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Toxicity of Chemical Constituents of PM10 in the South Coast Air Basin of California

Principal Investigator: D.K. Bhalla, Ph.D.
Co-Principal Investigator: M.T. Kleinman, Ph.D.
Investigator: R.F. Phalen, Ph.D.
Investigator: W.J. Mautz, Ph.D.
Investigator: D.B. Menzel, Ph.D.
Investigator: Y. Kikkawa, M.D.
Biostatistician: T.T. Taylor, Ph.D.
Chemist: G.R. Cass, Ph.D.
Sub Contractor: C. Nadziejko, Ph.D.
Sub Contractor: C. Basbaum, Ph.D.

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AIR POLLUTION HEALTH EFFECTS LABORATORY

Department of Community and Environmental Medicine

University of California, Irvine
Irvine, CA 92717

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ABSTRACT

Adverse respiratory effects are associated with PM10 exposure, although the attribute(s) of PM10 and biological mechanisms responsible are unclear. This study evaluated airway epithelial cell morphometry, macrophage function, breathing pattern, and ventilation in rats exposed for 4 hr/d, 4 d/wk for 8 weeks to components of PM10 (ammonium sulfate, ammonium nitrate, and road dust) singly, in combinations, and in mixtures including 0.20-ppm ozone (O_3). There were no changes in goblet cells with any exposure. High and low concentrations of PM10 components, singly and combined with 0.2 ppm O_3, reduced Fc-mediated phagocytic activity, suggesting an additive effect. Particles alone decreased respiratory burst activity, suggesting that these PM10 components can inhibit macrophage function, although when O_3 was included, respiratory burst activity increased. High concentrations of both sulfate and nitrate significantly increased airway permeability, but low concentrations and mixtures of particulates and O_3 had inconsistent effects. Changes in breathing pattern and minute ventilation consistent with mild pulmonary irritation occurred with exposure to all particle types at the high, but not the low, concentration. Addition of O_3 to the exposure mixture induced breathing pattern responses indicative of an O_3 effect, while exposure to high concentration multi-component mixtures of PM10 compounds and O_3 induced effects suggesting interaction between PM10 and O_3.

EXECUTIVE SUMMARY

In Southern California, the air quality standard for airborne particles less than 10 μm in diameter (PM10) is one of the most frequently violated of the State's Ambient Air Quality Standards. Epidemiological studies have associated inhalation of PM10 at concentrations near the California standard (50 $\mu g/m^3$, 24 hr average), and below the Federal standard (150 $\mu g/m^3$, 24 hr average) with increased hospital admissions and emergency room visits for respiratory illnesses, increased incidences of asthma attacks, reduced pulmonary function, and increased mortality for cardiorespiratory causes. These associations have not been previously tested in a systematic manner to determine whether these health outcomes might be attributable to specific components of ambient PM10. Furthermore, research has not established which specific aspects of the lung's defense systems are impaired by PM10 exposure.

The objectives of this study were: 1) to apply disease-related endpoints to assess the potential for PM10 components to injure human populations, and 2) to evaluate the toxicity of PM10, both alone, and in combination with ozone.

We fulfilled these objectives by:

a) selecting components of ambient PM10 that constitute significant fractions of total PM10;

b) developing an exposure scenario that simulated many of the characteristics of human exposure patterns; and

c) exposing laboratory animals to the selected particles, and evaluating the effects on critical lung defenses.

The project investigated the effects of three components of PM10 (ammonium sulfate, ammonium nitrate, and road dust), each at two different concentrations. These compounds were selected because they constitute a significant portion of the ambient PM10 found in Southern California. The low concentration of each type of particulate was selected to be representative of estimated peak 4-hr concentrations, based on extrapolations from ambient air monitoring data. The high concentration of each particulate type was approximately 3 times the low concentration. The three PM10 components were studied singly, in various combinations, and in mixtures including 0.20-ppm ozone (O_3).

Separate groups of rats were exposed for 4 hr/d, 4 d/wk for 8 weeks to each of the exposure conditions. The end-points used to detect adverse effects included changes in airway epithelial cell morphometry (goblet cell number and mucin production), macrophage function (phagocytosis, respiratory burst and release of inflammatory markers), and changes in breathing pattern and ventilation.

Analysis of goblet cells throughout the respiratory tract (trachea, lobar bronchi and bronchioles) did not change as a consequence of any of the various exposures.

Fc-mediated phagocytic activity was reduced in rats exposed to both concentrations of PM10 components when inhaled they were alone, and in combination with 0.2 ppm O_3. The data suggest that the effects of particulates and O_3 may be additive.

Particles alone tended to decrease respiratory burst activity. However, when particles were inhaled with O_3, there was an increase in respiratory burst activity. Thus, particles alone might increase potential risk of respiratory tract damage, but might also exacerbate ozone-induced lung injury. The amount of inflammatory mediators released by macrophages from pollutant exposed rats tended to increase, although none of the group means were elevated significantly ($P<0.05$) compared to controls. Overall, the data suggest that exposure to the studied fractions of PM10 can reduce the effectiveness of macrophages in the lung's defense system.

While a significant ($P<0.05$) increase in airway permeability occurred in rats exposed to high concentrations of sulfate and nitrate, no consistent change was observed in rats exposed to either low concentrations of the PM10 components or the mixtures of particulates and O_3.

Breathing pattern and minute ventilation responses consistent with mild pulmonary irritation were present with exposure to all particle types at the high concentration, but not at the low concentration. Two component mixtures of 0.2 ppm O_3 plus any of the PM10 components induced breathing pattern responses indicative of an O_3 effect. The multicomponent mixtures of PM10 compounds (at the higher total PM10 concentration) and O_3 induced more complex breathing pattern effects suggesting irritant interactions between PM10 and O_3 effects.

This is the first toxicological study to test the effects of PM10 components in a protocol designed to simulate essential characteristics of human exposures to ambient particulate pollutants. The study shows that critical components of respiratory system defenses can be impaired by PM10 exposure, and that such effects occur with exposure to environmentally relevant concentrations. This study tested only three of the possible components of PM10 in a limited set of combinations, but demonstrated significant ($P<0.05$) adverse health-related changes. Other factors which might influence PM10 toxicity, and which may be important for the evaluation of air quality standards, include such PM10 components as carbonaceous material, acidic aerosols and acidic vapors. The importance of particle size and the effects of normal activity patterns on exposure and dose should also be investigated in future studies.

CONCLUSIONS

Epidemiologic studies have shown that exposure to airborne particles is associated with significant ($P<0.05$) adverse effects on human health, including chronic lung diseases and mortality. Ambient airborne particles are a complex mixture, both in terms of particle size, and chemical composition. They also vary between locations, and even between different times at the same location. The mechanism(s) by which inhaled ambient air particles cause or aggravate diseases remains unclear. It is not known, whether particle chemical composition, size, mass, surface area, or some other factor is the basis of the harmful effects associated with PM10.

The results of this study found significant ($P<0.05$) impairment in several indices of lung defense in rats exposed to 3 representative compounds found in PM10 in Southern California cities. There were also significant ($P<0.05$) interactions of PM10 with concurrently inhaled ozone, another prevalent atmospheric pollutant in California's ambient air.

Evaluation of macrophage functions in rats exposed to PM10 showed that critical defenses against respiratory infections could be impaired. Exposure to some of the particulate pollutants in this study significantly ($P<0.05$) disrupted the airway epithelial barrier. This could allow antigens present in the environment to penetrate to sub-epithelial regions, leading to the release of histamine and induction of bronchoconstriction. Measurements of breathing patterns and ventilatory parameters in this study demonstrated that these pollutants had an irritant effect on the lung. This study provides toxicological support for earlier epidemiological findings that associate PM10 exposure with increased risks of pulmonary disease and respiratory infections.

INTRODUCTION

Airborne particles have been implicated in inducing human health problems. Epidemiologic studies have demonstrated associations between daily particulate concentrations and hospital admissions and emergency room visits for respiratory illnesses (Martin 1964, Greenberg et al. 1967, Knight et al. 1989). There are also associations between asthma attacks and airborne particle levels (Whittemore and Korn, 1980). Elevated levels of particles less than 10 μm aerodynamic diameter (PM10) have been linked with reduced pulmonary function in adults (Pope et al., 1991) and children (Stern et al., 1989). Ozkaynak and Spengler (1985), and Ozkaynak and Thurston (1987) analyzed the 1980 U.S. vital statistics and available ambient air pollution databases for sulfates, fine inhalable particles and total suspended particles. The analysis found significant ($P<0.05$) associations between particulate exposures and total mortality. Particle exposure measures related to the respirable and/or toxic fraction of the aerosols, such as fine particles and sulfates, were most consistently and significantly ($P<0.05$) associated with reported total annual mortality rates.

The principal objectives of this study were: 1) to apply disease-related endpoints in order to assess the potential of PM10 components for injuring human populations, and 2) to evaluate the toxicity of PM10, both alone and in combination with ozone. In order to fulfill these objectives, a series of inhalation exposures was undertaken using the rat, a reliable toxicologic model. These studies quantified aspects of the induction and exacerbation of disease by PM10 components. The study focussed on non-acidic inhaled pollutants, and interactions between inhalation of these particles and the oxidant gas, ozone (O_3). The choices of biological endpoints were based on knowledge of disease processes likely to be initiated by the inhalation of PM10 components.

Ambient Air Quality Standards and Characteristics of PM10

Both California and the Federal Government have established annual and 24-hour standards for PM10. These are size-defined mass standards that apply to PM10 in general. The standards do not consider the chemical nature of the particulate mixture. The current standards are listed in Table 1.

Table 1. PM10 Standard ($\mu g/m^3$)

	Annual	24-hour average
California	30	50
Federal	50	150

In 1990 the maximum 24-hour PM10 concentration in the South Coast Air Basin ranged from 88 $\mu g/m^3$ in the relatively clean-air city of El Toro, to 520 $\mu g/m^3$ in the more heavily polluted city of San Bernardino. Furthermore, the State 24-hour standard was exceeded

in all cities in the South Coast Air Basin between 20 to 70% of the days in which PM10 was monitored (PM10 samples are collected every 6 days).

The character of PM10 is variable, both with regard to sampling locations and with regard to time (season of the year, time of day, etc.). This study focused on Southern California PM10 at the request of the California Air Resources Board because of the large number of exceedances of the standards in this region, and because of the large population subject to potentially adverse impacts due to PM10 exposure (Hall et al., 1992).

Southern California PM10 is a complex mixture of primary emission particles such as combustion aerosols, particles generated by motor vehicles (tire wear, brake linings, elemental carbon from exhaust fumes), and fugitive dusts, as well as secondary aerosols such as nitrate and sulfate particles from atmospheric gas to particle conversion processes.

In selecting the composition of the test atmospheres to be used in this toxicological evaluation of PM10, we depended heavily on the results of an intensive air sampling campaign which was conducted in Southern California under the sponsorship of the California Air Resources Board. PM10 was sampled in Claremont, CA, as part of the South Coast Air Quality Sampling (SCAQS) program in the summer of 1987 (Wolff et al., 1991). The results are shown in Table 2.

Table 2. Concentrations ($\mu g/m^3$) of Particulate Components in Claremont, CA

	Mean ± SD	Max	% of PM10
PM10	60 ± 24	120	-
Fine Part. Matter (<2.5 μm)	36 ± 17	91	60.0
Coarse Part. Matter (>2.5 μm)	23 ± 10	47	38.0
Sulfate	6.5 ± 3.8	18	10.8
Nitrate	12.4 ± 6.5	28	20.7

Ozone (O_3), another pollutant whose concentration frequently exceeds Federal and State standards in Southern California, is often inhaled together with PM10 by exposed populations. This was taken into account in the design of this study by including combinations of PM10 components with ozone in the exposure regimens.

This study is the first to attempt a systematic evaluation of PM10 toxicity, although not all characteristics or components of PM10 could be examined. For example, nitrates and

sulfates, which together represent about 30% of PM10 were included, but elemental carbon, which represents about 10% of the fine particle fraction of PM10, was not studied.

Resuspended dust is a major contributor to the coarse fraction of PM10. Cass et al. (1982) determined that crustal materials contributed between 35 and 55% by mass of total suspended particulate matter (TSP) at Azusa, CA, and that direct highway emissions contributed between 12 and 25%. Gray et al. (1988) attributed about 50% of annual average PM10 at Rubidoux, CA, to fugitive dust, of which 73% was attributed to resuspension from paved roads.

Based upon the SCAQS Claremont data, the coarse particulate fraction represents about 40% of PM10. Given the sizable contribution of resuspended particulate matter from paved roads to these dust fractions (Chow et al., 1990), we have used road dust to simulate the coarse fraction of PM10. Nitrate and sulfate are major contributors to the fine particulate fraction of PM10. We used ammonium salts of these compounds to represent the fine PM10 aerosol fraction.

Two concentrations of each PM10 component were used. The low level was selected to be representative of estimated peak 4-hr concentrations, based on extrapolation from ambient air data. The high level was approximately 3 times the estimated peak 4-hr level. The effects of these PM10 components were evaluated alone, in various combinations, and in mixtures with ozone.

The particulate concentrations used for these inhalation exposure studies of PM10 health effects are shown in Table 3. The aerosol mixtures simulated some of the important size and chemical characteristics of Southern California PM10, and provided a coarse aerosol similar to that reported by Wolff et al. (1991) at Claremont, CA. The fractional contribution of sulfate to PM10 was approximately equal to that reported at Claremont, CA, during the summer. In the selected mixture, nitrate constitutes the remainder of the aerosol. The nitrate to sulfate ratio (5:1) is higher than that observed at Claremont, CA, but is within the range of values reported for other California cities, such as Long Beach (Wolff et al., 1991), and predicted for Fontana and Rubidoux, CA (Pilinis and Farber, 1991).

