560	ND	0.2272	4.5743	0.1406	0.0585	0.6954	ND	ND	0.0209	ND	0.0854	0.0299
12/1																					
12/6	487.1	ND	3.3019	27.5434	1.0528	1.4512	1.5331	0.1714	0.0751	ND	0.0347	3.2284	0.0408	0.0727	0.3820	ND	0.0602	0.1662	ND	ND	0.0480
12/7	219.2	ND	1.7473	20.4689	ND	0.6513	0.9925	0.0664	ND	ND	0.0699	2.1388	0.0059	0.0593	0.2303	ND	ND	0.0403	ND	0.1541	0.0631
12/8	165.5	0.5951	4.6023	10.1235	ND	0.9759	2.0253	0.1394	ND	0.0100	0.1615	7.3146	0.0357	0.1530	0.6839	ND	ND	ND	ND	0.8268	0.0136

<sup>a</sup> All dates are in 1999. All concentrations are in micrograms per cubic meter of air ( $\mu\text{g}/\text{m}^3$ ). ND = not detectable (below the limit of detection).

<sup>b</sup> On 5 days, filters were not analyzed due to insufficient particle mass or other technical difficulties.

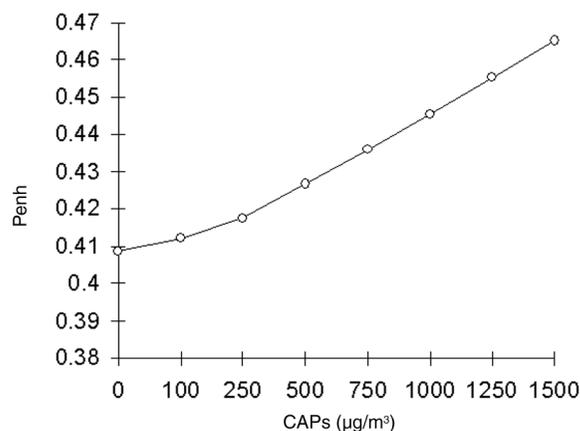
<sup>c</sup> As measured on the filter assembly used for trace metal concentrations.



**Figure 3. Pulmonary function tests performed immediately after pollutant exposure on each of 3 days.** Penh is an indicator of airway responsiveness. Data were collected at baseline and after exposure to 12 mg/mL MCh in a whole-body plethysmograph and are shown as the means  $\pm$  SEM for each exposure group ( $n = 60\text{--}72$  mice/group) from all 12 experiments.

every 100- $\mu\text{g}/\text{m}^3$  increase in CAPs was calculated to be 0.86% (see also the Discussion section). To put this increase in perspective, one can examine the slopes of the lines in Table 3. The slope of the line for OVA challenge in the unit [CAPs] analysis (ie, comparing control with asthmatic mice) is 0.27265060, whereas that for CAPs exposure is 0.00008645; this means that the effect of CAPs (or ambient particle) exposure is smaller by more than 3000-fold than the effect of challenge with the allergen OVA.

For the important subgroup of animals exposed to CAPs + O<sub>3</sub>, the results of this analysis were less clear. Specifically, cumulative concentration analysis showed no effect (Table 3). Use of individual days' unit [CAPs] (ie, noncumulative) showed a decrease in Penh, but no significant change when log [CAPs] was used. Due to this ambiguity



**Figure 4. Estimated increases in baseline Penh for increasing [CAPs].** Based upon multivariable statistical analysis showing increased airway responsiveness as a result of CAPs exposure, estimates of increases in Penh at baseline caused by specific [CAPs] were calculated. Baseline Penh value (= 0.40867) in these calculations was assumed to be the average baseline in the PBS-challenged air-exposed group for the 3 days of exposure from all 12 experiments. Note that the highest [CAPs] actually achieved during these exposures was  $\sim 1,500 \mu\text{g}/\text{m}^3$ . As a result, estimates were calculated for 0 to 1,500  $\mu\text{g}/\text{m}^3$  CAPs.

between linearized and nonlinearized [CAPs] and the resulting effects on the analysis, we concluded that this effect is not significant, and that high [CAPs] are skewing the data in the nonlinearized (unit) analyses.

### Analysis III: Multivariable Regression Analysis Considering the Elemental Composition of Concentrated Ambient Particles

Although CAPs is a complex mixture of environmental particles, the previous analyses that considered only the concentrations of CAPs failed to take into account the elemental complexity. As can be seen in Table 2, mice were exposed to many different elements at concentrations that varied daily. Hence, no two CAPs exposures can be considered to be identical. One way to account for these compositional differences during exposure would be to analyze the data considering all the concentrations of the various elements for each day. In practice, this can only be done with a large number of exposure days because so many elements (20) need to be taken into account. Our data set contained 36 dates of exposure (of which the data from only 31 were technically adequate or sufficient for analyzing elemental content) making it statistically impractical to do a full elemental analysis.

Instead, we considered that certain elements tend to occur together: Their concentrations are correlated, going up or down in concert. To determine which elements

**Table 3.** Statistical Results<sup>a</sup>

Significant Variable	Unit [CAPs]	Log [CAPs]
<b>CAPs Concentrations Considered on a Daily Basis</b>		
OVA	0.27265060 ( $P = 0.0013$ )	0.29293889 ( $P = 0.0257$ )
O <sub>3</sub>	0.05012191 ( $P = 0.0393$ )	NS
CAPs	0.00008645 ( $P < 0.0001$ )	0.02882399 ( $P < 0.0001$ )
CAPs + O <sub>3</sub>	-0.00015492 ( $P = 0.0223$ )	NS
<b>CAPs Concentrations Considered Cumulatively over 3 Days</b>		
OVA	0.27832301 ( $P = 0.0015$ )	0.3065659 ( $P = 0.0093$ )
O <sub>3</sub>	NS	NS
CAPs	0.00008966 ( $P < 0.0001$ )	0.02656284 ( $P < 0.0001$ )
CAPs + O <sub>3</sub>	NS	NS

<sup>a</sup> Data are presented as slopes of the regression line with  $P$  values in parentheses. Slopes represent changes in log Penh per unit [CAPs] or changes in log Penh per log [CAPs] as noted. These variables of interest were significant in influencing the magnitude of Penh at baseline after adjusting for these factors in the regression model: MCh; Buxco plethysmograph system; and the interactions of MCh and Buxco system, OVA and MCh, day and OVA, day and OVA and MCh, and MCh and day.

occurred together during the exposures, and could thus be considered as one compositional factor, we conducted a rotated factor analysis (Koutrakis and Spengler 1987; see the Statistical Analyses section). This analysis yielded six elemental groups that accounted for 99% of the composition of the particles to which the mice were exposed: an AlSi group, a BrSe group, a Cr group, a NiV group, a PbCu group, and a S group. Having only six compositional elements to consider in the statistical analysis of pulmonary responses to CAPs exposure made such an analysis feasible for the 31 days of data. It is worth noting that (1) each of the six groups includes a number of other associated elements, not simply those that name the group (eg, the AlSi group also includes S, K, Ca, Ti, Cr, Mn, Fe, and Cu; see Koutrakis and Spengler 1987), and (2) most elements are included in more than one group.

Because we analyzed only six different elemental groups, we chose to consider results to be statistically significant if the  $P$  value was less than 0.0083 (0.05/6 elemental groups). These significant effects are marked in Table 4. Other slopes,  $P$  values, and confidence intervals are shown for factors that are marginally significant but do not quite achieve the more stringent  $P$  value we chose.

When we considered elemental concentrations on a daily basis (rather than cumulatively), only two statistically significant relationships were found. In the first, asthmatic animals exposed to CAPs + O<sub>3</sub>, specifically on days when the concentration of AlSi was high, showed significant increases in baseline Penh. Note that the effects seen in this group of mice are synergistic; that is, the effect of the combined condition (asthma) and the exposure (CAPs + O<sub>3</sub>) is greater than additive. An estimate of the

magnitude of this response, generated by the regression equation in this analysis, is shown in Figure 5.

In the second, nonasthmatic (PBS-challenged) control animals exposed only to CAPs, specifically on days when the S concentration was high, showed significant increases in baseline Penh. Note that the slope of this line is less steep than that described for AlSi in the OVA + O<sub>3</sub> group (0.011637 and 0.068752, respectively).

When concentrations were considered cumulatively, the same positive correlations were found: AlSi increased responses in asthmatic mice exposed to CAPs + O<sub>3</sub>; and control mice exposed only to CAPs showed increased Penh due to S. In addition, significant effects were seen in other groups using this analysis. Some of these correlations were of marginal significance and some associations had negative slopes (eg, in asthmatic mice, the Cr factor is associated with a significant decrease in baseline Penh; see Table 4).