Table 3. Aerosol Composition for PM10 Inhalation Studies

Component	Low $\mu g/m^3$	High $\mu g/m^3$	Mixture $\mu g/m^3$	% Mass
Sulfate	20	70	70	10
Nitrate	90	350	350	48
Road Dust	300	900	300	42

The nitrate and sulfate components were generated by aerosolizing aqueous solutions. Road dust, which CALTRANS collected by vacuum sweeping freeway surfaces, was disaggregated, sieved, sterilized and elutriated to provide particles representative in size of the coarse fraction of PM10 (particles between 2 μm and 10 μm diameter). The freeway sampled was relatively new, and dust was collected from a region that had not been subject to any spills of hazardous materials.

It should be noted that this project represents only a reasonable start for assessing the effects of PM10 on health. Important questions remain to be answered, including the effects of other components of PM10 (for example, carbonaceous or acidic aerosols and acidic vapors), the effects of other mixtures of components, and the relative toxicity of particles of different sizes and chemical compositions.

Relevance of Sub-chronic PM10 Exposure to Lung Disease

This study was designed using endpoints that relate to human respiratory diseases. The selected endpoints measured changes in parameters of lung defenses that, if impaired, would be expected to significantly increase morbidity and mortality of human subpopulations that are "at risk." These subpopulations include asthmatics, chronic bronchitics, patients with chronic obstructive pulmonary disease (COPD), young children and other chronically ill individuals. The disease-related parameters measured included changes in: 1) the number of mucus secreting cells, and total mucus secretion (mucus hypersecretion is a hallmark of chronic bronchitis [e.g. Cherniack et al.,1972]); 2) macrophage functions (impaired macrophage function significantly ($P<0.05$) increases the risk of lung infections [e.g. Gardner, 1985]); 3) airway permeability (bronchial hyperreactivity and asthma are associated with increased permeability [e.g. Biagini et al., 1986]); and 4) breathing pattern response (reflex respiratory changes in response to inhaled irritants in humans and laboratory animals [e.g. Alarie, 1973]).[1] We focused on these endpoints because adverse changes in these parameters have the potential to affect large numbers of susceptible people, and alterations in these parameters have been linked to the pathogenesis of pulmonary diseases. Alterations in mucus secretion, immune defenses, epithelial permeability and breathing pattern, particularly in people with underlying lung diseases, have been linked to elevated levels of particles and pollutant gases in epidemiologic studies. Thus, our studies involving exposure of animals to components of PM10 and O_3 will help elucidate potential roles for PM10 to induce adverse health effects, and to exacerbate preexisting lung disease. The studies will also help clarify mechanisms for the actions of PM10 in the etiology of lung disease, and will provide plausible biological mechanisms that can be used in the interpretation of past and future epidemiology investigations.

[1] Two of the endpoints proposed in the original application had to be modified to accommodate the study design because of personnel changes due to Dr. Nadziejko's move from U.C. Irvine to New York University. The initially proposed measurements of bactericidal activity of macrophages and mucin gene expression in the lung were replaced by comparable measurements of respiratory burst and mucus glycoprotein in the bronchoalveolar lavage fluid.

Disease: Chronic Bronchitis

Parameter: Mucus secretion and secretory cell populations

Chronic bronchitis is a leading cause of pulmonary morbidity and mortality. Chronic bronchitis (defined as daily expectoration of sputum for more than 3 months a year for two successive years) can occur by itself, or more commonly in combination with airway hyperactivity and emphysema. Chronic hypersecretion of mucus, and subsequent chronic respiratory tract infection, has a devastating effect on the lung, eventually leading to hypoxia and severe derangements in gas exchange (Cherniack, Cherniack and Naimark, 1972). Individuals with advanced cases of chronic bronchitis/chronic obstructive pulmonary disease (COPD) are often so severely compromised that any further increase in the amount of secretions in the respiratory tract may result in hospitalization and/or death.

Increased mucus secretion is a non-specific response of the lung to a variety of inhaled irritants, including particulate matter found in polluted air. The Harvard Six-Cities study showed that the prevalence of bronchitis in children correlated with the annual mean PM15 (Dockery et al., 1989). Morphological changes indicative of chronic bronchitis (increases in goblet cell number and submucosal gland hypertrophy) have been reported in rats exposed to sulfur dioxide, cigarette smoke, formaldehyde, nitrous oxide (Lamb and Reid, 1968), and sulfuric acid particles (Schlesinger 1985).

The rat is the species of choice for studying mucus secretion because unlike mice, hamsters, rabbits and guinea pigs, rats have well-developed submucosal mucus glands as well as goblet cells (Reid, 1970). Two methods were used to detect changes in mucus secretion: 1) morphometric determination of goblet cell number, and 2) measurement of mucous glycoprotein in bronchoalveolar lavage fluid.

Increased numbers of goblet cells and submucosal gland hypertrophy are well-established, moderately sensitive indices of increased mucus production. Reid (1970) has shown that rats exposed to smoke from 5 cigarettes per day for five days a week for 6 weeks showed a doubling of goblet cell density (Lamb and Reid, 1969). A significant ($P<0.05$) increase in submucosal gland size was seen after three weeks of exposure to sulfur dioxide (Lamb and Reid, 1968). Thus, we hypothesized that significant changes in goblet cell density would be seen with some of our exposure conditions after 30 days of exposure.

Measurement of density and size of mucus secreting cells alone does not reflect the amount of mucus secreted by these cells. Also, morphometric parameters cannot be extrapolated to changes in the total mucus secreting cell population per lung. For example, a fifty- percent increase in the density of goblet cells in the proximal airways does not take into account exposure-induced extension of goblet cells into the peripheral

airways. Thus, the total increase in goblet cells per lung may be much higher than the 50% change observed in the proximal airways. Measurement of mucus glycoprotein concentrations in the BAL may be a better, or more sensitive, indicator of mucus hypersecretion and this assay was added to our original research plan.

Disease: Respiratory tract infection

Parameter: Defense functions of alveolar macrophages

Respiratory tract infections include upper respiratory tract infections (colds), acute and chronic bronchitis, and pneumonia. Respiratory tract infections have been associated with increased mortality in the elderly, absenteeism in younger populations, as well as exacerbation of asthma and incidence of bronchitis in the general population. A number of epidemiological studies have reported correlations between particulate pollution levels and incidence of respiratory tract infection in adults and children (Pope, 1989; Dockery et al., 1989).

Alveolar macrophages play a major role in lung defenses against infection, although other defense mechanisms are also present (Gardner, 1985). Exposure to sulfuric acid reduces the capacity of rabbit macrophages to adhere to glass (Nauman & Schlesinger, 1986) but does not suppress bacterial killing or reduce viability of macrophages recovered from mice (Coffin, 1972). Increased numbers of neutrophils and macrophages were lavaged from lungs of rabbits after exposure to sulfuric acid aerosol (Nauman & Schlesinger, 1986), indicating induction of an inflammatory reaction. Aerosolized sulfuric acid produced transient mobilization of macrophages and neutrophils, with little evidence of functional impairment (Schlesinger, 1985), in contrast to the effects of O_3 or NO_2 (Gardner, 1985). Brain (1971) reported that inert carbon aerosols induce macrophage migration to alveoli in proportion to particle loading. Bowden (1985) found that macrophage clearance of particles proceeds normally by way of the mucociliary route, unless particle loading exceeds macrophage clearance capacity, or toxic gases, such as O_3 or NO_2, injure the epithelium of the terminal bronchioles.

Inhalation of an atmosphere composed of sulfate aerosol and O_3 has been shown to increase the number of inflammatory cells and macrophages in acute focal lesions in rat lungs. The magnitude of the adverse effects was proportional to the level of acidity of the sulfate aerosol (Last et al., 1982, 1984). Aranyi et al. (1983) found increased susceptibility to infection in mice after exposure to O_3 or to a mixture of O_3 + SO_2 + ammonium sulfate, with the effect greater with the mixture of pollutants than with O_3 alone. Rats exposed to O_3 alone, O_3 + sulfuric acid, and O_3 + ammonium sulfate in our laboratory showed accelerated macrophage-related clearance (Phalen et al., 1980). The authors concluded that sulfuric acid aerosol alone in rats did not, while O_3 + sulfuric acid or ammonium sulfate did, increase macrophage migration into alveoli. The results also showed that alveolar macrophage migration was impaired by fine particulate carbon + acid.

Experiments with rats exposed to acidic atmospheres containing carbon soot have shown that soot particles accumulate in the cells of the deep lung, and that they persist for up to 7 days after exposure (Phalen and Kleinman, 1988). A high percentage of these soot-containing cells were macrophages. There was a delay in deep lung clearance in these animals after exposure to the acid/soot mixture, suggesting possible impairment in macrophage function. Studies with O_3 have shown that there is decreased binding of sheep red blood cells (SRBC) onto the surface of macrophages, as evidenced by decreased number of rosettes formed, indicating damage to Fc receptors (FcR), which play a role in the lung's immunological defenses (Prasad et al., 1988). Recent studies of diesel soot plus acid mixtures have shown, however, that macrophage phagocytic function may be depressed at high exposure levels, but accelerated at lower exposure levels (Kleinman and Mautz, 1989).

Based on these previous investigations, we have evaluated three aspects of macrophage function in rats exposed to ammonium nitrate, ammonium sulfate, and road dust particulate matter, alone, in various mixtures, and with O_3: 1) Fc mediated phagocytic activity, which was measured using a rosette assay described by Prasad et al. (1988). 2) Release of the inflammatory mediators leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2), which were measured using enzyme-linked immunosorbent assay (ELISA) methods. These mediators may play significant roles in control of the lung's inflammatory response to inhaled toxic or infectious agents. 3) The ability of macrophages to mount a respiratory burst, a mechanism by which macrophages produce biocidal compounds that kill bacteria. Respiratory burst was measured using an in-vitro assay for reduction of cytochrome C by macrophage-produced superoxide.

Disease: Asthma

Parameter: Airway Permeability

Asthma, an obstructive disease of the airways, is characterized by decreased vital capacity of the lungs, bronchial edema, increased mucus production and increased eosinophils in the airways. It is an immunologic response, believed to be mediated through production of immunoglobulin E (IgE) and its binding to mast cells following antigenic exposure. Although a relationship between air pollution and asthma in humans has not been conclusively demonstrated, epidemiologic studies suggest a possible relationship between air pollutants and asthma in children. Sultz et al. (1970) studied the records of 22 hospitals in Erie County, New York, and found increasing incidence rates for asthma in children under the age of 15 years with increasing levels of air pollution.

Animal studies have demonstrated anaphylaxis on sensitization with allergen following exposure to air pollutants. Biagini et al. (1986) showed increased bronchial hyperactivity in monkeys that inhaled 1 ppm O_3 and platinum aerosol for 12 weeks. Osebold et al. (1988) exposed specific pathogen-free Swiss-Webster mice for 4 days to O_3 concentrations ranging from 0.1 to 0.24 ppm, followed by ovalbumin aerosol inhalation. This exposure cycle was repeated 4 times, and the animals were tested one week after

the last exposure. A significant ($P<0.05$) allergic enhancement was found after exposure to O_3. An intact airway epithelial barrier appears to present a major defense against allergens and toxins in the air.

The barrier properties of the epithelium are attributed primarily to apical tight junctions, where the membranes of adjacent cells appose each other and obliterate the intercellular space. Increased airway permeability represents a breach in the barrier. Studies in our laboratory have shown increased airway permeability following exposure to oxidant air pollutants (Bhalla et al., 1985; 1988). O_3 increased epithelial permeability in the proximal airways immediately after exposure. Permeability remained elevated for up to 8-12 hours after an O_3 exposure, and returned to control values by 24 hrs after exposure. Exposure to a combination of O_3 and NO_2 produced greater permeability than either gas alone (Bhalla et al., 1986; 1989).

A disrupted epithelial barrier would allow allergens present in the environment to penetrate tight junctions and be deposited in the sub-epithelial space. Hence, allergens would have access to nerve endings and other cells of the lymphoid system. Subsequent release of IgE, and its binding to mast cells could result in release of histamine and other cellular mediators capable of inducing bronchoconstriction and other responses and symptoms of asthma.

Therefore, studies were performed to assess airway epithelial permeability characteristics in rats exposed to PM10 and O_3 atmospheres. Concentrations of total protein and albumin recovered in bronchoalveolar lavage fluid (BALF) were measured as indicators of permeability.

Parameter: Reflex Breathing Pattern Response

Inhalation of respiratory irritants produces reflex respiratory changes in breathing pattern, and can induce asthmatic attacks. Changes in respiratory frequency, tidal volume, and minute ventilation provide information as to the airway site which is primarily affected (Alarie, 1966, 1973; Coggins et al., 1982; Chang et al., 1981; Mautz et al., 1985b; Dallas et al., 1986). Breathing pattern and minute ventilation measurements provide measures of the relative irritancy of inhaled PM10 and O_3, and give an indication as to the mechanisms involved. Pulmonary irritants, like O_3, invoke a rapid-shallow breathing pattern that is a vagally-mediated reflex believed to originate with stimulation of lung C fibers (Alarie, 1973; Lee et al., 1979, 1980; Adams et al., 1981; Coleridge et al., 1983; Coleridge and Coleridge, 1984; McDonnell et al., 1983; Beckett et al., 1985). Compounds classified as sensory or upper airway irritants, such as formaldehyde (HCHO) and airborne acids, induce reflex responses believed to be mediated by trigeminal afferent pathways (Alarie, 1973; Ulrich et al., 1972). These responses generally involve a decrease in frequency accompanied by ventilatory depression, or by a compensatory increase in tidal volume with a low respiratory frequency (Chang et al., 1981; Davis et al.,

1967; Mautz et al., 1983). Respiratory frequency is thought by many investigators to be the most sensitive index of respiratory irritation (J. Mauderly, personal communication).

Breathing pattern and ventilatory parameters are easily measured in humans; thus, the irritancy responses measured in rodents exposed to particulate matter and O_3 can be related to human studies.

The eight-week time course of the experiments involved changes in body mass due to normal growth of control animals and possibly inhibited growth of exposed animals. Because metabolic rate and respiratory ventilation to support gas exchange are allometrically related to body mass (Mortola, 1984; Heusner, 1987), inhaled dose rate may vary between exposure groups both because of differences in body mass, and differences in reflex breathing pattern responses. Measurement of respiratory ventilation allowed us to monitor these possible changes.

Methods

Exposure Protocol and Animals

Exposure Protocol

Exposures were conducted for 4 hr per day, 4 days per week, followed by 3 clean air days for recovery. This weekly regimen was repeated for 8 weeks (32 exposures). This represents a reasonable simulation of human exposure scenarios in the South Coast Air Basin. Weekend pollution levels are frequently lower than mid-week levels, and typical diurnal variations in PM10 and oxidant concentrations show a mid-day peak of 4 to 6 hours duration. An 8-week exposure period was chosen as sufficient to elicit changes from repeated injury/repair cycles in the biological endpoints selected, and to represent a time period of peak seasonal exposure. All 4 experimental series utilized the same exposure protocol.

We studied 3 individual particulate substances--California Road Dust (combined crustal and organic carbon compounds), ammonium nitrate, and ammonium sulfate. Two exposure concentrations, one equivalent to the estimated maximum 4 hr concentration observed in the city of Azusa, CA, in the South Coast Air Basin, and the second level of approximately three times the 4 hr maximum concentration, were used in this study. These are referred to as low and high concentrations (see Table 3). The four experiments are summarized in the table below.

Table 4: Atmospheres Tested

Experiment 1: High Concentration:	A. Road Dust (900 $\mu g/m^3$) B. Ammonium Sulfate (70 $\mu g/m^3$) C. Ammonium Nitrate (350 $\mu g/m^3$) D. Clean Air
Experiment 2: Low Concentration	A. Road Dust (300 $\mu g/m^3$) B. Ammonium Sulfate (20 $\mu g/m^3$) C. Ammonium Nitrate (90 $\mu g/m^3$) D. Clean Air
Experiment 3: Aerosols + O_3	A. Road Dust (300 $\mu g/m^3$) + O_3 (0.2 ppm) B. Ammonium Sulfate (70 $\mu g/m^3$) + O_3 (0.2 ppm) C. O_3 (0.2 ppm) D. Clean Air
Experiment 4: Aerosols + O_3	A. Ammonium Nitrate (350 $\mu g/m^3$) + O_3 (0.2 ppm) B. Ammonium Nitrate (350 $\mu g/m^3$) + Ammonium Sulfate (70 $\mu g/m^3$) + Road Dust (300 $\mu g/m^3$) C. Ammonium Nitrate (350 $\mu g/m^3$) + Ammonium Sulfate (70 $\mu g/m^3$) + Road Dust (300 $\mu g/m^3$) + O_3 (0.2 ppm) D. O_3 (0.2 ppm) E. Clean Air

Animal Housing and Exposure

Exposure subjects were male Fischer 344 rats (Simonsen Laboratories, Inc., Gilroy, CA). They were barrier reared and maintained in laminar flow isolation units supplied with filtered air. The rats were held in the laboratory for 1 week after delivery, and exposures began when the rats were 11 weeks old. Different groups, each containing 6-10 rats, were exposed to each of the various exposure conditions. The rats were exposed nose-only to a continuous stream of the atmospheres. Nose-only exposures prevent artifacts due to airborne dander, ammonia and dried excreta. Exposures were 4 hours per day, on 4 consecutive days per week. The rats were exposed on a total of 32 days over an 8-week period.

Between exposures, the rats were housed in a purified air-barrier environment. They were given clean water and dry laboratory chow *ad libitum*. Animals were handled by personnel wearing clean lab coats, head covers, shoe covers and gloves to prevent the

spread of infections to the rats.[2] Exposure tubes were thoroughly machine-washed in hot soapy water and disinfected with bleach daily. Animal quarters were cleaned twice weekly.

Pollutant generation

Ozone was generated by passing medical grade oxygen through two corona-discharge ozonizers (Sander ozonizer, Type III, Osterberg, Germany). Sulfate and nitrate aerosols (about 0.3 and 0.6 μm mass median aerodynamic diameter, respectively) were generated by nebulizing aqueous solutions of ammonium sulfate (Ho et al., 1980) or ammonium nitrate. The aerosol was dried with dilution air, brought to Boltzmann charge equilibrium by passage through a ^{85}Kr aerosol neutralizer, and was then introduced into the exposure system airstream. The chambers were maintained at 60% relative humidity.

California road dust was obtained from freeways in South Orange County with the cooperation of CALTRANS. Particles in the PM10 range were isolated using a combination of sieving and elutriation. The material was sterilized, to eliminate possible pathogens, by heating for 4 hr at 70°C prior to use. Sterilization may have altered some of the organic constituents of the road dust, although as will be described later, there was no significant ($P<0.05$) loss of mutagenic activity associated with the sterilization process. The sterilized particles were aerosolized using a continuous feed fluidized bed generator elutriated to remove particles greater than 10 μm, and then diluted, discharged and introduced into the chamber airstream. Characterization of the road dust included determination of total and extractable organic carbon contents, mutagenicity using a Salmonella-strain assay system, inorganic sulfate and nitrate concentrations.

Atmosphere Characterization

Chemical Analyses

Samples of aerosol particles were collected daily on preweighed and equilibrated (50% R.H.) Pallfex T60A20 Teflon-coated glass fiber filters. The samples were collected during the entire exposure period. After sample collection and re-equilibration to 50% R.H., the filters were weighed to obtain a total gravimetric measurement. Pallflex filters were leached with water, and the leachate analyzed for sulfate and nitrate by ion chromatography. Elemental carbon was determined from samples collected on quartz-fiber filters that were combusted in pure oxygen, and the resulting carbon dioxide (CO_2) quantitated using a Dasibi infra-red absorption monitor. Samples of road-dust collected on membrane filters from the breathing zones of rats during exposures were analyzed for elemental components by X-ray fluorescence spectrometry. Ozone was monitored using a Dasibi UV absorption monitor.

[2]Respiratory infections are tightly controlled in our laboratory because they could seriously compromise the validity of many of our endpoints.

Particle size measurements and atmosphere stability

Samples were collected for particle size analyses using cascade impactors (Sierra Model 226 and Andersen Model 298). Each particle-containing atmosphere was tested bi-weekly. A real-time aerosol monitor (MIE RAM-1) was used to provide real-time mass concentration data to assess the stability of the aerosol component of the atmospheres during each day of study.

Extraction of Road Dust Samples for Mutagenicity Testing

Unsterilized and sterilized road dust samples (134.6 and 134.9 mg, respectively) were extracted with dichloromethane followed by methanol using sonication and shaking. The extracts were dried under a stream of nitrogen and resuspended in dimethyl sulfoxide (DMSO) to an approximate concentration of 3.4 mg equivalent of road dust extracted. This concentration was serially diluted in DMSO to develop the dose-response curve.

Microsuspension Mutagenicity Assay

A microsuspension procedure reported by Kado et al. (1983, 1986), was used to evaluate mutagenicity of the road dust extract. The assay, which is a modification of the Salmonella/microsome test, is approximately 10 times more sensitive than the standard Ames Salmonella test based on absolute amounts of material added per determination.

Tester strain TA98 was kindly provided by Dr. B.N. Ames, Berkeley, CA. Bacteria were grown overnight in Oxoid Nutrient Broth No. 2 (Oxoid Ltd., Hants, England) to approximately 1-2 X 10^9 cells/ml, and harvested by centrifugation (5,000 x g, 4°C, 10 minutes). Cells were resuspended in ice-cold phosphate-buffered saline (PBS, 0.15M, pH 7.4) to a concentration of approximately 1X10^{10} cell/ml (determined spectrophotometrically at 550 nm).

The S9 and S9 mix were prepared according to the procedure of Ames et al. (1975). The S9 from livers of Aroclor 1254 pretreated male Sprague-Dawley rats contained 52.4 mg protein/ml, as determined using the modified Biuret method of Ohnishi and Bar (1978).

For the microsuspension assay, the following ingredients were added, in order, to 12 x 75 mm sterile glass culture tubes on ice: 0.1 ml S9 mix, 0.005 ml PM10 extract in DMSO, and 0.1 ml concentrated bacteria in PBS (1 x 10^{10}/ml PBS). The mixture was incubated in the dark at 37°C with rapid shaking. After 90 minutes the tubes were placed in an ice bath and taken out one at a time, immediately before adding 2 ml molten agar (Ames et al., 1975) containing 90 nmoles of histidine and biotin. The combined solutions were vortex-mixed and poured onto minimal glucose plates. Plates were incubated at 37°C in the dark for 48 hours. Strain markers were routinely determined for each experiment.

The spontaneous number of revertants was based on plates which had only DMSO added. All procedures were carried out in a room fitted with yellow fluorescent lights (G.E. F40Go) to minimize potential photo-oxidation.

Morphometric Determination of Goblet Cell Density

Tissue preparation

Fixation and sectioning of lung tissue were performed as described by Lamb and Reid (1969). Rats were anesthetized with IP pentobarbital, and the tracheas tied off just below the larynx. The lung and trachea were removed, 2.5 ml of buffered neutral formalin was injected via the trachea, and the tissues were immersed in fixative. After fixation the left lung was embedded in paraffin in one block. The lung was sectioned longitudinally through the left main airway and major intrapulmonary airways. Sections (5 μm thick) were cut from the blocks (1 section per block per animal) and stained with alcian blue/periodic acid Schiff (ABPAS) reagent.

Morphometry

Goblet cell density in the proximal (non-cartilaginous, pseudostratified columnar epithelium) intrapulmonary airways was determined as number of goblet cells per unit airway length. Approximately 20 high-power magnification fields were surveyed per animal to measure goblet cell density.

Determination of changes in macrophage function

Alveolar lavage procedure

The rats were anesthetized, the abdominal aortas were severed, and the tracheas were exposed. A catheter was inserted into the trachea and tied in place. The diaphragm was cut away from the anterior rib cage to allow the lungs to expand fully during lavage. The lungs were lavaged with Hank's Balanced Salt Solution (HBSS) without Ca^{2+} or Mg^{2+} (GIBCO) (to avoid cell clumping) at 37°C. The lavage volume was 7 ml and it was instilled and aspirated three times at a rate of about 0.5 ml/second. The lavage was repeated three times per animal and reserved (on ice). The lavage from each animal was centrifuged at 1000 RPM, at 4°C for 10 minutes. The fluid from the first lavage was reserved for protein and biochemical assays. The pelleted cells were resuspended in HBSS with Ca^{2+} and Mg^{2+} (GIBCO) at 37°C and cells were counted using a bright line hemocytometer. Viability was assessed using the Trypan Blue exclusion method. The volume of the cell suspension was adjusted to 1 million viable cells per ml. The yield by this lavage procedure was at least 1 million cells per rat, of which more than 95% were macrophages, with an average viability greater than 90%. A 0.1 ml aliquot of cells was plated onto a glass microscope slide using a cytocentrifuge (Shandon Southern). The

cells were stained with Wright-Giemsa stain and a differential count was made (Nadziejko et al., 1992; Kleinman et al., 1993).

Phagocytosis

Macrophage Phagocytosis

Phagocytic activity was measured using a suspension assay (Schlesinger et al., 1985). In brief, 1 ml of cell suspension was added to polypropylene culture tubes containing 0.5 ml of medium and 1 ml of a suspension of latex microspheres (1.1-1.4 μm diameter, 10^8 ml^{-1}). The suspensions were incubated for 60 minutes with gentle agitation. An aliquot of the suspension (0.1 ml) was removed and plated onto a microscope slide using a cytocentrifuge. The cells were then washed with calcium- and magnesium-free PBS (Phosphate Buffered Saline) (pH 7.2) to remove the free latex particles. The slides were air dried under HEPA-filtered laminar flow air and the cells stained with a Wright-Giemsa monochromatic cytological stain (Diff-Quick, American Hospital Supply). The slides were immersed in xylene for 8 hr to remove the polystyrene particles; phagocytized spheres were visualized as unstained "ghosts" in the cell cytoplasm. The percentage of latex positive cells (minimum of 2 spheres/cell) was determined.

Fc-mediated phagocytosis

A rosette assay (Rao et al., 1980; Prasad et al, 1988; Kleinman et al., 1993) was used to determine the effect of exposure on Fc receptor-mediated phagocytosis. Lab-Tek chambers, each containing 1 x 10^5 cells in 0.5 ml of HBSS, were incubated for 1 hr at 37°C. Non-adherent cells were removed by washing, and 0.1 ml of anti-Sheep Red Blood Cell (SRBC) antibody, at a previously determined antibody concentration, was added to each chamber. Plates were then incubated for 30 minutes at 37°C. After incubation, the macrophages were washed with HBSS to remove excess antibody and 0.1 ml of SRBC (1 x 10^7) was added. The chambers were incubated for 30 minutes at 37°C, the unbound SRBCs were washed away gently with HBSS, and the number of cells with three or more SRBC attached were counted as positive rosettes (a total of 300 cells were counted).