Given that some elemental factors have positive slopes and some have negative slopes, one can conceive of days on which the various factors occur in such concentrations as to allow cancellation of one another. This would result in no CAPs effect overall. For example, the percentage of increase in baseline Penh in asthmatic mice exposed to 50  $\mu\text{g}/\text{m}^3$  AlSi (the highest concentration of AlSi achieved during these experiments was 52  $\mu\text{g}/\text{m}^3$ ) compared with that of animals exposed to no AlSi is 63% (see Figure 5). This is deceiving, however, because exposure exclusively to AlSi did not occur. On the days of exposure, the mice were exposed to other elements in addition to AlSi. Thus, increases in Penh attributable to AlSi may be offset by decreases in Penh attributable to

**Table 4.** Changes in Penh Analyzed by Compositional Groups in CAPs<sup>a</sup>

	Total CAPs	AlSi	NiV	PbCu	S	Cr
<b>Concentrations Considered on a Daily Basis</b>						
PBS						
Slope <sup>b</sup>	NS	NS	NS	NS	0.011637 <sup>c</sup>	NS
P value					0.0063	
95% CI <sup>d</sup>					0.0033, 0.0200	
OVA						
Slope	0.018306 <sup>e</sup>	NS	NS	NS	NS	NS
P value	0.0267					
95% CI	0.0021, 0.0345					
PBS + O <sub>3</sub>						
Slope	0.019550 <sup>e</sup>	NS	0.038598	NS	NS	NS
P value	0.0218		0.0154			
95% CI	0.0028, 0.0363		0.0069, 0.0702			
OVA + O <sub>3</sub> <sup>f</sup>						
Slope	NS	0.068752 <sup>c</sup>	NS	-0.045785	NS	NS
P value		< 0.0001		0.0154		
95% CI		0.0357, 0.1018		-0.0828, -0.0088		
<b>Concentrations Considered Cumulatively over 3 Days</b>						
PBS						
Slope	NS	NS	NS	NS	0.009840 <sup>c</sup>	
P value					0.0046	
95% CI					0.0030, 0.0166	
OVA						
Slope	0.015645 <sup>e</sup>	NS	NS	NS	NS	-0.032110 <sup>c</sup>
P value	0.0331					0.0038
95% CI	0.0021, 0.0345					-0.0538, -0.0104
PBS + O <sub>3</sub>						
Slope	0.015997 <sup>e</sup>	NS	NS	NS	NS	-0.047553 <sup>c</sup>
P value	0.0350					0.0003
95% CI	0.001, 0.0309					-0.0736, -0.0215
OVA + O <sub>3</sub> <sup>f</sup>						
Slope	NS	0.077772 <sup>c</sup>	NS	-0.063506	NS	NS
P value		0.0001		0.0099		
95% CI		0.0468, 0.1087		-0.1117, -0.0153		

<sup>a</sup> Factor analysis was conducted on the 31 days of exposure for which x-ray fluorescence (elemental) analysis was available (see Table 2). Total CAPs analysis was conducted on the same 31 days of exposure. The BrSe elemental group is not included in this table because we found no significant associations. NS = not significant.

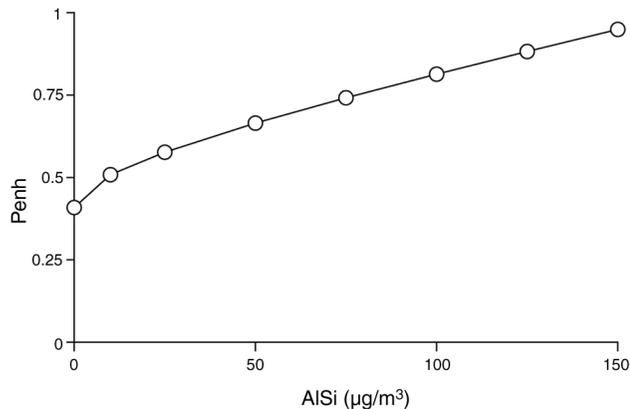
<sup>b</sup> Slope of the regression line.

<sup>c</sup> Statistically significant at  $P < 0.0083$ .

<sup>d</sup> 95% CI for the slope of regression line.

<sup>e</sup> Statistically significant at  $P < 0.05$ .

<sup>f</sup> This row represents the *synergistic* effects of OVA challenge combined with exposure to O<sub>3</sub>; ie, are the effects of asthma plus O<sub>3</sub> exposure greater than the additive effects of challenge with OVA and exposure to O<sub>3</sub> as individual factors?



**Figure 5. Estimated increases in baseline Penh for increasing concentrations of AlSi.** Based upon multivariable statistical analysis showing increased airway responsiveness in asthmatic animals exposed to  $O_3$  resulting from AlSi exposure, estimates of increases in Penh at baseline caused by specific concentrations of AlSi were calculated. Baseline Penh (= 0.40867) in these calculations was assumed to be the average baseline in the PBS-challenged air-exposed group for the 3 days of exposure from all 12 experiments. Note that the highest concentration of the AlSi factor actually achieved during these exposures was  $\sim 150 \mu\text{g}/\text{m}^3$ . As a result, estimates were calculated for 0 to  $150 \mu\text{g}/\text{m}^3$  AlSi.

other elements found in the CAPs mixture. The resulting effect of CAPs in these animals ultimately falls somewhere between the extremes of the negative and positive effects from different elements.

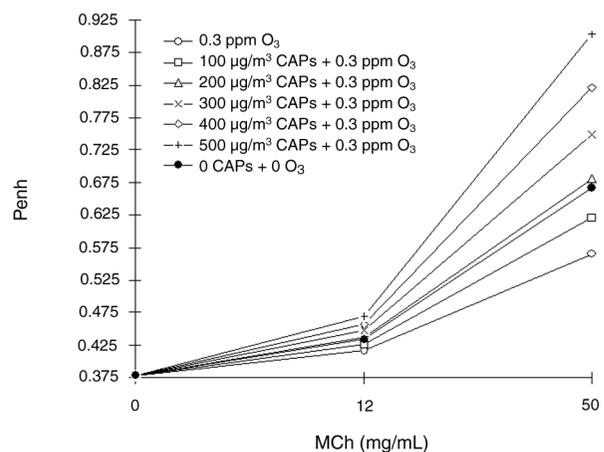
#### Analysis IV: Concentrations of Ambient Particles and Methacholine Concentration-Response

In the final five experiments, two important changes were made to the protocol based on our evaluation of interim data. First, to improve our ability to detect airway responsiveness, pulmonary responses to 50 mg/mL MCh were assessed each day immediately after pollutant exposures, in addition to baseline (no MCh) and 12 mg/mL MCh as had been done in experiments 1 through 7. Second, because no effects had been detected in pulmonary function tests performed starting 24 hours after the last OVA or PBS challenge in the first seven experiments (see below), this step was discontinued. Instead, lavage analysis was performed beginning at 24 hours after OVA or PBS challenge to detect any changes in BAL fluid that were not seen when we performed them starting at 48 hours after the last challenge.

When these results were analyzed, clear statistically significant increases in Penh were seen in animals exposed to CAPs +  $O_3$  as a function of MCh concentration ( $P = 0.0045$ ). Of particular interest, exposure to  $O_3$  alone caused airway hyporesponsiveness ( $P = 0.0012$ ), suggesting that the animals in the combined exposure group showed airway hyperresponsiveness regardless of the negative effects of  $O_3$

exposure alone. Increases in Penh of borderline statistical significance were seen in response to CAPs exposure alone as a function of MCh concentration ( $P = 0.1075$ ). The differences in statistical outcome when the last five experiments were compared with all 12 experiments may reflect (1) the inclusion of two MCh concentrations in the model; and (2) the absence of relatively high outlier CAPs concentrations in the last five experiments (see Table 1).

For exposures to CAPs +  $O_3$  for both asthmatic and control animals, estimates of the effect of increasing [CAPs] on airway responsiveness were generated from the equations that resulted from Analysis II and are shown in Figure 6. Although the increase in Penh is statistically significant, it is relatively small given the increases in particle concentrations found in real-world exposures, as was also seen in Figure 4. For example, exposure to  $300 \mu\text{g}/\text{m}^3$  CAPs +  $O_3$  (0.3 ppm) elevated Penh after 12 or 50 mg/mL MCh by 3.2% and 12.0%, respectively, over responses calculated for unexposed animals (no CAPs, no  $O_3$ ; see also the Discussion section). On the other hand, if one compares the effects of combined exposure to CAPs +  $O_3$  with those of  $O_3$  exposure alone, increased airway responsiveness is more readily apparent at lower [CAPs] because exposure to  $O_3$  alone depressed the Penh response. For example, if mice were exposed to  $100 \mu\text{g}/\text{m}^3$  CAPs +  $O_3$ , their Penh values at 12 and 50 mg/mL MCh were increased by 2.3%



**Figure 6. Estimated increase in Penh as a function of MCh concentration and its interaction with  $O_3$  and different concentrations of CAPs.** Based upon multivariable statistical analysis showing increased airway responsiveness as a result of interactions between MCh and  $O_3 \pm$  CAPs exposures, estimated increases in Penh for specific exposure conditions were calculated. Baseline Penh (= 0.378) at 0 MCh in these calculations was assumed to be the average baseline in the PBS-challenged air-exposed group for the 3 days of exposure from the five experiments (8 through 12) that included the broader MCh concentration-response. Note that the highest concentration of CAPs actually achieved during these exposures was  $\sim 500 \mu\text{g}/\text{m}^3$ . As a result, estimates were calculated for 0 to  $500 \mu\text{g}/\text{m}^3$  CAPs.

and 9.8%, respectively, over responses calculated for animals exposed only to O<sub>3</sub>.

As seen with other analytic approaches, the model of asthma was effective in this subset of five experiments. Penh increased as a function of both MCh ( $P < 0.0001$ ) and as a function of MCh and day (1, 2, or 3;  $P < 0.0001$ ) in the OVA-challenged groups; and Penh decreased significantly in both asthmatic and control animals exposed only to O<sub>3</sub> as a function of MCh concentration ( $P = 0.0012$ ).