Respiratory Burst

Macrophages, on contact with antigenic material or during the act of phagocytosis, will mount a respiratory burst response in which reactive oxygen species (ROS) are synthesized and released. These compounds are highly toxic and act to preserve the sterility of the lung in the presence of inhaled pathogens. A major component of the ROS is superoxide anion, which is a free radical. Superoxide production was measured using a cytochrome C reduction method (Kemmerich et al., 1987). Aliquots of (100 μl) of cell suspensions (10 macrophages per ml) from each exposed rat were added to the wells of a 96 well microtiter plate. To these cells, 50 μl of cytochrome C (200 μm in HBSS) and 50 μl of freshly opsonized zymosan (5 mg/ml) were added, and the cultures were

incubated in the dark at 37°C for 60 min. Exactly 180 μl of each solution was transferred to a corresponding well of a sterile 96 well microtiter plate and the absorbances at 540 nm and 550 nm were read using a microtiter plate reader. The difference in absorption (A_{550}-A_{540}) was multiplied by the molar extinction coefficient for reduced cytochrome C and the results were reported as n moles/mg protein/45 minutes.

Release of Inflammatory Mediators

The mediators LTB_4 and PGE_2 play important roles in regulating inflammatory responses. These were measured using ELISA kits purchased from Cayman Chemical (Ann Arbor, MI). Aliquots (500 μl) of macrophage cell suspension (10^6 macrophages per ml) were added to the wells of a 24 well culture plate. The cells were incubated for 60 minutes at 37° to permit adherence, after which the medium containing non-adherent cells was removed. Sample was transferred to microcentrifuge tubes and centrifuged at 300 g for 10 min. The cell-free medium was returned to the macrophages in the wells and was incubated (37°C) for an additional 4 hr. The medium was withdrawn and stored frozen (-70°C) until samples were analyzed. Analyses were performed using ELISA kits (Cayman Chemical) and calibration relationships were prepared using authentic LTB_4 and PGE_2.

Permeability Measurements

Protein measurement

Total protein in the lavage was measured by the bicinchononic acid (BCA, Pierce Chemical Co, Rockford, IL) procedure (Bhalla et al., 1992). A set of protein standards concentrations ranging from 0.05 mg/ml to 0.25 mg/ml were prepared by diluting a stock solution of bovine serum albumin (BSA).

Protein standards, unknown samples, and diluents used for blanks (0.01 ml each) were pipetted into wells of a microtiter plate, followed by 2 ml of BCA working reagent. All samples were incubated at 60°C for 30 min, then cooled, followed by measurement of absorbance at 560 nm using a microtiter plate reader (Titertek Multiscan MC). Absorbance of blanks was subtracted from that for standards and unknown samples. A standard curve was prepared by plotting the net absorbance at 560 nm vs. protein concentration, and the curve was used to determine the protein concentrations of the unknown samples.

Albumin Assay

An enzyme-linked immunosorbant assay (ELISA) for detecting albumin in BALF samples was used (Bhalla et al., 1992). Polystyrene nonflexible 96 well microtiter plates (Costar) were coated with 100 μl of 2 ng/μl goat anti-rat antibody (GAR) to albumin (Cappel) in carbonate buffer, pH 9.6. The plates were covered and refrigerated overnight (18 hours). The plates were washed two times with freshly made carbonate buffer and rapped on a

blotting towel to remove excess wash material or antibody that was not firmly bound. To block non-specific binding, 150 μl of a gelatin-carbonate buffer (4 mg gelatin per ml carbonate buffer) was added to each well. The plates were covered and placed in a humid chamber at room temperature for 1 to 2 hours, and then washed two times with a solution of PBS-Tween 20-gelatin (0.5 ml TWEEN-20/L PBS + 1.0 mg Gelatin per ml). Serial dilutions of a standard (15 mg/ml) rat albumin (Sigma Chemical Co.) from 1:128,000 to 1:4096,000 and rat lavage fluid from 1:800 to 1:3200 were made using PBS-Tween 20-gelatin solution. Each well received 100 μl of the diluted standard or lavage fluid. The plates were covered and incubated in a humid chamber for 1 to 2 hours at room temperature and then washed three times with PBS-Tween-20-gelatin. A 1:2000 dilution of rabbit anti-rat IgG-peroxidase conjugated albumin (5.0 ml/ml) was made and 100 μl was added to each well. The plates were incubated in a humid chamber at room temperature for 1 hour, washed 2 times with PBS-Tween-2-gelatin and once with PBS-Tween-20. The color was developed by the addition of 100 μl per well of citrate-phosphate buffer (pH 5.0) which contained 1 mg/ml O-Phenylenediamine dihydrochloride (OPD) and 1 μl/2 ml of 30% H_2O_2. The plates were covered and incubated in the dark at room temperature for 20 minutes. The reaction was stopped by the addition of 50 μl per well of 2 N H_2SO_4. The plates were read at 492 nm with a Titertek Multiscan MC plate reader.

Breathing Pattern and Ventilatory Responses

Breathing Pattern and Ventilation

Standard nose-only exposure tubes were modified to function as flow plethysmographs for measurement of breathing frequency, tidal volume, and minute ventilation. Breathing pattern and minute ventilation were measured on the first and fourth exposure day of weeks 1, 3, 5, 7 and 8 of each experimental series. The rats (n=8 per exposure group) were held in 57 mm diameter acrylic plastic tubes with aluminum nose cones similar to the nose-only exposure tubes described by Raabe et al. (1973). These tubes were modified to function as flow plethysmographs during exposure by clamping a membrane collar of latex dental dam between the aluminum nose cone and body tube. The latex collar fit snugly around the rat's head just behind the ears, thus separating respiratory orifices from the body. Thoracic displacement of air during respiration was measured with a rodent pneumotachograph (Dynasciences, Bluebell, PA) connected between the body tube and a port in the aluminum nose cone opening at the rat's nose. Pressure across the pneumotachograph was measured with a differential pressure transducer (Validyne PM-45, Northridge, CA). The flow signal was electrically integrated and counted to display tidal volume and breath frequency on a chart recorder (Gould Model 2800S, Cleveland, OH). Eight plethysmographs were plugged into ports of the 1 m^3 stainless steel exposure chambers. Within each chamber, the exposure atmosphere was conducted through stainless steel ducts past the ports providing an individual nose-only exposure to each rat. Three sets of recordings were made during each of the 4 hour

exposures, with the three data recordings averaged to yield an estimate of breath frequency, tidal volume, and minute ventilation at each hour of exposure.

Statistical Analysis

This study consisted of four separate experiments, each with 4 or 5 exposure conditions, including a clean air control exposure. Data for each of the four experiments was analyzed separately. Data for each biological endpoint was analyzed by analysis of variance (ANOVA) to test for significant atmosphere effects. In all cases, tests were two-sided and the significance level was 0.05. *A Posteriori* multiple comparison tests (Tukey or Dunnett procedures; Snedecor and Cochran, 1985) were used to test for significant differences between individual group means. The Dunnett test was used if only comparisons vs. a single control atmosphere were being made. The Tukey test was used otherwise. Statistical power calculations on data from a previous experiment indicated adequate power to detect biologically significant changes ($ß \geq 0.8$; $\alpha \leq 0.05$) could be obtained with n=8 animals for breathing pattern data, and n=10 animals for all other endpoints.

RESULTS

Atmosphere Characterization

Analyses

The concentration of PM10 components (ammonium sulfate, ammonium nitrate and road dust), ozone and relative humidity data are summarized in Table 5 for the 4 experiments conducted during this 3 year program (mean and standard error). These values represent averages of approximately 32 daily measurements for each component.

Mass median aerodynamic diameters (MMAD) for each of the three particle components are also summarized in Table 5, which also includes the geometric standard deviation (GSD) for each particle type. The GSD is representative of the dispersivity of the aerosol. On the average, a GSD of about 2 indicates that the aerosol was polydisperse and that 70% of the particle mass was in the range of 1/2xMMAD to 2xMMAD. These values are comparable to measurements of these compounds in Southern California ambient air.

The concentrations of elemental carbon, nitrate and sulfate in road dust samples were determined in representative samples. Overall, carbon represented less than 1%, nitrate represented about 0.5%, and sulfate represented about 0.3% of road dust by mass. Combined, these minor constituents of road dust contributed about 15 $\mu g/m^3$ in the high (900 $\mu g/m^3$) atmospheres, and 5 $\mu g/m^3$ in the low (300 $\mu g/m^3$) atmospheres. Atmospheric stability for the road dust atmosphere was monitored using a real-time analyzer. As an example, data recorded at 30-min intervals are shown in Figure 1A for the low concentration of individual components exposure study.

Table 5: Atmosphere Composition: Concentration ($\mu g/m^3$, Mean ± S.D. of weekly average concentrations)

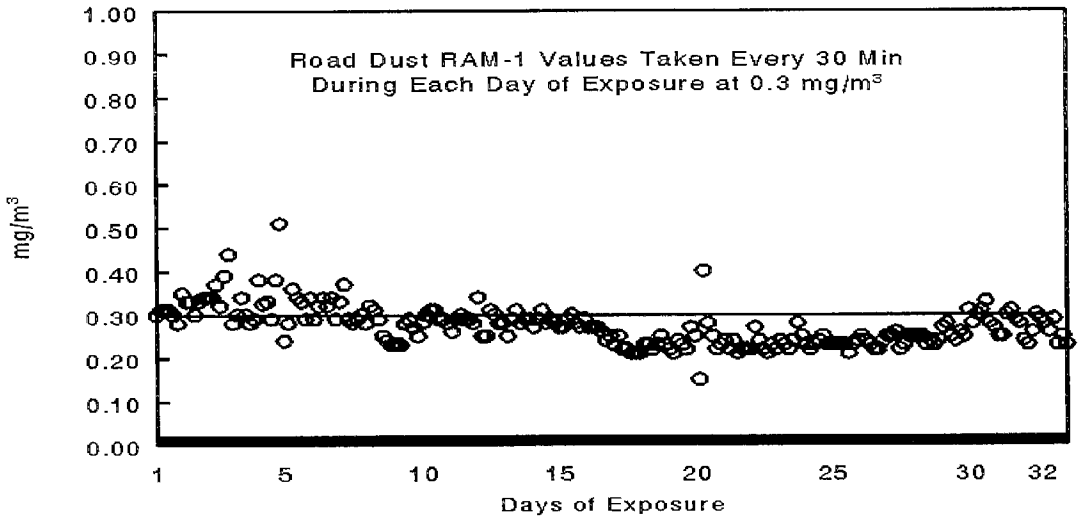
	Indv. Comp.		Mixtures[e]		Average Particle Size	
	High Conc.	Low Conc.	+ O_3	+ O_3	MMAD (μm)	GSD
Road Dust[f]	930±80	318±26	302±25	350±75[g]	5.0±0.3	1.8±0.1
Sulfate	71±3	19±1	70±4	65±14	0.28±0.03	2.6±0.2
Nitrate	353±10	91±2	---	365±35	0.6±0.1	2.2±0.2
Ozone	--	---	0.19±0.01	0.20±0.01	---	---
RH	59±1	61±1	58±2	62±2	---	---

[e]Determined by mass balance after subtraction of nitrate and sulfate mass estimates from gravimetric results.

[f]Elemental carbon, analyzed by combustion, represented about 1% by mass. Extractable nitrate and sulfate concentrations represented less than 0.5% by mass. In road dust-containing atmospheres at 900 $\mu g/m^3$, these three components combined represented less than 15 $\mu g/m^3$.

[g]The relatively large standard deviation is due to inclusion of variance due to nitrate and sulfate components in the total variance of the mixture (which represents about 50% of the total variance).

Figure 1A. Atmospheric Stability Monitored Using A Real-Time Analyzer



Mutagenicity Testing

Dr. Norman Kado (of U.C. Davis) performed mutagenicity testing of samples of road dust. The results, summarized in Table 6, indicate that on the basis of revertants/mg extracted, road dust mutagenicity (15 rev/mg road dust) was similar to that found for extracted ambient PM10 aerosols (5 rev/mg) and for NIST 1649 "Urban Dirt" standard reference material (20 rev/mg).

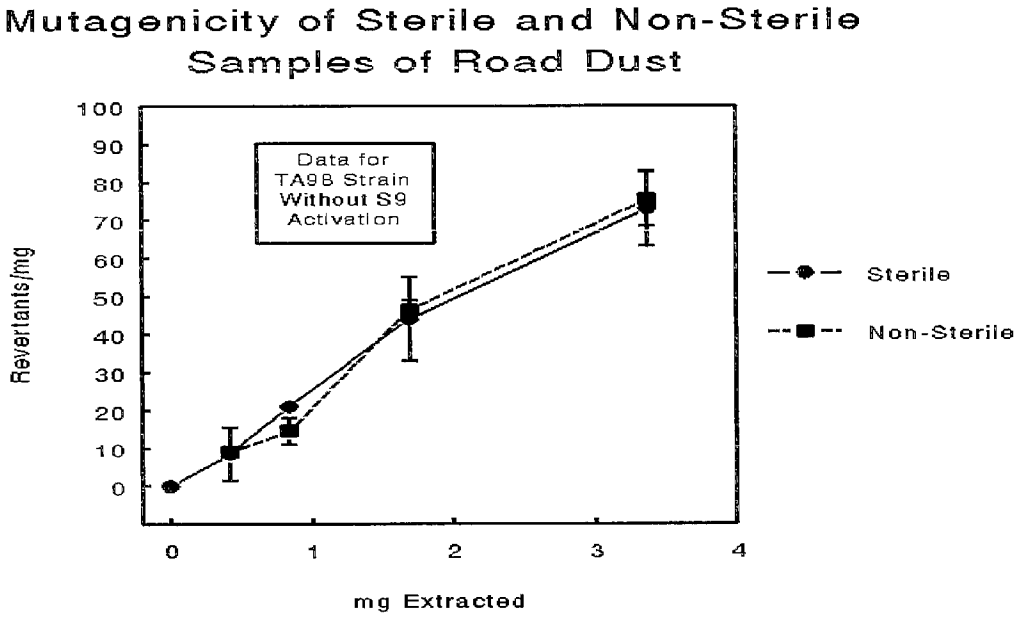
There was concern that the sterilization process used in preparing the road dust would alter mutagenicity. To test this, dose-response curves were prepared using sterilized and non-sterilized samples. There were no differences between these materials when using tester strain TA98 (Figure 1B). There was some divergence using tester strain TA100 at the two highest dose levels, but at levels consistent with ambient air samples, there was no difference.

Table 6. Mutagenicity Testing of Road Dust Samples

Revertants/μg, Mean ± S.D.

	-S9		+S9	
	TA98	TA100	TA98	TA100
Sterilized Road Dust	22±2.1	11±4.6	40±2.5	11±7.3
Unsterilized Road Dust	23±1.4	19±5.3	46±7.6	14±1.0
NIST 1649 Reference Material	20		---	
Ambient Air PM10	6.1 ± 3.6		---	

Figure 1B. Mutagenicity of Sterile and Non-sterile Road Dust



Permeability

Protein

Bronchoalveolar lavage fluid (BALF) from clean air exposed control rats and rats exposed to the various atmospheres was analyzed for total protein and albumin as indicators of epithelial disruption and increased mucosal permeability. The results of these studies are shown in Figures 2A - 2H. Altered permeability was observed in rats exposed to the high concentrations of the PM10 components. Total protein concentration in the BALF was significantly ($P<0.05$) higher in the rats exposed to sulfate and nitrate when compared to the controls. Albumin, considered to be a more accurate indicator of transmucosal transport, was elevated in BALF of rats exposed to all of the atmospheres, however the difference was significant only for nitrate ($p<0.05$) and near-significant for sulfate and road dust ($p<0.08$). Protein concentrations in the BALF of rats exposed to individual PM10 components in combination with O_3 were similar to the protein concentrations in the control rats. When the albumin concentrations in the BALF of these rats were determined, a slight, but nonsignificant increase ($P>0.05$) was observed in the groups exposed to O_3 or sulfate + O_3. Total protein, but not albumin, measurements, revealed a similar trend of nonsignificant ($P>0.05$) elevation in the group of rats exposed to nitrate + sulfate + road dust + O_3 in comparison to the group exposed to nitrate + sulfate + road dust.

Total Protein in BAL of Rats Exposed to:

Fig. 2A Filtered Air, Dust (900 $\mu g/m^3$), SO_4 (70$\mu g/m^3$), or NO_3 (350 $\mu g/m^3$)

Fig. 2B Filtered Air, Dust (300 $\mu g/m^3$), SO_4 (20 $\mu g.m^3$), or NO_3 (90 $\mu g.m^3$)

Fig. 2C Filtered Air, O_3 (0.2 ppm), Dust (300 $\mu g/m^3$) + O_3 (0.02 ppm), or SO_4 (70 $\mu g/m^3$) + O_3 (0.2 ppm)

Fig. 2D Filtered Air, O_3 (0.2 ppm), NO_3 (90 $\mu g/m^3$) + O_3 (0.02 ppm), NO_3 (350 $\mu g/m^3$) + SO_4 (70 $\mu g/m^3$) + Dust (300 $\mu g/m^3$), or NO_3 (350 $\mu g/m^3$) + SO_4 (70$\mu g/m^3$) + Dust (300 $\mu g/m^3$) + O_3 (0.02 ppm)

Figure 2A: Total protein in BAL of rats exposed to air, dust (900 mg/m3), SO4 (70 mg/m3) or NO3 (350 mg/m3)

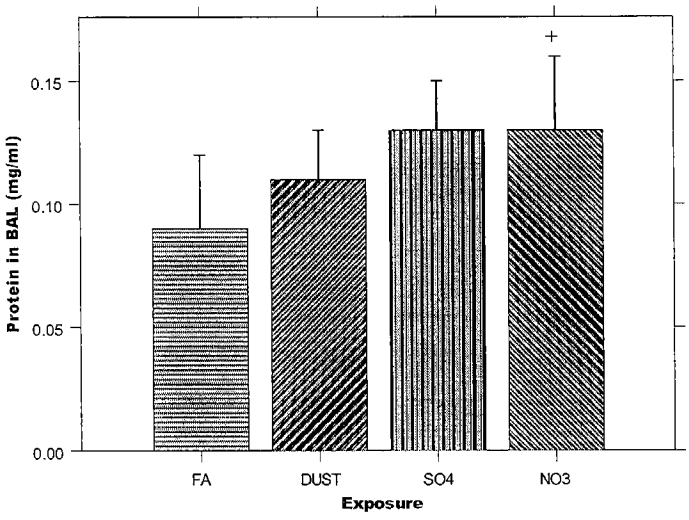


Figure 2B:Total protein in BAL of rats exposed to air, dust (300 mg/m3, SO4 (20 mg/m3) or NO3 (90 mg/m3)

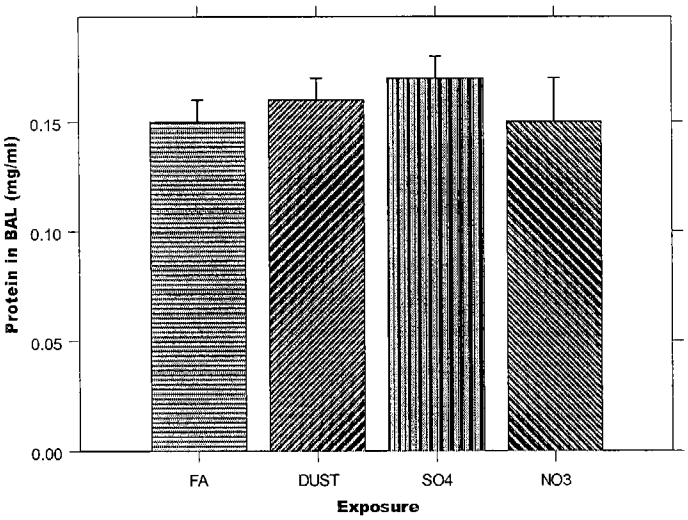


Figure 2C: Total protein in BAL of rats exposed to air, dust (300 mg/m3) + O3 (0.2 ppm), SO4 (70 mg/m3) + O3 (0.2 ppm) or O3 (0.2 ppm)

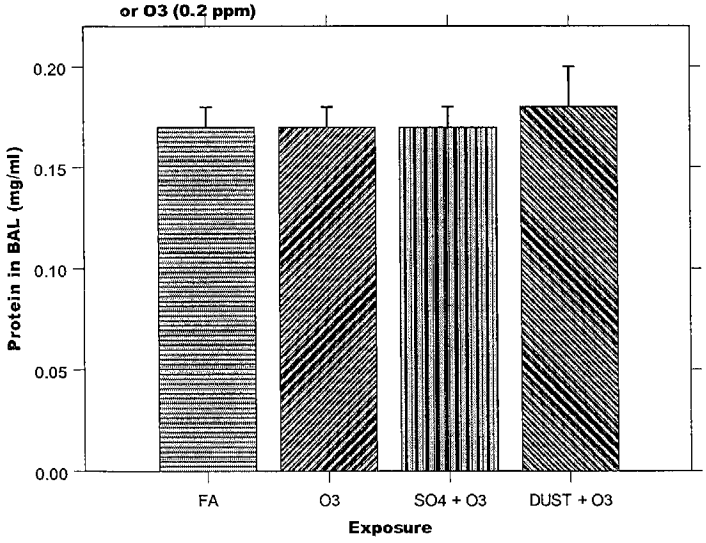
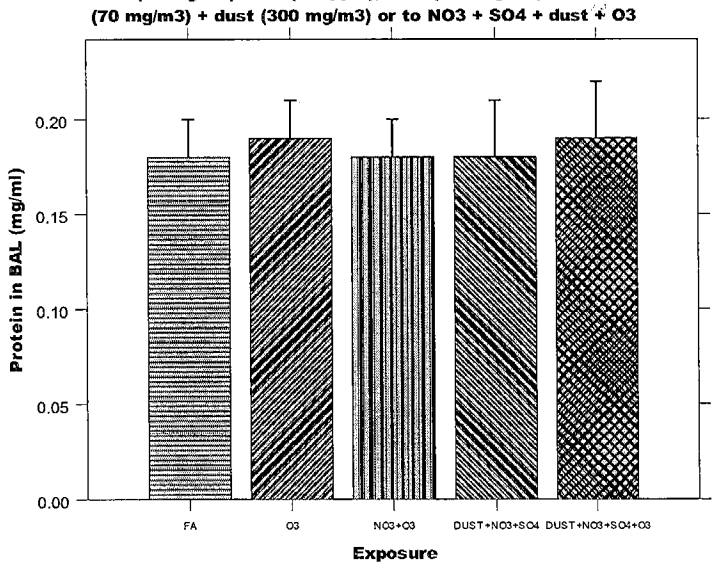


Figure 2D: Total protein in BAL of rats exposed to air, O3 (0.2 ppm), NO3 (90 mg/m3) + O3 (0.2 ppm), NO3 (350 mg/m3) + SO4 (70 mg/m3) + dust (300 mg/m3) or to NO3 + SO4 + dust + O3



Total Albumin in BAL of Rats Exposed to:

Fig. 2E Filtered Air, Dust (900 $\mu g/m^3$), SO_4 (70 $\mu g/m^3$), or NO_3 (350 $\mu g/m^3$)

Fig. 2F Filtered Air, Dust (300 $\mu g/m^3$), SO_4 (20 $\mu g.m^3$), or NO_3 (90 $\mu g.m^3$)

Fig. 2G Filtered Air, O_3 (0.2 ppm), Dust (300 $\mu g/m^3$) + O_3 (0.02 ppm), or SO_4 (70 $\mu g/m^3$) + O_3 (0.2 ppm)

Fig. 2H Filtered Air, O_3 (0.2 ppm), NO_3 (90 $\mu g/m^3$) + O_3 (0.02 ppm), NO_3 (350 $\mu g/m^3$) + SO_4 (70 $\mu g.m^3$) + Dust (300 $\mu g/m^3$), or NO_3 (350 $\mu g/m^3$) + SO_4 (70$\mu g/m^3$) + Dust (300 $\mu g/m^3$) + O_3 (0.02 ppm)

Figure 2E: Total albumin in BAL of rats exposed to air, dust (900 mg/m3), SO4 (70 mg/m3) or NO3 (350 mg/m3)

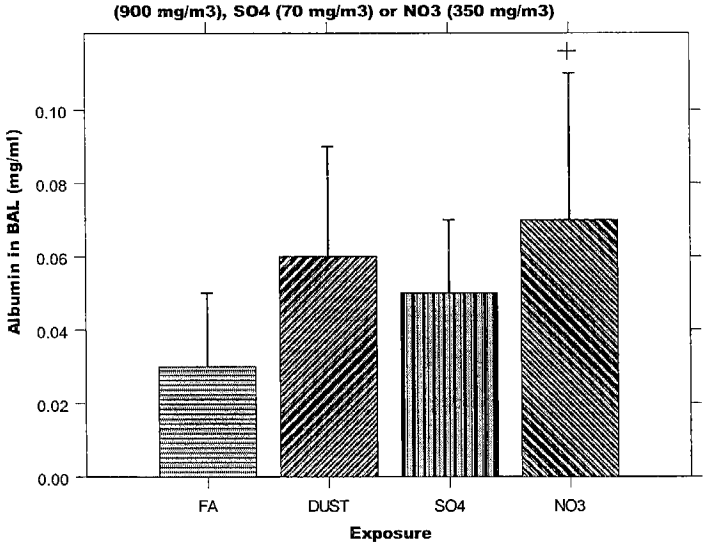


Figure 2F: Total albumin in BAL of rats exposed to air, dust (300 mg/m3), SO4 (20 mg/m3) or NO3 (90 mg/m3).