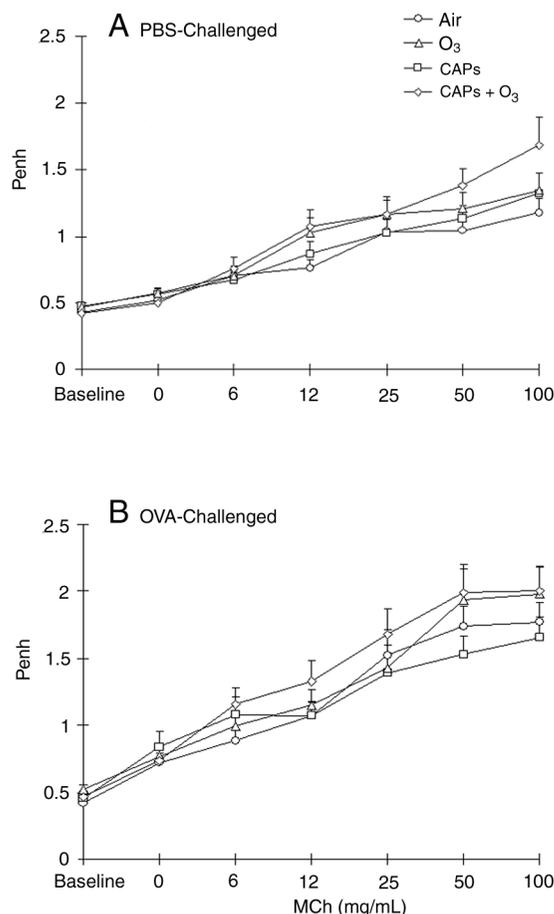
#### AIRWAY RESPONSIVENESS: STARTING 24 HOURS AFTER OVA OR PBS CHALLENGE

In experiments 1 through 7, pulmonary function tests were performed starting 24 hours after the last OVA or PBS challenge. This allowed us to evaluate a full MCh concentration-response curve including baseline (no aerosol given to animals), 0 (PBS vehicle only), 6, 12, 25, 50, and 100 mg/mL MCh. Using the statistical approach described in Analysis IV, which accounts for [CAPs] and MCh challenge in the model, none of the pollutant exposures resulted in significant effects compared with the negative controls (Figure 7). As expected, animals challenged with OVA (asthmatic mice) showed statistically different curves from those challenged with PBS ( $P < 0.001$ ).

#### ASSESSMENT OF CELLS IN BRONCHOALVEOLAR LAVAGE FLUID

Animals were killed starting at either 24 hours (experiments 8 through 12;  $n \geq 25$  animals per exposure group) or 48 hours (experiments 1 through 7;  $n \geq 35$  animals per exposure group) after the final OVA or PBS challenge. Lung lavage was conducted and total and differential cell counts were done. These data from lavage fluid analysis are shown in Table 5. Histopathologic evaluation showed changes consistent with allergic inflammation in the OVA-challenged group: mucous cell hyperplasia; and peribronchial and perivascular mononuclear cell infiltrates that contain scattered eosinophils. No differences among exposure groups were seen.

When animals were killed 24 hours after exposure, all asthmatic animals had higher numbers of total cells than control animals, as we had expected; these increases were attributable to higher numbers of both macrophages and eosinophils in the lavage fluid. By 48 hours, the profile had changed. Animals exposed to CAPs, alone or in combination with O<sub>3</sub>, had decreased numbers of total cells and macrophages in their lavage fluid compared with the same values at 24 hours. Again, as expected, the asthmatic animals showed increased total cell numbers attributable to higher numbers of eosinophils in the lavage fluid.



**Figure 7.** MCh concentration-response conducted starting 24 hours after the last OVA or PBS challenge (experiments 1 through 7). Mice were evaluated in a whole-body plethysmograph and Penh values recorded at baseline (no aerosol), in response to vehicle alone (PBS), and to increasing concentrations of MCh in PBS. Values shown are means  $\pm$  SEM for each exposure group ( $n \geq 35$ –47 mice/group) and each concentration of MCh.

#### IN VITRO CYTOKINE (TNF- $\alpha$ AND MIP-2) PRODUCTION CAUSED BY PARTICLE SAMPLES

In experiments 8 through 12, a portion of the CAPs flow for animal exposures was collected onto filters and used for in vitro experiments. Particles were released from these filters into aqueous solution as described in the Methods section. These particle suspensions were used to stimulate in vitro cytokine production (TNF- $\alpha$  and MIP-2) by rat alveolar macrophages (as previously described by Imrich et al 1999). The alveolar macrophages were exposed to the same particle concentration (100  $\mu$ g/mL) collected in all 15 CAPs samples (3 days of exposure and sampling in each of 5 experiments). The responses of three different cell groups were analyzed: Some alveolar macrophages were used as control cells; others were primed for 3 hours with either 250 ng/mL LPS or 25 U/mL IFN- $\gamma$  to model the

**Table 5.** Total and Differential Cell Counts from Lavage Fluid Analysis<sup>a</sup>

Exposure	Total Cells ( $\times 10^5$ )	Macrophages ( $\times 10^5$ )	Eosinophils ( $\times 10^3$ )
<b>24 Hours After Pollutant Exposure</b>			
PBS / Air	1.70 $\pm$ 0.11	1.69 $\pm$ 0.11	0.60 $\pm$ 0.19
PBS / O <sub>3</sub>	2.02 $\pm$ 0.14	1.99 $\pm$ 0.14	2.08 $\pm$ 0.62
PBS / CAPs	2.28 $\pm$ 0.25	2.26 $\pm$ 0.25	1.67 $\pm$ 0.51
PBS / CAPs + O <sub>3</sub>	2.22 $\pm$ 0.17	2.21 $\pm$ 0.17	1.00 $\pm$ 0.27
OVA / Air	2.43 $\pm$ 0.12 <sup>b</sup>	2.26 $\pm$ 0.11 <sup>b</sup>	17.10 $\pm$ 3.13 <sup>b</sup>
OVA / O <sub>3</sub>	2.60 $\pm$ 0.18 <sup>b</sup>	2.46 $\pm$ 0.18 <sup>b</sup>	13.90 $\pm$ 2.29 <sup>b</sup>
OVA / CAPs	2.43 $\pm$ 0.17 <sup>b</sup>	2.27 $\pm$ 0.16 <sup>b</sup>	16.15 $\pm$ 1.93 <sup>b</sup>
OVA / CAPs + O <sub>3</sub>	2.72 $\pm$ 0.18 <sup>b</sup>	2.49 $\pm$ 0.18 <sup>b</sup>	21.99 $\pm$ 4.19 <sup>b</sup>
<b>48 Hours After Pollutant Exposure</b>			
PBS / Air	2.22 $\pm$ 0.24	2.18 $\pm$ 0.24	1.41 $\pm$ 0.55
PBS / O <sub>3</sub>	2.74 $\pm$ 0.33	2.73 $\pm$ 0.33	1.16 $\pm$ 0.29
PBS / CAPs	1.77 $\pm$ 0.15 <sup>c</sup>	1.76 $\pm$ 0.15 <sup>c</sup>	0.79 $\pm$ 0.27
PBS / CAPs + O <sub>3</sub>	1.81 $\pm$ 0.14 <sup>c</sup>	1.79 $\pm$ 0.14 <sup>c</sup>	1.27 $\pm$ 0.42
OVA / Air	2.31 $\pm$ 0.17 <sup>b</sup>	2.01 $\pm$ 0.15	29.08 $\pm$ 6.78 <sup>b</sup>
OVA / O <sub>3</sub>	2.85 $\pm$ 0.26 <sup>b</sup>	2.56 $\pm$ 0.24	28.30 $\pm$ 5.20 <sup>b</sup>
OVA / CAPs	2.27 $\pm$ 0.17 <sup>b, c</sup>	1.96 $\pm$ 0.14 <sup>c</sup>	27.80 $\pm$ 7.66 <sup>b</sup>
OVA / CAPs + O <sub>3</sub>	2.25 $\pm$ 0.14 <sup>b, c</sup>	1.95 $\pm$ 0.12 <sup>c</sup>	28.98 $\pm$ 5.53 <sup>b</sup>

<sup>a</sup> Values are expressed as means  $\pm$  SEM. Neutrophil and lymphocyte numbers each contributed  $< 1.0\%$  of the total cell population and, therefore, are not shown for ease of viewing and interpretation.

<sup>b</sup>  $P < 0.05$ , effects of OVA challenge.

<sup>c</sup>  $P < 0.05$ , effects of CAPs exposure when comparing 48-hour data with 24-hour data.

effects of preexisting inflammation. Cells were incubated with particles for 20 hours. At this point, supernatants were collected for cytokine analysis and cells were analyzed in the flow cytometer for viability and particle binding (as evidenced by right-angle light scatter; see the Materials and Methods section). Results are shown in Table 6.

Particle samples for in vitro analysis were collected during the mouse exposures in the fall of 1999. On the dates that particles were collected, however, the CAPs aerosols had few, if any, in vivo effects on the mice. Hence, the original goal of comparing in vitro activity with in vivo effects could not be achieved. Nevertheless, the results of the in vitro analyses confirm prior observations that priming lung macrophages substantially augments cytokine production in response to CAPs exposure. The data also show substantial variability in the responses caused by different CAPs samples.