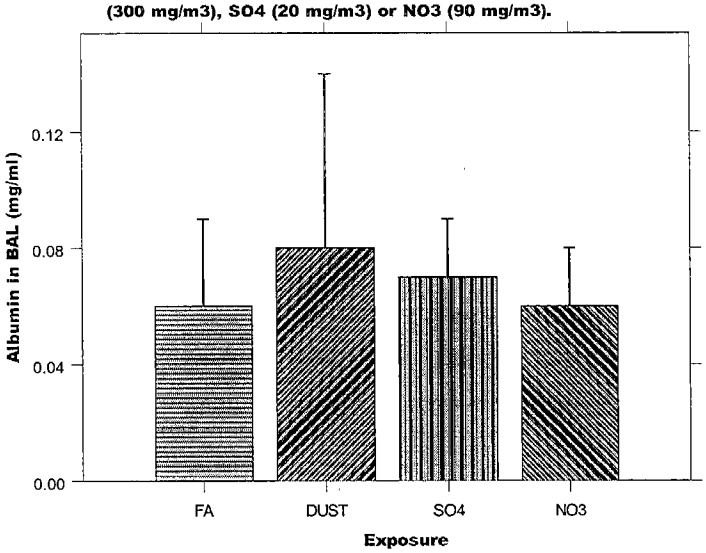


Figure 2G: Total albumin in BAL of rats exposed to air, dust (300 µg/m3) + O3 (0.2 ppm), SO4 (70 µg/m3) + O3 (0.2 ppm) or O3 (0.2 ppm)

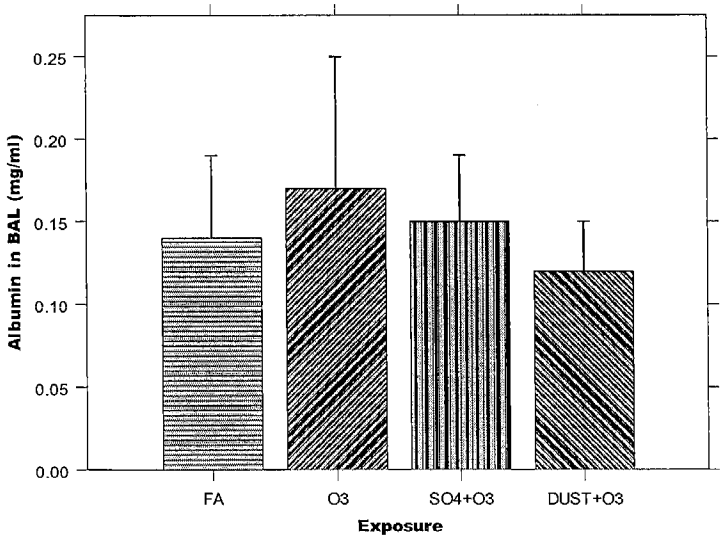
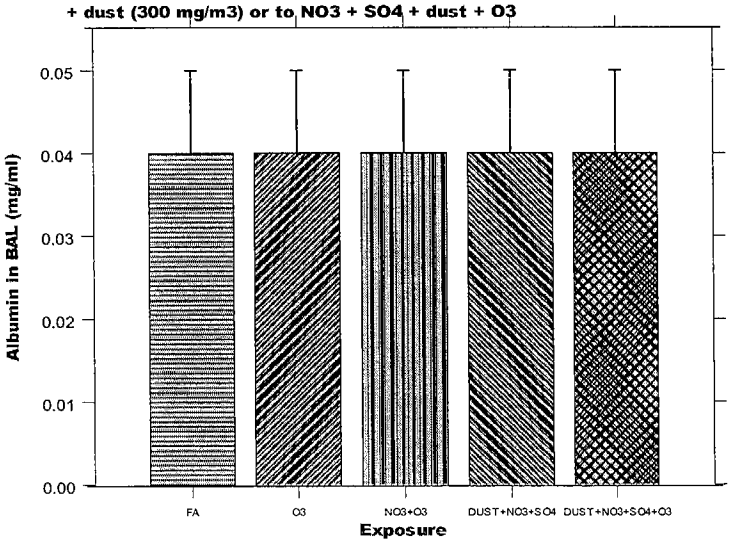


Figure 2H: Total albumin in BAL of rats exposed to air, O3 (0.2 ppm), NO3 (90 mg/m3) + O3 (0.2 ppm), NO3 (350 mg/m3) + SO4 (70 mg/m3) + dust (300 mg/m3) or to NO3 + SO4 + dust + O3



Rats exposed to the low concentrations of the various particles, both singly and in mixtures, did not have an increase ($P>0.05$) in BALF albumin concentration as was observed with the high concentration exposures. Only protein content of BALF from rats exposed to sulfate was significantly ($P<0.05$) elevated in comparison to the controls. Albumin levels in all of the exposure groups were similar to those in the control groups ($P>0.05$).

Morphology

Goblet Cell Quantitation

The number of goblet cells in the trachea, lobar bronchi and brochioles are shown in Figures 3A – 3J. The first experimental series involved exposure to high concentrations of road dust, sulfate and nitrate. Goblet cell number in the trachea was calculated by Dr. Nadziejko, and was expressed as the number of cells per field. This analysis revealed that the PM10 components studied in this project did not affect the goblet cell population ($P>0.05$).

The mode of analysis was modified in later experiments to improve the sensitivity. The modified analysis involved determination of goblet cell number as a fraction of total cell number in different airway regions (i.e., trachea, lobar bronchus and bronchioles). There were no differences between the FA exposed control animals, and any of the exposed groups ($P>0.05$).

In one experimental series the goblet cell fractions in the Particle + O_3-exposed group (Figure 3I) were lower in the bronchiolar epithelium than at the corresponding site in the air-exposed controls. However, the goblet cell number in the group exposed to the mixture containing O_3 (Figure 3J) was higher than in the O_3-exposed group ($P>0.05$).

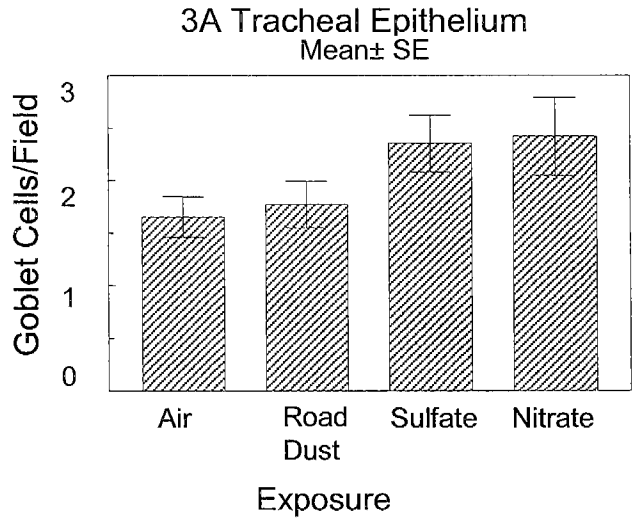
Goblet Cells in Trachael Epithelium:

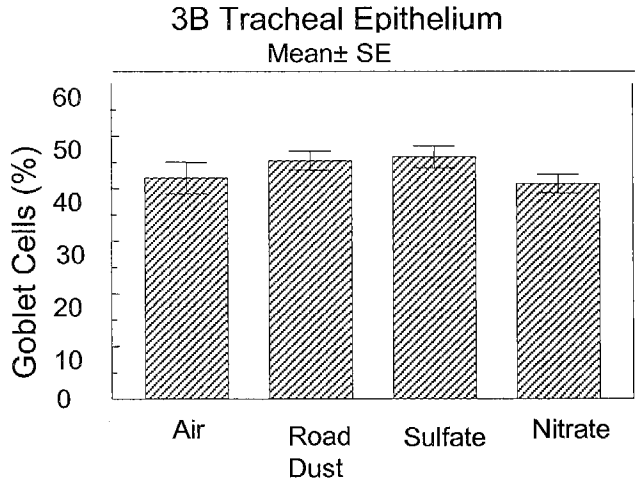
Fig. 3A Filtered Air, Road Dust (900 μg/m^3), SO_4 (70 μg/m^3), NO_3 (350 μg/m^3)

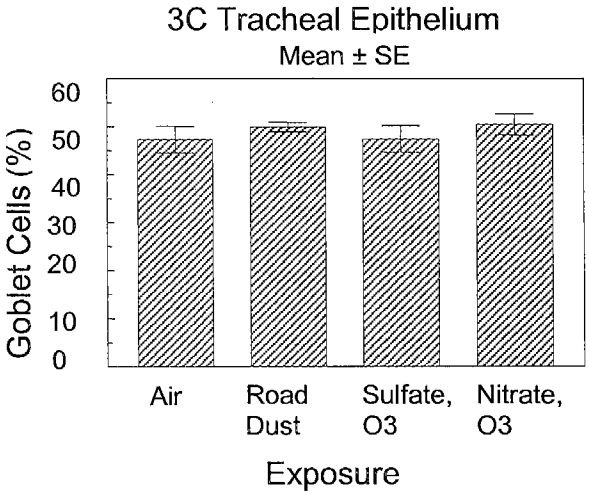
Fig. 3B Filtered Air, Road Dust (300 μg/m^3), SO_4 (20 μg/m^3), NO_3 (90 μg/m^3)

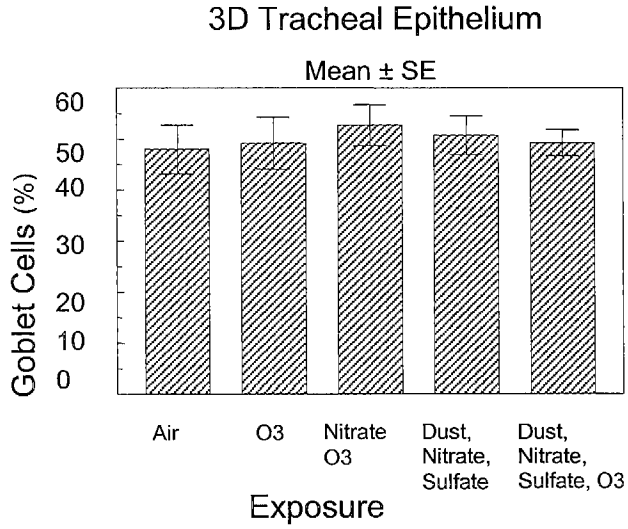
Fig. 3C Filtered Air, Road Dust (300 μg/m^3) + O_3 (0.2 ppm), SO_4 (70 μg/m^3) + O_3 (0.2 ppm), NO_3 (350 μg/m^3) + O_3 (0.2 ppm)

Fig. 3D Filtered Air, O_3 (0.2 ppm), NO_3 (350 μg/m^3) + O_3 (0.2 ppm), NO_3 (350 μg/m^3) + SO_4 (70 μg/m^3) + Road Dust (300 μg/m^3), NO_3 (350 μg/m^3) + SO_4 (70 μg/m^3) + Road Dust (300 μg/m^3) + O_3 (0.2 ppm)







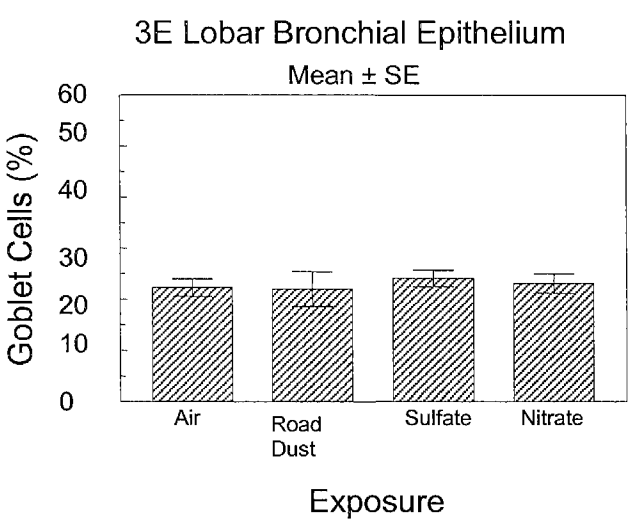


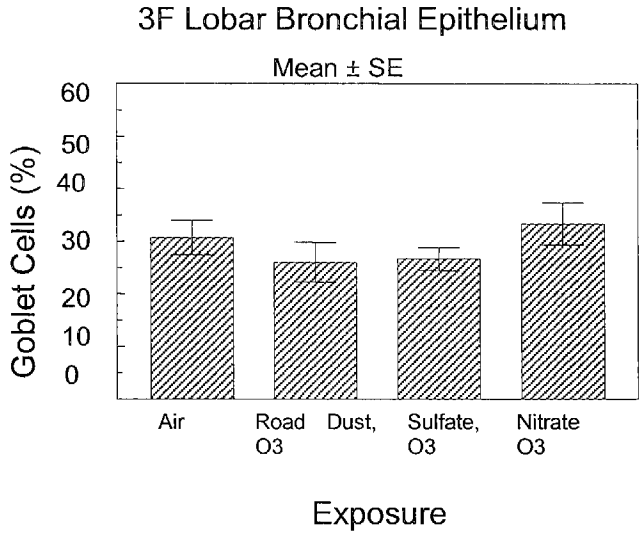
Goblet Cells in Lobar Bronchial Epithelium:

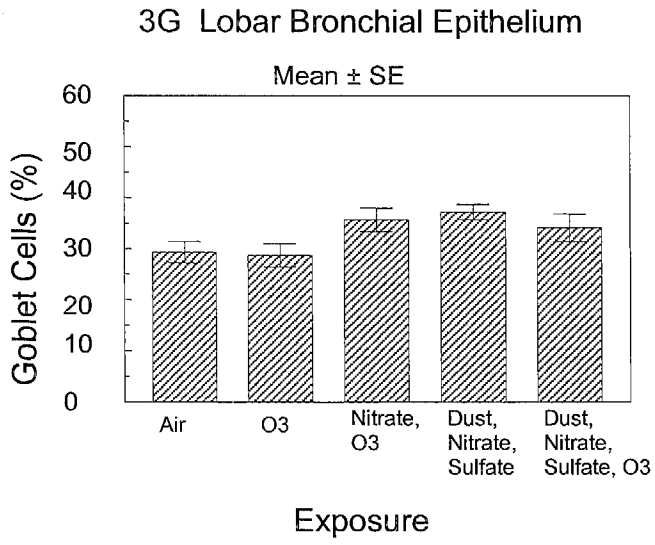
Fig. 3E Filtered Air, Road Dust (300 μg/m^3), SO_4 (20 μg/m^3), NO_3 (90 μg/m^3)

Fig. 3F Filtered Air, Road Dust (300 μg/m^3) + O_3 (0.2 ppm), SO_4 (70 μg/m^3) + O_3 (0.2 ppm), NO_3 (350 μg/m^3) + O_3 (0.2 ppm)