Using the elemental analysis employed for the mouse physiologic responses, statistical analyses of the in vitro data were conducted. However, only 11 of the 15 days of in vitro data could be used for this analysis because (1) on

3 days the particle concentrations were so low that the amount collected onto the filter precluded elemental analysis by x-ray fluorescence (11/15/99–11/17/99), and (2) on the fourth day the filter was damaged (12/1/99). In this analysis, with limited statistical power, no associations between elemental content and either TNF- $\alpha$  or MIP-2 production were found.

## DISCUSSION

These studies produced several major findings. We found that exposure to CAPs alone caused an increase in airflow obstruction (as measured by changes in Penh) in both control and asthmatic mice. The magnitude of this effect was small (approximately 0.9% for each increase of 100  $\mu\text{g}/\text{m}^3$  CAPs) and the effect was transient (seen immediately after exposure but not 24 hours later). Furthermore, the composition of the particles was an important determinant of effects on airway physiology. The analyses we conducted were complicated; a summary of the major associations found using the various analytic approaches is provided in Table 7.

**Table 6.** In Vitro Production of Cytokines TNF- $\alpha$  and MIP-2 by Alveolar Macrophages Exposed to Concentrated Ambient Particles<sup>a</sup>

Sample	Control			Primed with Lipopolysaccharide			Primed with Interferon- $\gamma$		
	% Viable	Right-Angle Light Scatter	Cytokine (ng/mL)	% Viable	Right-Angle Light Scatter	Cytokine (ng/mL)	% Viable	Right-Angle Light Scatter	Cytokine (ng/mL)
<b>TNF-<math>\alpha</math> Production</b>									
Control	94.5	220	0.001	85.1	257	3.142	94.4	232	0.001
10/18	93.6	305	0.11	79.5	303	5.413	84.6	294	0.743
10/19	91.2	340	0.193	69.8	330	9.659	77.9	342	1.094
10/20	91.4	291	0.079	74.3	296	7.72	78.8	304	0.753
10/25	91.4	375	0.235	71.6	371	10.769	72.7	387	1.218
10/26	94	232	0.119	77.4	322	6.384	81.2	324	0.825
10/27	94.7	291	0.096	79.9	298	4.395	83.4	291	0.698
11/15	86.1	387	0.866	71.5	383	11.404	68.6	388	2.916
11/16	85	363	1.544	69.1	362	12.629	69.6	380	6.24
11/17	83.8	392	2.101	69.9	375	12.268	72.5	381	6.122
11/29	91.7	308	0.272	76.4	316	6.191	80.2	328	0.601
11/30	88.4	348	0.301	69	333	8.775	72.7	355	1.254
12/1	80.1	427	2.624	62.8	385	14.84	66.5	417	7.265
12/6	95.2	273	0.071	86.7	292	3.684	92.2	269	0.295
12/7	95	283	0.054	82.5	294	4.532	89.6	279	0.347
12/8	92.6	326	0.157	75.9	320	6.586	80.1	326	0.532
UAP	76.4	339	1.34	65.3	345	20.489	70.8	365	2.932
LPS	85.5	261	19.487	79.6	274	19.06	66.3	276	80.761
<b>MIP-2 Production</b>									
Control	94.5	220	0.001	85.1	257	7.516	94.4	232	0.001
10/18	93.6	305	2.653	79.5	303	18.59	84.6	294	3.354
10/19	91.2	340	5.508	69.8	330	29.55	77.9	342	5.651
10/20	91.4	291	4.07	74.3	296	26.94	78.8	304	4.503
10/25	91.4	375	8.41	71.6	371	27.29	72.7	387	6.162
10/26	94	232	6.07	77.4	322	26.39	81.2	324	5.084
10/27	94.7	291	3.812	79.9	298	22.17	83.4	291	3.774
11/15	86.1	387	17.33	71.5	383	36.58	68.6	388	8.521
11/16	85	363	19.13	69.1	362	38.72	69.6	380	13.1
11/17	83.8	392	24.86	69.9	375	39.28	72.5	381	12.97
11/29	91.7	308	5.492	76.4	316	25.45	80.2	328	4.589
11/30	88.4	348	6.2	69	333	33.13	72.7	355	6.536
12/1	80.1	427	26.98	62.8	385	36.84	66.5	417	15.05
12/6	95.2	273	1.528	86.7	292	17.79	92.2	269	1.212
12/7	95	283	2.475	82.5	294	20.53	89.6	279	1.966
12/8	92.6	326	4.331	75.9	320	25.07	80.1	326	4.512
UAP	76.4	339	10.33	65.3	345	27.57	70.8	365	10.79
LPS	85.5	261	101.4	79.6	274	109.1	66.3	276	89.55

<sup>a</sup> Cells were exposed to 100  $\mu$ g/mL CAPs.

**Table 7.** Summary of Major Findings

Linear Regression Analysis	Significant Outcomes	Strength of Effect
I: CAPs treated as a category	Exposure to CAPs or to CAPs + O <sub>3</sub> increased Penh	Example: On day 2 of exposure, control and asthmatic mice exposed to CAPs + O <sub>3</sub> showed increased Penh at baseline of 31% and 15%, respectively, compared with air-exposed controls
II: [CAPs] considered	Exposure to CAPs increased baseline Penh	~0.9% per 100- $\mu\text{g}/\text{m}^3$ increase in CAPs
III: Elemental composition of CAPs considered	[AlSi] in CAPs associated with increased Penh in asthmatic (OVA-challenged) mice exposed to O <sub>3</sub> [S] in CAPs associated with increased Penh in control mice	Statistically significant (Table 4), but cannot be considered in isolation (see Figure 5 and Analysis IV: CAPs Concentrations and Methacholine Concentration-Response section under Results)
IV: [CAPs] and [MCh] considered	As a function of [MCh]: Exposure to CAPs + O <sub>3</sub> increased Penh	Example: Mice exposed to 300 $\mu\text{g}/\text{m}^3$ CAPs + O <sub>3</sub> showed 3% and 12% increase in Penh at 12 and 50 mg/mL MCh, respectively, compared with air-exposed controls

The context for these studies is the epidemiologic observation that elevations in ambient air particle concentrations or O<sub>3</sub> are associated with adverse effects on people with asthma, a disorder with relatively high prevalence. Although numerous effects have been reported, the proportion of all people with asthma who experience measurable outcomes (eg, visits to emergency rooms) has not been well established but is likely to be small. Hence, one interpretation of our data is that the small effect we found is consistent with these epidemiologic observations.

In addition, if one examines the epidemiologic literature to calculate the changes in respiratory parameters that result from exposure to ambient particles or to surrogate particles, the small changes we found in this study are historically consistent. Much of the literature utilizes reports of hospital admissions, school absenteeism, or self-reported worsening of symptoms (such as shortness of breath or use of bronchodilatory agents) as negative outcomes that are associated with particulate pollution. In those studies that examined pulmonary function parameters as endpoints, often no association between particle pollution and pulmonary function was found. For example, Dockery and coworkers conducted one of the most extensive studies of the effects of particles (and other pollutants) on pulmonary function as part of the Harvard Six Cities Study of Air Pollution and Health. Although an association between certain air pollution components (particle components, SO<sub>2</sub>, and NO<sub>2</sub>) and increased respiratory illness or symptoms (eg, chronic cough, bronchitis, chest illness) was found, no associations were found

between pollutants and measures of pulmonary function (Ware et al 1986; Dockery et al 1989). More recently, using human volunteers, Nightingale and colleagues (2000) found no changes in FEV<sub>1</sub>, FVC, or responsiveness to MCh at 4 or 24 hours after a 2-hour exposure to 200  $\mu\text{g}/\text{m}^3$  diesel exhaust. Other studies that showed no significant association between particle concentrations and pulmonary function include Dodge (1983), Hiltermann and coworkers (1998), Roemer and associates (1998), and Titanen and colleagues (1999).

When associations between pulmonary function changes and particle pollution were detected, relatively small declines in pulmonary function were found. For example, Pope and colleagues (1991) found declines of ~2–6% in peak expiratory flow (PEF) in asthmatic subjects ages 8 through 72 that were associated with PM<sub>10</sub> concentrations of greater than 50  $\mu\text{g}/\text{m}^3$ . Interestingly, same-day PM<sub>10</sub> concentrations had the largest effect on PEF, which we also found to be true for changes in Penh in this study. Also of note is that both asthma patients and age-matched control subjects showed declines in PEF associated with increasing concentrations of PM<sub>10</sub>; again, this is similar to what we report here for mice. Other research by these investigators showed that, for PM<sub>10</sub> concentrations of 150  $\mu\text{g}/\text{m}^3$ , PEF declined ~1.8% and 1.3% for asthmatic and healthy children, respectively (Pope and Dockery 1992). Changes in PEF in that study were more closely associated with 5-day moving averages than with same-day concentrations of PM<sub>10</sub>.