Fig. 3G Filtered Air, O_3 (0.2 ppm), NO_3 (350 μg/m^3) + O_3 (0.2 ppm), NO_3 (350 μg/m^3) + SO_4 (70 μg/m^3) + Road Dust (300 μg/m^3), NO_3 (350 μg/m^3) + SO_4 (70 μg/m^3) + Road Dust (300 μg/m^3) + O_3 (0.2 ppm)





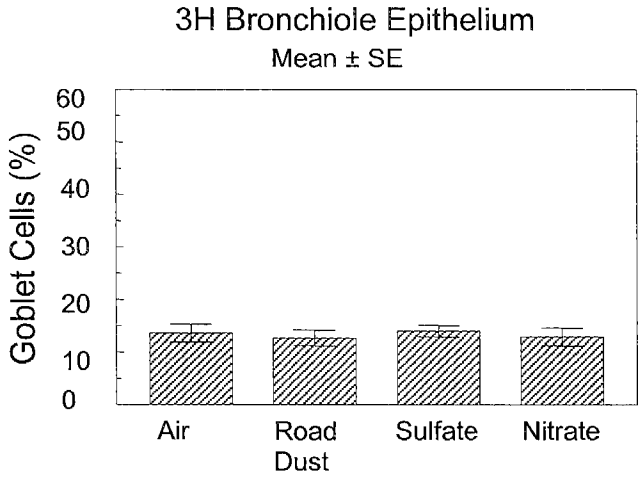


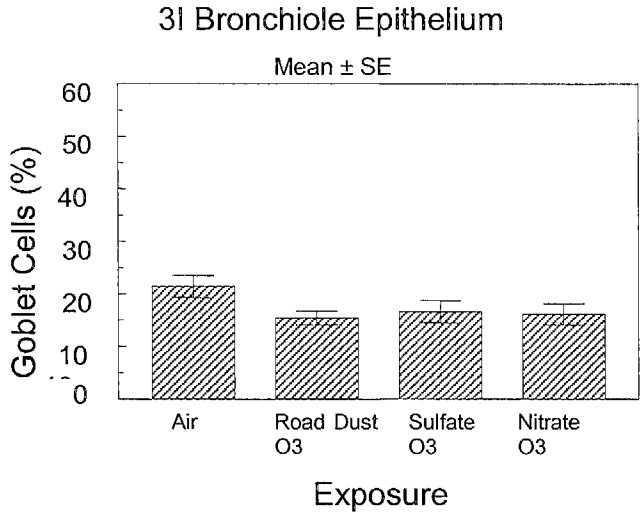
Goblet Cells in Bronchiolar Epithelium:

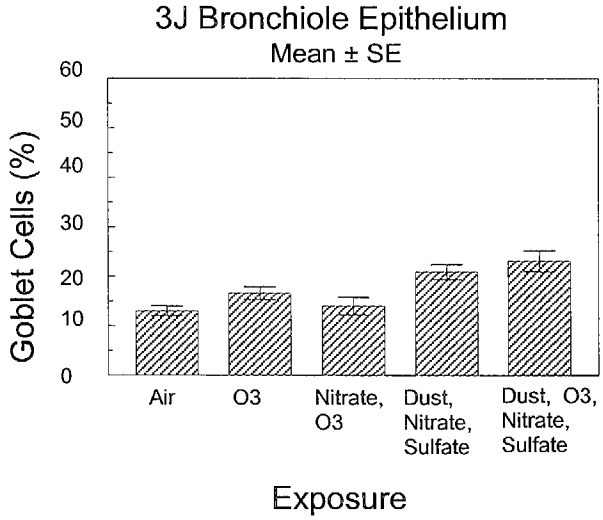
Fig. 3H Filtered Air, Road Dust (300 μg/m^3), SO_4 (20 μg/m^3), NO_3 (90 μg/m^3)

Fig. 3I Filtered Air, Road Dust (300 μg/m^3) + O_3 (0.2 ppm), SO_4 (70 μg/m^3) + O_3 (J.2 ppm), NO_3 (350 μg/m^3) + O_3 (0.2 ppm)

Fig. 3J Filtered Air, O_3 (0.2 ppm), NO_3 (350 μg/m^3) + O_3 (0.2 ppm), NO_3 (350 μg/m^3) + SO_4 (70 μg/m^3) + Road Dust (300 μg/m^3), NO_3 (350 μg/m^3) + SO_4 (70 μg/m^3) + Road Dust (300 μg/m^3) + O_3 (0.2 ppm)







Histopathology

A detailed morphometric analysis of lung lesions did not constitute a part of this project, but in a pilot study, lungs from the high exposure groups were examined for adverse effects on cell morphology. The results indicate a substantial effect of the PM10 atmospheres. The changes included an increase in alveolar nuclear density, an increase in alveolar septal wall thickness, a decrease in alveolar surface area and a decrease in alveolar chord length. The magnitude of these effects was consistently in the order of nitrate>sulfate> road dust.

Macrophage Responses

Fc Mediated Phagocytic Activity (FcR)

In general, as shown in Figures 4A to 4D, macrophages from rats exposed to PM10 components exhibited depressed ability to attack antigenic material via Fc receptor-mediated processes. The effects were small, but at the high concentration, the effects of nitrate were statistically significant ($p<0.05$) and at the low concentration, significant ($P<0.05$) reductions were seen after exposures to nitrate and to sulfate. Ozone alone (0.2 ppm) significantly ($P<0.05$) reduced FcR activity, and combined exposures to ozone with both dust (low concentration) and sulfate (high concentration) showed reductions which were slightly, but not significantly ($P>0.05$) greater than ozone alone. The four component mixture (high nitrate, high sulfate and low dust plus O_3) showed significant ($P<0.05$) reduction vs. control, while ozone alone did not ($P>0.05$). The degree of reduction induced by the mixture was approximately equal to the sum of the reductions caused by the three component mixture and ozone atmospheres. The data support the hypothesis that PM10 components can reduce macrophage function.

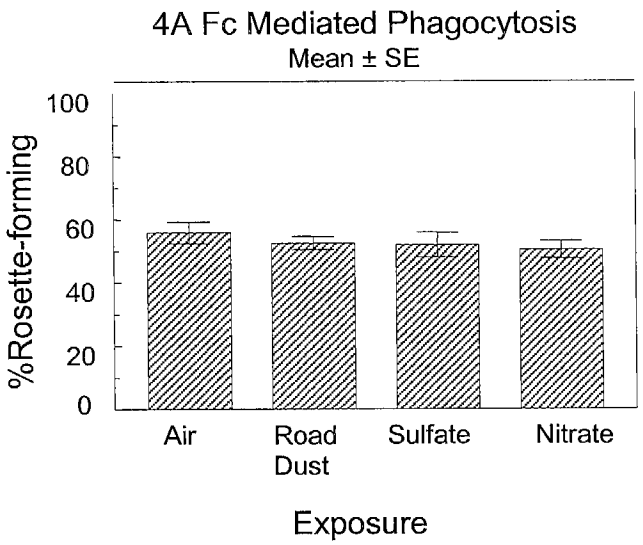
FcR-Mediated Phagocytosis:

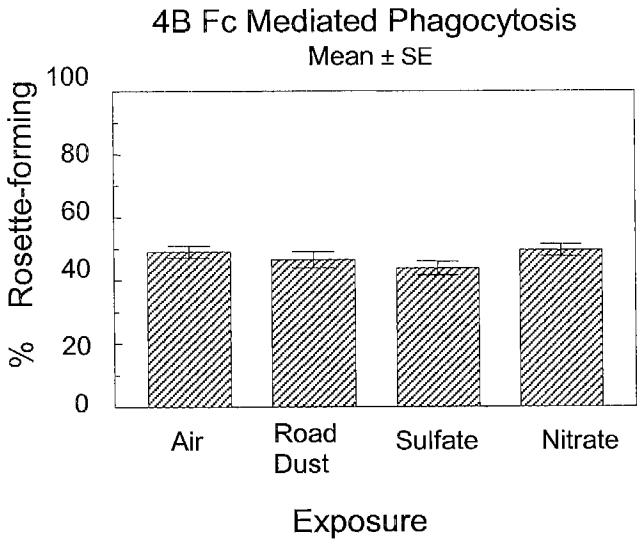
Fig. 4A Filtered Air, Road Dust (900 $\mu g/m^3$), SO_4 (70 $\mu g/m^3$), NO_3 (350 $\mu g/m^3$)

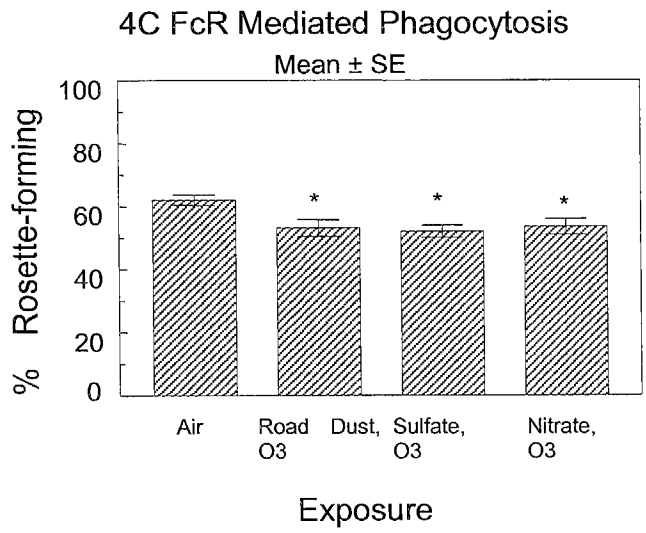
Fig. 4B Filtered Air, Road Dust (300 $\mu g/m^3$), SO_4 (20 $\mu g/m^3$), NO_3 (90 $\mu g/m^3$)

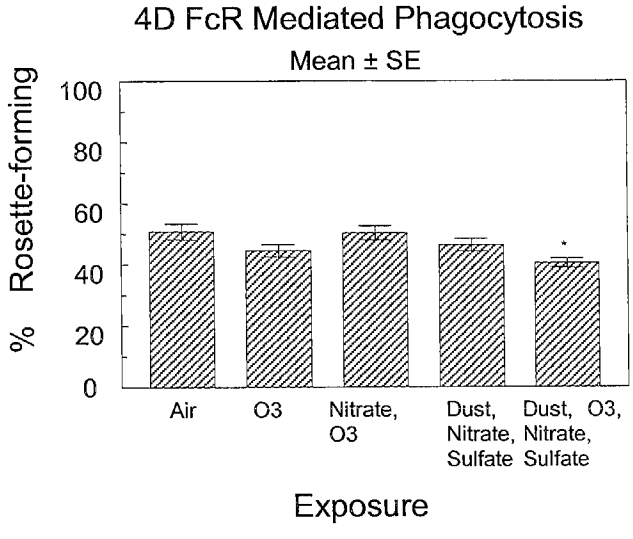
Fig. 4C Filtered Air, Road Dust (300 $\mu g/m^3$) + O_3 (0.2 ppm), SO_4 (70 $\mu g/m^3$) + O_3 (0.2 ppm), NO_3 (350 $\mu g/m^3$) + O_3 (0.2 ppm)

Fig. 4D Filtered Air, O_3 (0.2 ppm), NO_3 (350 $\mu g/m^3$) + O_3 (0.2 ppm), NO_3 (350 $\mu g/m^3$) + SO_4 (70 $\mu g/m^3$) + Road Dust (300 $\mu g/m^3$), NO_3 (350 $\mu g/m^3$) + SO_4 (70 $\mu g/m^3$) + Road Dust (300 $\mu g/m^3$) + O_3 (0.2 ppm)









Phagocytosis

The clearance of insoluble particles from the alveolar region of the lung is largely dependent on the phagocytic activity of resident macrophages. This activity is not dependent upon Fc receptors and is essentially a non-specific response. The results indicate that macrophages from road dust-exposed rats show decreased ($P<0.05$) phagocytic activity

Respiratory Burst Activity

Respiratory burst data for individual components at high and low concentrations are summarized in Figures 4E and 4F, respectively. The production of superoxide was significantly depressed by high concentrations of sulfate alone ($p<0.05$) and by road dust alone ($p<0.05$). Mixtures of sulfate + O_3 (Figure 4G), O_3 alone and O_3 + nitrate (Figure 4H) significantly increased respiratory burst activity ($p<0.05$). Effects of ozone alone were equivocal in that significant ($P>0.05$) changes from control were not seen in one of the experiments (Figure 4G), but were seen in another experiment ($P<0.05$; Figure 4H). On the other hand, the mixture of road dust + O_3 (Figure 4G) and the three and four component road dust-containing mixtures (Figure 4H) were not different from control ($P>0.05$).

The data demonstrate interesting phenomena. First, the fine particle components (nitrate and sulfate) when administered alone, reduced respiratory burst activity, which might tend to reduce the biocidal competence of the macrophages. In the presence of O_3, however, both compounds significantly ($P<0.05$) increased superoxide production, and thus may tend to exacerbate inflammatory responses to injury caused by oxidant pollutants. Second, atmospheres containing the large particle component, road dust at a concentration of 300 $\mu g/m^3$, did not induce significant ($P<0.05$) changes in respiratory burst activity either with or without O_3, or with or without nitrate and sulfate.

Release of inflammatory mediators

The production of PGE_2 and LTB_4 by macrophages stimulated with opsonized zymosan or phorbol myristate acetate is summarized in Figures 4I to 4L. Individual analyses of variance for each exposure experiment were performed. There were no significant ($P>0.05$) effects of exposure atmosphere, however in all but two cases, PGE_2 and LTB_4 production were upregulated in macrophages from PM10-exposed rats. This finding supports the hypothesis that particle exposures can influence inflammatory processes, and is in concordance with our findings on respiratory burst.