Other researchers have observed similar declines in pulmonary function. Roemer and colleagues (1993) found declines in morning and evening PEF in children with chronic respiratory symptoms; the declines ranged from 0.5% to 2.6% for every 100- $\mu\text{g}/\text{m}^3$  increase in  $\text{PM}_{10}$  and were dependent upon time of day and on whether the  $\text{PM}_{10}$  concentrations were analyzed on the same day as, or averaged for up to 7 days after, the PEF measurements. In other studies that measured morning PEF in children, declines associated with increasing concentrations of  $\text{PM}_{10}$  ranged from 0.27% associated with a 10- $\mu\text{g}/\text{m}^3$  increase in  $\text{PM}_{10}$  (Timonen and Pekkanen 1997) to 1.5% for an increase of 20  $\mu\text{g}/\text{m}^3$   $\text{PM}_{10}$  (Koenig et al 1993). Many other studies have shown intermediate effects (Wjst et al 1993; Hoek and Brunekreef 1994; Yang and Yang 1994; Romieu et al 1996; Peters et al 1997).

The experimental design for the experiments reported here included collecting data that might allow the correlation of physiologic effects with the composition of CAPs. The idea that the composition of the particles elicits physiologic changes is not new. Many studies have been conducted, both in vivo and in vitro, that emphasize the importance of particle composition when analyzing the physiologic and biochemical effects of particle pollution. Metals, endotoxin, and polycyclic aromatic hydrocarbons have all been targeted as likely suspects for causing the adverse effects attributed to particles (Davison et al 1974; Natusch et al 1974; Natusch and Wallace 1974; Linton et al 1976; Castranova et al 1984; Hatch et al 1985; Dreher et al 1996; Kodavanti et al 1997; Vincent et al 1997; Bonner et al 1998; Goldsmith et al 1998; Dye et al 1999; Ghio et al 1999; Imrich et al 1999; Adamson et al 2000; Jimenez et al 2000). Although compositional data were available for 31 of our 36 experimental days, the number of elements we found in ambient particles (20) prevented statistical analysis of the data considering each element separately. Instead, we exploited the fact that certain elements tend to occur together: Their concentrations are correlated, going up or down in concert. We conducted a rotated factor analysis (Koutrakis and Sioutas 1996) and analyzed the elemental groups for associations with airway responsiveness. Our data indicated that the aluminum-silicon fraction of particles was most detrimental to the animals on the days of our exposures. Because composition varies daily and from location to location, it is likely that particle samples from other geographic areas would have other components that have negative physiologic impact.

A number of advantages and limitations of the experimental design merit discussion. The concentration and composition of ambient air particles vary from day to day, as reflected in our data. The 12 weeks studied included

summer, fall, and winter periods and evaluated 36 exposure days. However, we cannot exclude the possibility that the data underestimate (or overestimate) effects of CAPs that might occur on other days. The susceptibility of mouse strains to inhaled toxicants also varies (Ichinose et al 1997; Miyabara et al 1998d), which raises the possibility that the BALB/c strain we used is not susceptible to air pollution. Our previous findings that OVA-challenged BALB/c mice showed increased airway responsiveness and allergic inflammation in response to residual oil fly ash aerosols argue against this possibility (Goldsmith et al 1999b).

One substantial advantage of this study was that we examined the pulmonary function of mice throughout their exposures by using whole-body plethysmography. Conventional methods of pulmonary function assessment would have resulted in the death of the mice and would have precluded using such large numbers of mice because they are much more time consuming. The large number of mice we used gives power to the statistical analysis. Given that the changes in pulmonary function were relatively small, it is unlikely the data would have reached statistical significance if fewer animals had been studied. One may argue that Penh has not yet been rigorously tested; but Penh has been shown to correlate well with changes in pulmonary resistance (Hamelmann et al 1997). However, even with the increased ability to handle large numbers of animals, in the last five experiments we could challenge animals with only two concentrations of MCh at the end of each exposure day. This was practicable only because we had fewer groups to analyze in these experiments. With the second MCh concentration in these experiments, airway responsiveness could be better studied. With this analysis, it was apparent that the combination of CAPs plus  $\text{O}_3$  caused an increase in airway responsiveness.

To further investigate whether particle composition correlated in some way with physiologic effects, we used suspensions of CAPs collected from the last five experiments (15 days) to evaluate the ability of the particles to stimulate proinflammatory cytokine production by alveolar macrophages in vitro. Because the effects of CAPs exposure on the mice was relative small when analyzed using data from all the experiments, exposure to particles (alone or in combination with  $\text{O}_3$ ) on only these 15 days did not produce an identifiable increase in physiologic parameters over controls. This then precluded our ability to correlate in vitro bioactivity with in vivo effects. However, the in vitro analysis did allow us to confirm the day-to-day variability in the bioactivity of CAPs. The data also argue against the possibility that the bioactive components of CAPs are present in a constant proportion and that the health effects result from simple increases in the amount of particles in the air.

This conclusion stems from the fact that the 15 CAPs samples showed markedly different bioactivity when assayed at a single, constant concentration.

The underlying hypothesis for this work (that CAPs plus O<sub>3</sub> would have synergistic effects on asthmatic mice) was not strongly supported by this study. Although synergistic effects were seen in one analysis (when composition of the particles was considered), synergistic effects were generally lacking. On the other hand, the effect of CAPs on the pulmonary function of all mice, regardless of asthmatic condition, was consistent for all analyses. The change in pulmonary function associated with particle exposure was small but significant and is likely reflective of what is seen in the human population.

The results illustrate some of the difficulties in accurately modeling pollutant effects using animal models and concentrations that approach realistic ambient levels. We used a concentration of O<sub>3</sub> (0.3 ppm) that is substantially greater than that commonly encountered in ambient air (< 0.08 ppm), but lower than that often used to elicit pulmonary effects in laboratory animals (≥ 0.8 ppm). The majority of experimental days resulted in CAPs concentrations in the low 100s of µg/m<sup>3</sup>. These exposures (for 5 hours) did yield some effects as noted, but they were of relatively small magnitude. Occasional exposure days resulted in larger effects on Penh when CAPs concentrations were nevertheless in the typical range (~60 to 200 µg/m<sup>3</sup>). Our results indicate that these responses can be attributed to the composition rather than the concentration of the particles on these days. It is possible that longer exposures or higher concentrations would result in greater effects; this remains to be investigated.

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#### ABOUT THE PRIMARY AUTHORS

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**Lester Kobzik** is a clinical and experimental pulmonary pathologist. He received his MD from Tufts University in 1979. He completed training in anatomic pathology and a fellowship in pulmonary pathology at the Brigham and Women's Hospital in Boston in 1985. He is associate professor of pathology at the Harvard Medical School and the Harvard School of Public Health.

**Carroll-Ann W Goldsmith** is a biologist with expertise in physiology and toxicology. She received her ScD from the Harvard School of Public Health and is currently a research associate there. Her research focuses on the effects of chemicals and pollutants on the lung, with particular emphasis on susceptible populations such as those with asthma.

**Paul J Catalano** is a biostatistician. He received his ScD from the Harvard School of Public Health and is currently

associate professor of biostatistics there. Dr Catalano's major research interests involve methods for analyzing multiple outcomes and repeated measures and their application to environmental dose-response modeling and quantitative risk assessment.

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#### ABBREVIATIONS AND OTHER TERMS

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Al(OH) <sub>3</sub>	aluminum hydroxide
ANOVA	analysis of variance
BAL	bronchoalveolar lavage
CAPs	concentrated ambient particles
EPA	Environmental Protection Agency (US)
FEV <sub>1</sub>	forced expiratory volume in 1 second
FVC	forced vital capacity
IFN-γ	interferon-γ
IL	interleukin
LPS	lipopolysaccharide
MCh	methacholine
MIP-2	macrophage inflammatory protein-2
NAAQS	National Ambient Air Quality Standard (US)
NO <sub>2</sub>	nitrogen dioxide
O <sub>3</sub>	ozone
OVA	ovalbumin
PBS	phosphate-buffered saline
Penh	enhanced (enh) pause (P)
PEF	peak expiratory flow
PEP	peak expiratory pressure
PIP	peak inspiratory pressure
PM <sub>2.5</sub>	particulate matter 2.5 μm or smaller
R1%	RPMI medium containing 1% fetal bovine serum
SO <sub>2</sub>	sulfur dioxide
SRM	Standard Reference Material
TNF-α	tumor necrosis factor α
UAP	urban air particle SRM



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## INTRODUCTION

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Particulate matter (PM\*) is a complex mixture of particles in the atmosphere. The size, chemical composition, and other physical and biological properties of these particles are highly variable from place to place and time to time because of differences in the sources (which may be natural in origin or generated by human activities such as driving vehicles and operating power or manufacturing plants), geographical conditions, weather, and seasonal patterns. Many epidemiologic studies have reported that short-term increases in low levels of PM are associated with short-term increases in morbidity and mortality. The strongest of these associations are found with older persons and with individuals who have compromised cardiac or airway function (reviewed in US Environmental Protection Agency [EPA] 1996a). People with asthma are affected by exposure to higher levels of PM. Their increased symptoms have been documented by an increase in hospital visits and by an increase in self-medication on high-pollution days (Dockery et al 1989; Pope et al 1991; Forsberg et al 1993; Yang and Yang 1994).

Plausible biological mechanisms that would link exposure to low levels of PM with increased morbidity and mortality, however, have not been established. In 1998, HEI issued RFA 98-1, "Characterization of Exposure to and Health Effects of Particulate Matter," to address gaps in our knowledge about PM's effects. Proposed areas of study included evaluating biologic mechanisms of exposure in appropriate animal models and the role of copollutants in modifying the possible effects of PM. In response to this RFA, Dr Lester Kobzik and colleagues at Harvard School of Public Health proposed to study the effects of PM exposure on airways using a mouse model of asthma. A particle concentrator that delivers particles concentrated to several times their level in ambient air would be used to maximize possible PM effects. Kobzik also proposed to evaluate the effects of PM in the presence of ozone (O<sub>3</sub>), a gaseous pollutant known to induce airway inflammation and to compromise respiratory function in many species (reviewed in US EPA 1996b). Kobzik hypothesized that the effects of PM plus O<sub>3</sub> would be synergistic, that is, greater than additive. The HEI Research Committee recommended funding the

proposal because they thought this approach to assessing the effects of PM and O<sub>3</sub> in a potentially relevant animal model was well thought out and innovative.<sup>†</sup>

This Critique is intended to aid HEI sponsors and the public by highlighting the strengths of the study, pointing out alternative interpretations, and placing the research into scientific perspective.

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## SCIENTIFIC BACKGROUND

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Asthma is a chronic disease of the lower airways characterized by inflammation, reversible airway obstruction, mucus hypersecretion, and airway hyperresponsiveness (which is exaggerated airway responsiveness to an agent that narrows the lumen of a bronchus or bronchiole). The most prevalent form of asthma in children and young adults is allergic asthma (American Thoracic Society Workshop 1999), which is associated with hypersensitivity to allergens, or atopy, and a tendency to airway hyperresponsiveness. It is characterized by the production of cytokines (such as interleukin [IL]-4, IL-5, and IL-10) that are synthesized by the T<sub>H</sub>2 subset of CD4<sup>+</sup> T lymphocytes, which trigger both the production of immunoglobulin E by B cells and the recruitment of eosinophils into the airways.

The prevalence and severity of asthma has increased worldwide in the last 20 years, especially in children. The reason for this upsurge of cases has not been established, but is likely to involve an interplay of genetic, socioeconomic, behavioral, and environmental factors. Several hypotheses have been put forward, including changes in exercise habits, increased exposure to airborne allergens derived from dust mites, pollens, and cockroaches (Platts-Mills 1989; von Mutius et al 1994; Malveaux and Fletcher-Vincent 1995; Rosenstreich et al 1997), and the decreased incidence of children developing bacterial or viral infections at an early age, which may skew their immune response toward a T<sub>H</sub>2 cytokine pattern (Shirakawa et al 1997).

Numerous epidemiologic studies have indicated that short-term exposure to particulate pollution exacerbates the symptoms of asthma (eg, Schwartz et al 1993; Lipsett et al 1997; Medina et al 1997) and can decrease lung function

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\* A list of abbreviations and other terms appears at the end of the Investigators' Report.

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.

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<sup>†</sup> The total costs for Dr Kobzik's 15-month study were \$215,202. The study began in September 1998 and was completed in February 2000. A draft report was received for review in March 2000 and a revised report was accepted for publication in November 2000. During the review process, the HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Report and in the Review Committee's Critique.

in people with asthma (eg, Pope et al 1991; Romieu et al 1996; Peters et al 1997). In addition, studies in animal models have shown that exposure to some components of PM can affect asthma-associated endpoints; for example, inhaling diesel exhaust particles increases allergic inflammation and airway hyperresponsiveness (Ichinose et al 1997; Miyabara et al 1998); and rats exposed by intratracheal instillation to residual oil fly ash, derived from power plant emissions, develop airway hyperresponsiveness and inflammation (Pritchard et al 1996; Dreher et al 1997).

Short-term exposure to O<sub>3</sub>, a prominent component of the ambient pollutant mixture in many locations, also decreases pulmonary function in people with asthma as well as in healthy subjects and induces an airway inflammatory response (reviewed in US EPA 1996b). Similar effects of O<sub>3</sub> have been observed in many animal models of asthma (US EPA 1996b).

Because the effects of fresh ambient particles on airway function had not been evaluated in an animal model of asthma, Kobzik and colleagues proposed to analyze the responses of mice to concentrated ambient particles (CAPs). The effects of O<sub>3</sub>, in the presence or absence of CAPs, on airway function were also to be evaluated. To more closely model responses of young children, they proposed to evaluate the effects in very young mice (3 weeks old). To explore the mechanism by which PM might affect the airways, the investigators proposed to measure the effects of CAPs on the production of inflammatory mediators by airway cells *in vitro*.

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## TECHNICAL EVALUATION

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### AIMS AND OBJECTIVES

This 15-month study had two objectives:

1. To determine whether exposure by inhalation to CAPs, O<sub>3</sub>, or the combination of CAPs plus O<sub>3</sub> increased airway responsiveness and inflammation in an animal model of asthma: young mice sensitized intraperitoneally and subsequently challenged intranasally with ovalbumin (OVA).
2. To determine whether resuspended CAPs induced cytokine and chemokine synthesis in rat airway cells *in vitro*.

The investigators originally planned to use two different concentrators to generate two different CAPs concentrations each day. However, the second concentrator was not operational during the study, so Kobzik and colleagues used one concentrator to generate two daily CAPs concentrations during part of the study period. The original

application also proposed to evaluate three O<sub>3</sub> concentrations, which would have required a large number of exposure groups; HEI recommended that the investigators use only two concentrations. Ultimately, only one O<sub>3</sub> concentration was evaluated, a decision made on the basis of preliminary results from the early part of the study and approved by HEI.

The study design is described in Figure 1 of the Investigators' Report.

### METHODS

#### Sensitization and Aerosol Challenge of Mice

BALB/c mice were sensitized with 10 µg OVA by intraperitoneal injection on days 7 and 14 after birth. Half of the mice, referred to as "asthmatic" or "OVA-challenged" mice in the Investigators' Report, were challenged with 3% OVA as a nebulized aerosol (median particle diameter 2.75 µm) for 10 minutes on days 21, 22, and 23 after birth. The other half of the mice received a phosphate-buffered saline (PBS) aerosol challenge ("nonasthmatic" or "PBS-challenged" mice).

#### Exposure to CAPs and O<sub>3</sub>

Mice were exposed to CAPs in a chamber connected to the Harvard Ambient Particle Concentrator (Sioutas et al 1995a,b; Godleski et al 2000). The concentrator preferentially concentrates ambient particles in the size range of 0.15–2.5 µm in median aerodynamic diameter (a subfraction of PM<sub>2.5</sub>). In some experiments, two concentrations of CAPs per day were used for exposure: the high concentration used the undiluted concentrated airstream; to achieve the low concentration, the investigators diluted the concentrated airstream with filtered air at the entry port to the exposure chamber. Ambient PM and CAPs concentrations varied widely from day to day (CAPs range was 63–1569 µg/m<sup>3</sup>) over the course of the study (May through December 1999), as did the factor by which the PM was concentrated (12-fold to 67-fold range). In addition, individual chemical components of the PM mixture varied on a daily basis as shown in Table 2 of the Investigators' Report.

O<sub>3</sub> was generated by passing 100% oxygen through a high-voltage discharge device and diluting it with filtered room air (< 10 ppb O<sub>3</sub>) to produce a concentration of 0.3 ppm O<sub>3</sub>. This mixture was pumped into the exposure chamber.

The protocols for measuring pollutant exposure and for evaluating airway responses are described in detail in Figure 1 of the Investigators' Report. On each of the 3 days that mice were challenged with OVA or PBS aerosol (days 21, 22, and 23 after birth), the mice were subsequently

exposed for 5 hours to CAPs, O<sub>3</sub>, CAPs plus O<sub>3</sub>, or filtered air in groups of five or six. Twelve experiments were performed, each comprising 3 consecutive days of pollutant exposure (ie, 36 different exposure days). In five of the twelve experiments, mice were exposed to one of six conditions: high CAPs concentration, 0.3 ppm O<sub>3</sub>, high CAPs concentration plus 0.3 ppm O<sub>3</sub>, filtered air, low CAPs concentration, or low CAPs concentration plus 0.3 ppm O<sub>3</sub>.

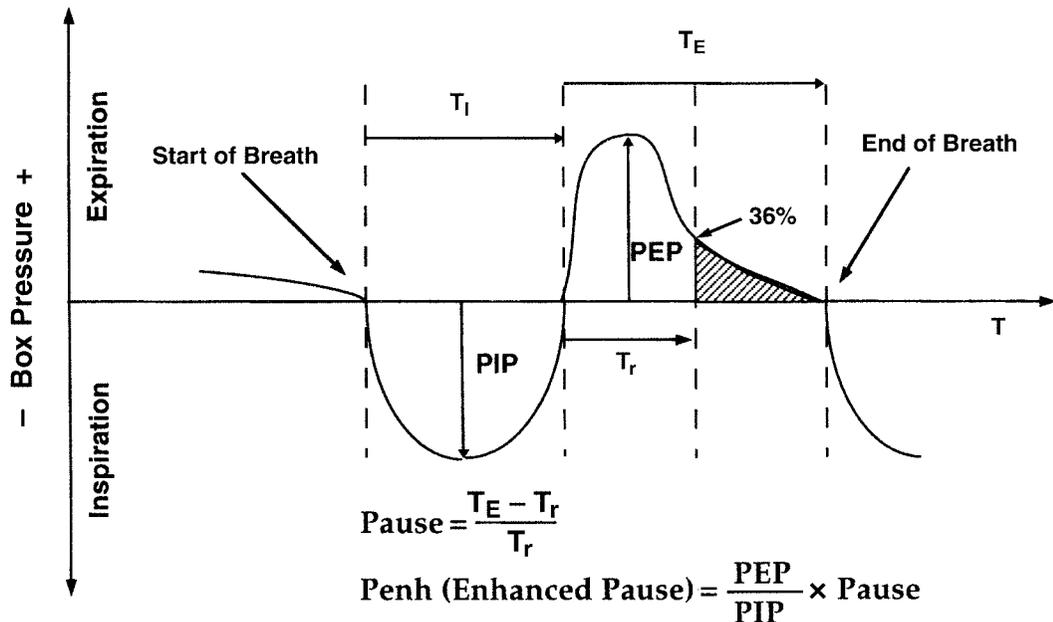
The investigators' initial analyses of these experiments suggested that low CAPs concentrations had no effect on the parameters they were testing. After discussions with HEI, the low CAPs exposures were discontinued and data from these groups were excluded from the final analyses. In the remaining seven of twelve experiments, the investigators exposed both sets of mice to one of four conditions: the highest CAPs concentration on each particular day, 0.3 ppm O<sub>3</sub>, CAPs plus 0.3 ppm O<sub>3</sub>, or filtered air.

**Measurement of Airway Responsiveness**

The investigators used a technique known as whole-body plethysmography to monitor changes in airway responsiveness after challenge with different aerosolized doses of the bronchoconstrictor methacholine (MCh). Airway responsiveness was quantified as enhanced (enh) pause (P) (or Penh), a parameter defined recently by Gelfand and colleagues (Hamelmann et al 1997).

The derivation of Penh for an individual animal is shown in Critique Figure 1, adapted from Hamelmann and associates (1997). It illustrates the changes in pressure that occur over the time course of a single breath. To measure Penh, a mouse was placed in an exposure chamber that was connected to a reference chamber; as the mouse breathed the MCh entering the exposure chamber, the difference in pressure between the two chambers (the "box pressure") was recorded. Penh is unitless and reflects changes in the waveform of the box pressure signal from both inspiration and expiration (PIP and PEP, respectively, in the diagram), combined with a measure of the duration of expiration.

In all twelve experiments, airway responsiveness testing was performed each day starting 30 to 120 minutes after the end of the 5-hour exposure to the pollutant atmosphere. In experiments 1 through 7, airway responsiveness testing was performed before (baseline) and after an aerosol challenge with 12 mg/mL MCh; in experiments 8 through 12, the challenges also included a 50 mg/mL MCh concentration. Furthermore, in experiments 1 through 7, airway responsiveness was also assessed on the day following the final exposure to the pollutant (starting approximately 19 hours after the end of the exposure period [24 hours after the final OVA or PBS challenge]). Airway responsiveness was assessed at baseline and after challenge with 6, 12, 25, 50, and 100 mg/mL MCh. In each



**Critique Figure 1. Respiratory parameters used to determine Penh during whole-body plethysmography.** The box pressure wave shows inspiration (curve down) and expiration (curve up). T<sub>1</sub> = inspiration time (seconds); T<sub>E</sub> = expiration time (seconds); PIP = peak inspiratory pressure (mL/sec), which is the maximal negative box pressure in one breath; PEP = peak expiratory pressure (mL/sec), which is the maximal positive box pressure in one breath; and T<sub>r</sub> = relaxation time (seconds), which is the time during exhalation until the box pressure drops to 36% of the PEP.

experiment, the investigators used two plethysmography systems (each containing 6 plethysmographs) to shorten the time needed to make airway responsiveness measurements. Possible differences between readings made in the different plethysmography systems were addressed by statistical analysis.

### Bronchoalveolar Lavage

To obtain cells in bronchoalveolar lavage fluid, the investigators killed mice either 24 hours (experiments 8 through 12) or 48 hours (experiments 1 through 7) after the start of the last OVA or PBS challenge and lavaged the lungs. They counted the total number of lavaged cells and evaluated how many were alveolar macrophages, eosinophils, neutrophils, or lymphocytes. Of these cell types, only macrophage and eosinophil numbers were shown in the Investigators' Report because the numbers of lymphocytes and neutrophils were very low in all groups.

### Physical and Chemical Characterization of PM Components

The investigators collected samples of particles in unconcentrated and concentrated ambient air on parallel sets of filters for the duration of each exposure period throughout the twelve experiments. They used Teflon filters to collect particles subsequently assessed for PM<sub>2.5</sub> mass concentration, particle size distribution, and sulfate, nitrate, and trace metal concentrations. A conductivity meter measured sulfate and nitrate; trace metals were analyzed by x-ray fluorescence. Black carbon was measured with an aethalometer on samples collected on quartz-fiber filters.

### In Vitro Effects of Resuspended Concentrated Particles on Rat Cells in Bronchoalveolar Lavage Fluid

Particles collected on Teflon filters from experiments 8 through 12 (11 total days due to technical difficulties with some of the filters) were used for the in vitro experiments. The particles were resuspended, sonicated, and added to cultures of lavaged cells (> 95% alveolar macrophages) prepared from CD rats to make a final concentration of 100 µg/mL. The investigators resuspended urban air particle Standard Reference Material 1649, collected in Washington DC, as one positive control for the effects of CAPs on the rat cells. To enhance the synthesis of proinflammatory cytokines and chemokines by alveolar macrophages in the rat lavage fluid, some aliquots of cells were pretreated with lipopolysaccharide from *Escherichia coli* or with interferon- $\gamma$  (IFN- $\gamma$ ) for 3 hours before adding particles. After incubating cells and particles for 20 hours, the investigators collected cell-free supernatants to measure

concentrations of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and macrophage inflammatory protein-2 (MIP-2).

### Statistical Analysis

To evaluate pollutant-dependent changes in numbers of lavaged cells, the investigators used multiway analysis of variance (ANOVA) on both total and differential cell counts and on log counts (Table 5 of the Investigators' Report).

The investigators used several statistical approaches to evaluate the effects of CAPs plus O<sub>3</sub> on airway responsiveness. For the main analyses, they used a general linear mixed model with a random effect to induce correlation in the replicate measures made in the same animal over a series of days (Analysis I). The investigators also constructed multiway ANOVA models in the context of a mixed-effects model. This provided a more quantitative evaluation of CAPs concentration during exposure and of the interaction of the various experimental factors (day, OVA or PBS challenge, O<sub>3</sub> or CAPs exposure or both, MCh challenge, and plethysmography system) on airway responsiveness (Analysis II). To account for day-to-day variation in the chemical composition of particles, the investigators performed a multivariable regression that considered the component elements of the CAPs. To simplify this analysis, they used a rotated factor analysis (Analysis III), originally described by Koutrakis and Spengler (1987), to characterize the sources of pollutants. Kobzik and colleagues derived associations between Penh and six groups of elements whose concentrations varied in concert. These elemental groups were: aluminum/silica (Al/Si), bromine/selenium (Br/Se), chromium (Cr), nickel/vanadium (Ni/V), lead/copper (Pb/Cu), and sulfur (S). It is worth noting that (1) each of the six groups includes a number of other associated elements, not simply those that name the group (eg, the aluminum/silica group also includes S, K, Ca, Ti, Cr, Mn, Fe, and Cu; see Koutrakis and Spengler 1987), and (2) most elements are included in more than one group. The investigators also performed an analysis that included MCh concentration (Analysis IV) on data from their last five experiments, in which they used more than one concentration of MCh immediately after exposure. The investigators used log and square-root transformations and a Bonferroni correction for multiple testing where appropriate.

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## RESULTS

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### **Exposure to CAPs, With or Without O<sub>3</sub>, Induced a Small Increase in Penh**

Using a statistical approach that considered all CAPs-exposed animals as a single category (Analysis I), rather than taking into account daily variations in CAPs concentrations, the investigators reported that exposure to CAPs alone or CAPs plus O<sub>3</sub> increased Penh, but exposure to O<sub>3</sub> alone did not. Performing an analysis that considered CAPs concentrations (Analysis II), the investigators reported that CAPs exposure resulted in a very small but significant increase in Penh and that the effects of combined CAPs plus O<sub>3</sub> exposure were not clear.

### **Factor Analysis Identified Associations Between Components of CAPs and Changes in Penh**

When the concentrations of the elements in CAPs were considered both daily and cumulatively (over the 3 days of exposure), factor analysis (Analysis III) produced three results: First, OVA-challenged animals exposed to CAPs plus O<sub>3</sub> showed increased Penh on days of high aluminum/silica levels. This increase in Penh in response to CAPs plus O<sub>3</sub> was more than additive compared with exposure to CAPs or O<sub>3</sub> alone. Second, on days of high exposure to lead/copper, Penh *decreased* in these mice, but this association was just below statistical significance. Third, a significant association was seen in PBS-challenged mice, who showed increased Penh in response to CAPs on days of high sulfur levels.

When CAPs component concentrations were considered cumulatively, chromium levels were associated with *decreased* Penh in OVA-challenged mice and in O<sub>3</sub>-exposed mice.

### **CAPs Plus O<sub>3</sub> Synergistically Increased Penh in an Analysis That Included Bronchoconstrictor Concentration**

When the investigators used more than one concentration of MCh immediately after exposure, CAPs plus O<sub>3</sub> resulted in increased Penh as a function of MCh dose (Analysis IV). Exposure to O<sub>3</sub> alone decreased Penh in this analysis, whereas exposure to CAPs alone had little or no effect. When a similar analysis was applied to the seven experiments in which they evaluated Penh at a range of MCh doses starting 24 hours after the last OVA or PBS challenge, they found no pollutant-related effects.

### **Exposure to CAPs With or Without O<sub>3</sub> Had Little or No Effect on Numbers of Cells in Lavage Fluid from OVA-Challenged Mice 24 or 48 Hours After the Last Challenge**

In OVA-challenged mice, exposure to CAPs or CAPs plus O<sub>3</sub> significantly decreased total cell and macrophage numbers at 48 hours after the last OVA challenge compared with the same values at 24 hours (Table 5 in the Investigators' Report). However, the number of total cells and macrophages in the control group (OVA-challenged mice exposed to filtered air) also decreased at 48 hours. Thus, this reported effect of CAPs exposure may be somewhat misleading. In fact, by examining the last four lines of Table 5, which show data for OVA-challenged mice at 48 hours, one sees that the means and standard errors of total cell and macrophage numbers in mice exposed to filtered air and those in mice exposed to CAPs or CAPs plus O<sub>3</sub> are not different. This comparison of means indicates that exposure to CAPs or CAPs plus O<sub>3</sub> had no effect on cell numbers. The value of this approach is underscored by comparing means of total cell and macrophage values for O<sub>3</sub>-exposed and air-exposed OVA- and PBS-challenged mice at both 24 and 48 hours: O<sub>3</sub>-exposed mice have higher values, which would be expected because O<sub>3</sub> is known to induce inflammation.

### **Resuspended CAPs Samples Stimulated Mediator Production in Lavaged Rat Cells in Vitro**

In the absence of CAPs exposure, levels of MIP-2 and TNF- $\alpha$  detected in supernatants from unprimed and IFN- $\gamma$ -primed alveolar macrophages were approximately 1 pg/mL. Resuspended CAPs samples (at 100  $\mu$ g/mL, the single concentration tested) stimulated synthesis of these mediators at a level much higher than background. Samples collected on different days gave somewhat different results, but CAPs-induced mediator production was detected in the picogram-to-nanogram range. For example, TNF- $\alpha$  ranged from 50–2600 pg/mL in supernatants from unprimed lavaged cells and MIP-2 ranged from 1–15 ng/mL in supernatants from IFN- $\gamma$ -primed cells. The magnitude of the effect of CAPs on mediator production in lavaged cells was frequently as high as the response to the positive control particles, Standard Reference Material 1649. Priming cells with lipopolysaccharide stimulated background production of both mediators to 3–7 ng/mL; CAPs enhanced these levels 3-fold to 10-fold.

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## DISCUSSION

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In this study, OVA-sensitized asthmatic mice exposed to CAPs exhibited a small increase in Penh, a recently

described measure of airway responsiveness. The investigators placed this small CAPs-induced Penh increase (calculated to be 0.9% for a 100- $\mu\text{g}/\text{m}^3$  CAPs concentration) in context by comparing it with the effect on Penh of the allergen OVA, which was 3000-fold larger. The investigators also showed that the increase in Penh was transient: It was detected when Penh was measured on the same day as the pollutant exposure but was not detected 24 hours later. This suggests that CAPs effects on pulmonary mechanical function occur rapidly after exposure. These results extend the authors' previous findings, which indicated that CAPs had no effect on Penh 24 to 48 hours after exposure (Goldsmith et al 1999). Together, these results suggest that the timing after PM exposure is critical when measuring particular health outcomes. Variations in the timing of such measurements may help to explain differences in the results of studies that have reported either small (eg, Pope et al 1991; Roemer et al 1993; Romieu et al 1996) or no (eg, Dockery et al 1989; Hiltermann et al 1998; Tittanen et al 1999) effects of PM on pulmonary function.

The plethysmographic technique used by Kobzik and colleagues to monitor airway responsiveness in small animals has many advantages: It is noninvasive and can be used for repeated and long-term measurements of large numbers of animals. In addition, the animals are conscious and unrestrained, and can be exposed to aerosolized, rather than intravenous, bronchoconstrictors such as MCh. Questions remain, however, about what Penh is actually measuring. The original study showed a good correlation between Penh and total lung resistance (Hamelmann et al 1997). Because the latter is composed of both airway and lung tissue resistance, however, it is not clear if Penh is an index of combined airway resistance and changes in lung compliance or of just airway resistance.

Kobzik and colleagues showed that the composition and effects of ambient air and CAPs differed qualitatively and quantitatively on different days during the study. The results of their factor analysis (which indicated significant associations between the aluminum/silica component of CAPs and increased Penh, and between chromium and decreased Penh) support the notion that different health outcomes may occur on different days. These positive and negative associations also imply that the magnitude of a specific outcome on a particular day is a function of the aggregate composition and level of pollutants in ambient air on that day. Similar findings that associate specific outcomes with components of the pollutant mix by factor analysis have been shown in recent controlled-exposure and epidemiologic studies (Clarke et al 2000; Laden et al 2000).

The original hypothesis for the study was that coexposure to CAPs and  $\text{O}_3$  would result in a synergistic increase

in Penh. This was based in part on the results of Vincent and colleagues (1997), who had found that  $\text{O}_3$  and the particles they had evaluated (resuspended PM from ambient air in Ottawa, Ontario) had synergistic effects on the proliferation of airway epithelial cells. The factor analysis of the data from the current study (Analysis III) suggested synergy between CAPs and  $\text{O}_3$  on Penh. The authors are rightly cautious, however, in interpreting this analysis because other analyses did not show such a synergy. In addition, because the number of animals tested in each group was small, it is likely that the study has limited power to detect meaningful synergistic effects.

In vivo, the investigators found little or no effect of CAPs in the presence or absence of  $\text{O}_3$  on airway inflammation in BALB/c mice. The findings with CAPs are similar to those the investigators reported previously in this strain (Goldsmith et al 1999). It is worth noting that in their earlier study, exposing the mice to PM from a specific source (residual oil fly ash) resulted in increased Penh but did not change the inflammatory parameters in bronchoalveolar lavage fluid (Goldsmith et al 1999). This suggests that some agents might modify one aspect of the allergic or asthmatic phenotype (for example, airway responsiveness) without affecting another (for example, inflammation). Because the increase in Penh was so small in response to CAPs in the current study, the investigators appropriately do not draw any conclusions about the effectiveness of CAPs in changing one or both endpoints associated with the asthmatic phenotype.

Resuspended CAPs did induce in vitro synthesis of inflammatory mediators by lavaged cells, similar to the earlier findings of Kobzik and colleagues (Imrich et al 1999). Thus, the current study indicates that CAPs induced inflammatory effects in vitro but not in vivo. Comparing in vivo and in vitro inflammatory effects of CAPs is difficult, however, because the investigators measured different endpoints in the two studies (cell numbers in vivo, mediator production in vitro).

### Statistical Design

The statistical design was appropriate, but it would have been helpful to include a formal presentation of the statistical model and a discussion and analysis of possible carryover effects in the longitudinal design. If present, such carryover effects might have complicated the interpretation of the results, and they are of independent scientific interest. However, the current study was unlikely to have had sufficient power to assess these effects because a substantially larger sample size is needed to assess interactions than is needed to assess main effects. A direct interpretation of the statistical model in the original scale could

have been obtained by using a generalized linear model, such as implemented by SAS Proc GENMOD, as an alternative to transforming the dependent variable. The investigators also should have used robust standard errors to ensure valid inferences, an approach also available in SAS. These refinements might not have changed the conclusions of the study, but they would have increased our confidence in the investigators' interpretations.

The use of factor analysis or principal components analysis to reduce the number of regressors was appropriate and well implemented, provided the resulting factors have some degree of scientific validity. However, investigators using this approach must be careful to avoid allowing outliers to have undue influence by setting them aside before computing the factors.

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## SUMMARY AND CONCLUSIONS

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In this well-designed study, exposing allergen-sensitized and challenged BALB/c mice to CAPs over a series of days resulted in a small transient change in Penh, a measure of airway responsiveness, and little or no change in inflammation in the airways. O<sub>3</sub> appeared to synergize with CAPs in increased Penh in only one of the four different statistical data analyses. Thus, the effects of CAPs plus ozone did not achieve synergy that could be convincingly demonstrated.

These results indicate that PM<sub>2.5</sub>, even when concentrated to levels higher than normally found in ambient air, had little effect on the airway mechanical and inflammatory parameters measured in this small-animal model of asthma. This model, OVA-challenged young mice, mimics some of the characteristics of human asthma (most notably, allergen-induced airway inflammation) but not all (for example, mucus hypersecretion). Because the model is limited in representing the complex illness of human asthma, we cannot be certain to what extent these results may or may not predict the effects of ambient PM and ozone exposure on people with asthma.

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## ACKNOWLEDGMENTS

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5	Formation and Characterization of Particles: Report of the 1996 HEI Workshop		1997
<b>HEI Program Summaries</b>			
	Research on Particulate Matter		1999

\* Reports published since 1990, with exceptions.

Copies of these reports can be obtained from the Health Effects Institute and many are available at [www.healtheffects.org](http://www.healtheffects.org).



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HEALTH  
EFFECTS  
INSTITUTE

Charlestown Navy Yard  
120 Second Avenue  
Boston MA 02129-4533 USA  
+1-617-886-9330  
*www.healtheffects.org*

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