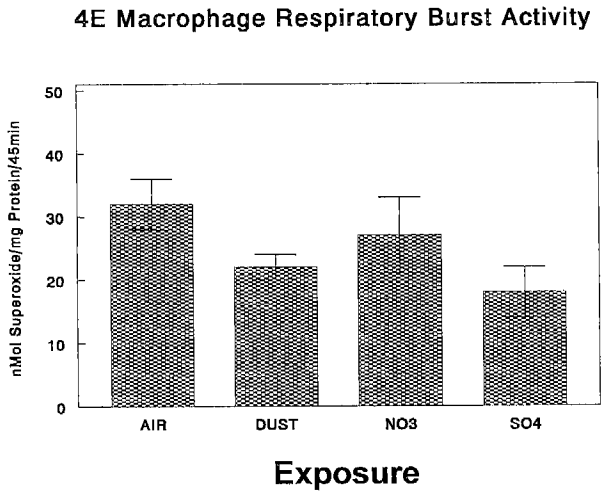
Macrophage Respiratory Burst Activity:

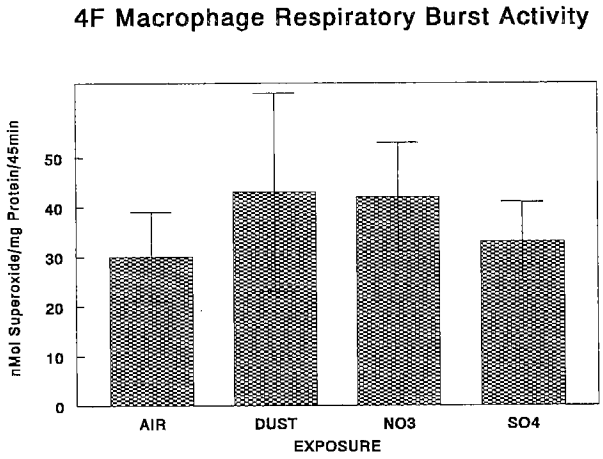
Fig. 4E Filtered Air, Road Dust (900 $\mu g/m^3$), SO_4 (70 $\mu g/m^3$), NO_3 (350 $\mu g/m^3$)

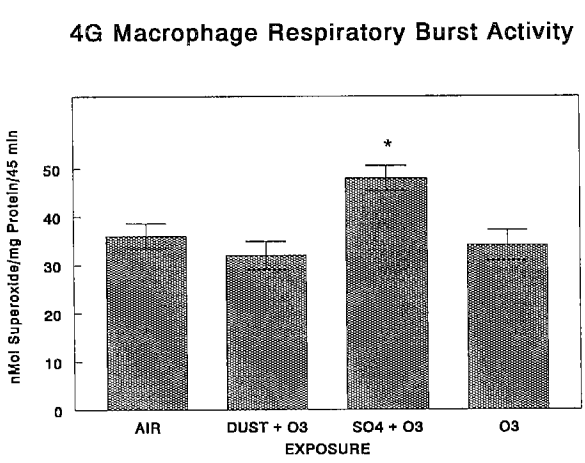
Fig. 4F Filtered Air, Road Dust (300 $\mu g/m^3$), SO_4 (20 $\mu g/m^3$), NO_3 (90 $\mu g/m^3$)

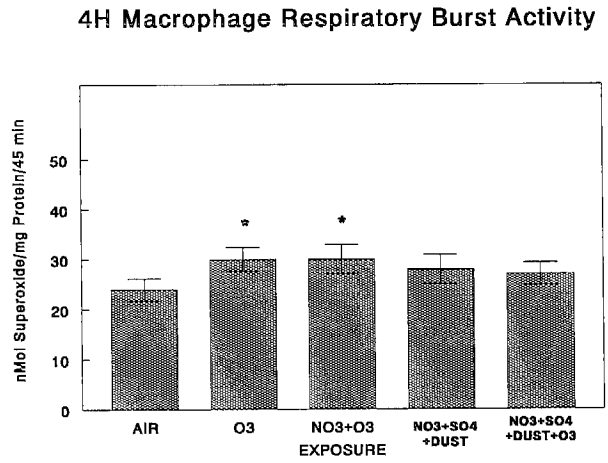
Fig. 4G Filtered Air, Road Dust (300 $\mu g/m^3$) + O_3 (0.2 ppm), SO_4 (70 $\mu g/m^3$) + O_3 (0.2 ppm), NO_3 (350 $\mu g/m^3$) + O_3 (0.2 ppm)

Fig. 4H Filtered Air, O_3 (0.2 ppm), NO_3 (350 $\mu g/m^3$) + O_3 (0.2 ppm), NO_3 (350 $\mu g/m^3$) + SO_4 (70 $\mu g/m^3$) + Road Dust (300 $\mu g/m^3$), NO_3 (350 $\mu g/m^3$) + SO_4 (70 $\mu g/m^3$) + Road Dust (300 $\mu g/m^3$) + O_3 (0.2 ppm)





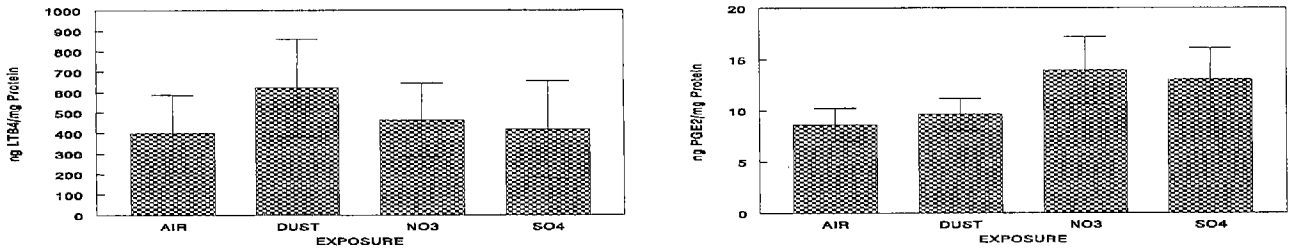




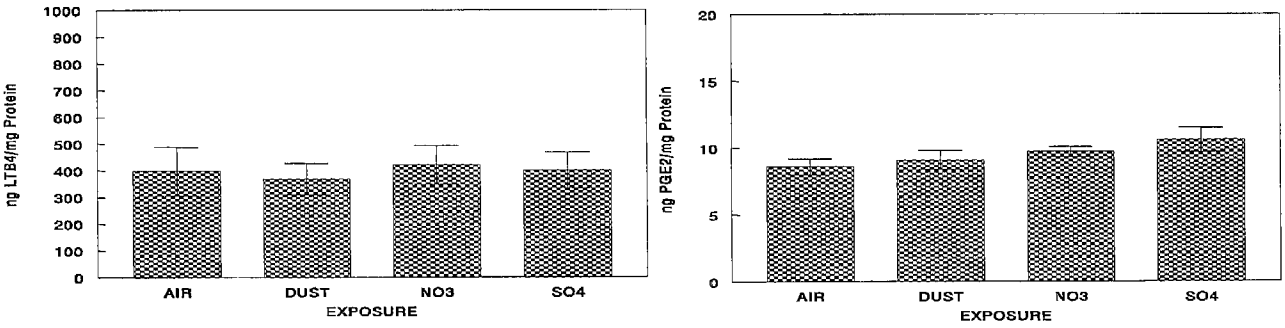
Arachidonic Acid Metabolites:

Fig. 4I Filtered Air, Road Dust (900 μg/m^3), SO_4 (70 μg/m^3), NO_3 (350 μg/m^3)
Fig. 4J Filtered Air, Road Dust (300 μg/m^3), SO_4 (20 μg/m^3), NO_3 (90 μg/m^3)

4 I Macrophage - Generated Arachidonic Acid Metabolites



4J Macrophage - Generated Arachidonic Acid Metabolites

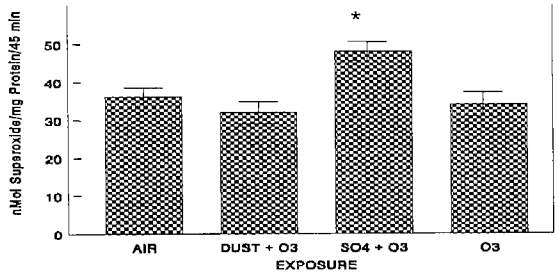


Arachidonic Acid Metabolites:

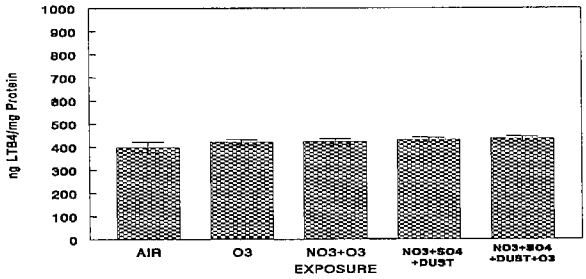
Fig. 4K Filtered Air, Road Dust (300 $\mu g/m^3$) + O_3 (0.2 ppm), SO_4 (70 $\mu g/m^3$) + O_3 (0.2 ppm), NO_3 (350 $\mu g/m^3$) + O_3 (0.2 ppm)

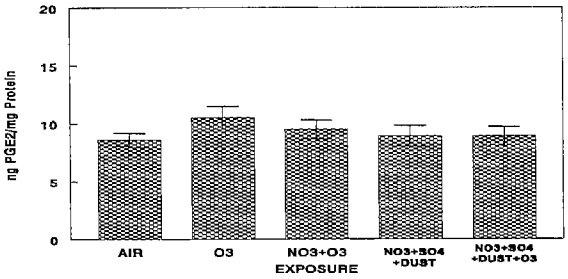
Fig. 4L Filtered Air, O_3 (0.2 ppm), NO_3 (350 $\mu g/m^3$) + O_3 (0.2 ppm), NO_3 (350 $\mu g/m^3$) + SO_4 (70 $\mu g/m^3$) + Road Dust (300 $\mu g/m^3$), NO_3 (350 $\mu g/m^3$) + SO_4 (70 $\mu g/m^3$) + Road Dust (300 $\mu g/m^3$) + O_3 (0.2 ppm)

4K Macrophage – Generated Arachidonic Acid Metabolites



4L Macrophage - Generated Arachidonic Acid Metabolites

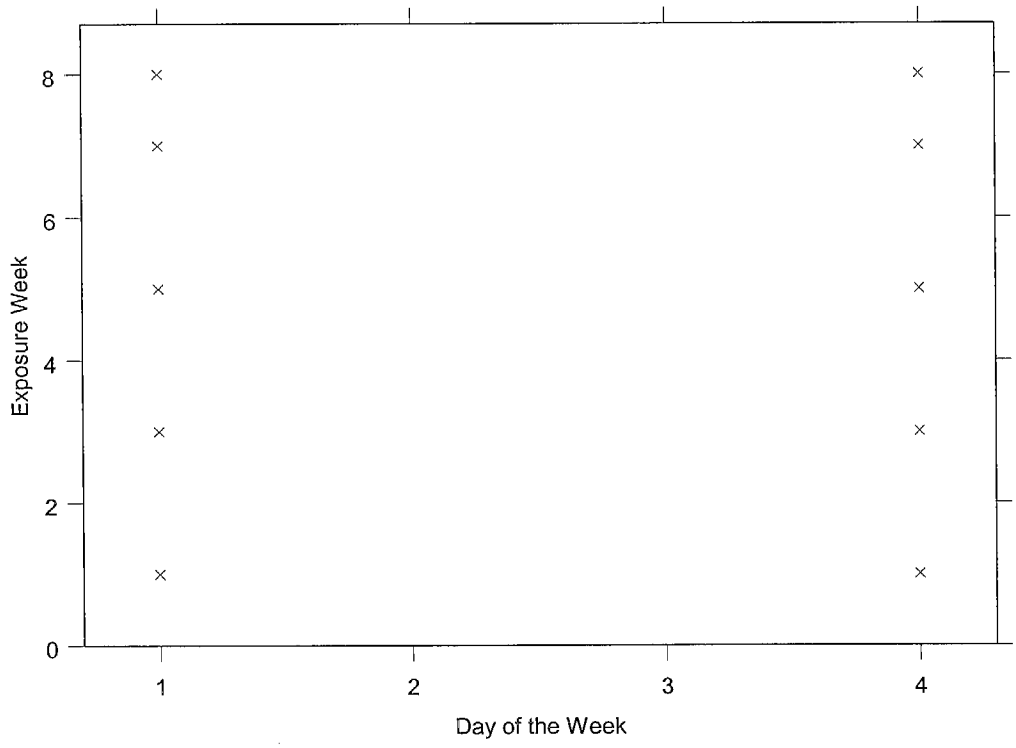




Breathing Pattern and Minute Ventilation

Breathing pattern and minute ventilation were measured on the same animals on the first and fourth days of every other week during the course of the 8-week experiments (Figure 5A). Data from the fourth hour of exposure, when breathing pattern responses were expected to be developed, were analyzed by repeated measures analysis of variance. Because responses on the first day of each week (the beginning of each weekly exposure episode) could differ from responses on the fourth day of each weekly exposure episode (the end of each weekly episode), data for the first days of weeks (days 1, 9, 17, 25, and 29) were analyzed separately from data for the fourth days of weeks (days 4, 12, 20, 28, and 32). The effects of upper airway irritants were usually observed after the first hour, and those of pulmonary irritants were observed during the third and fourth hour of exposure.

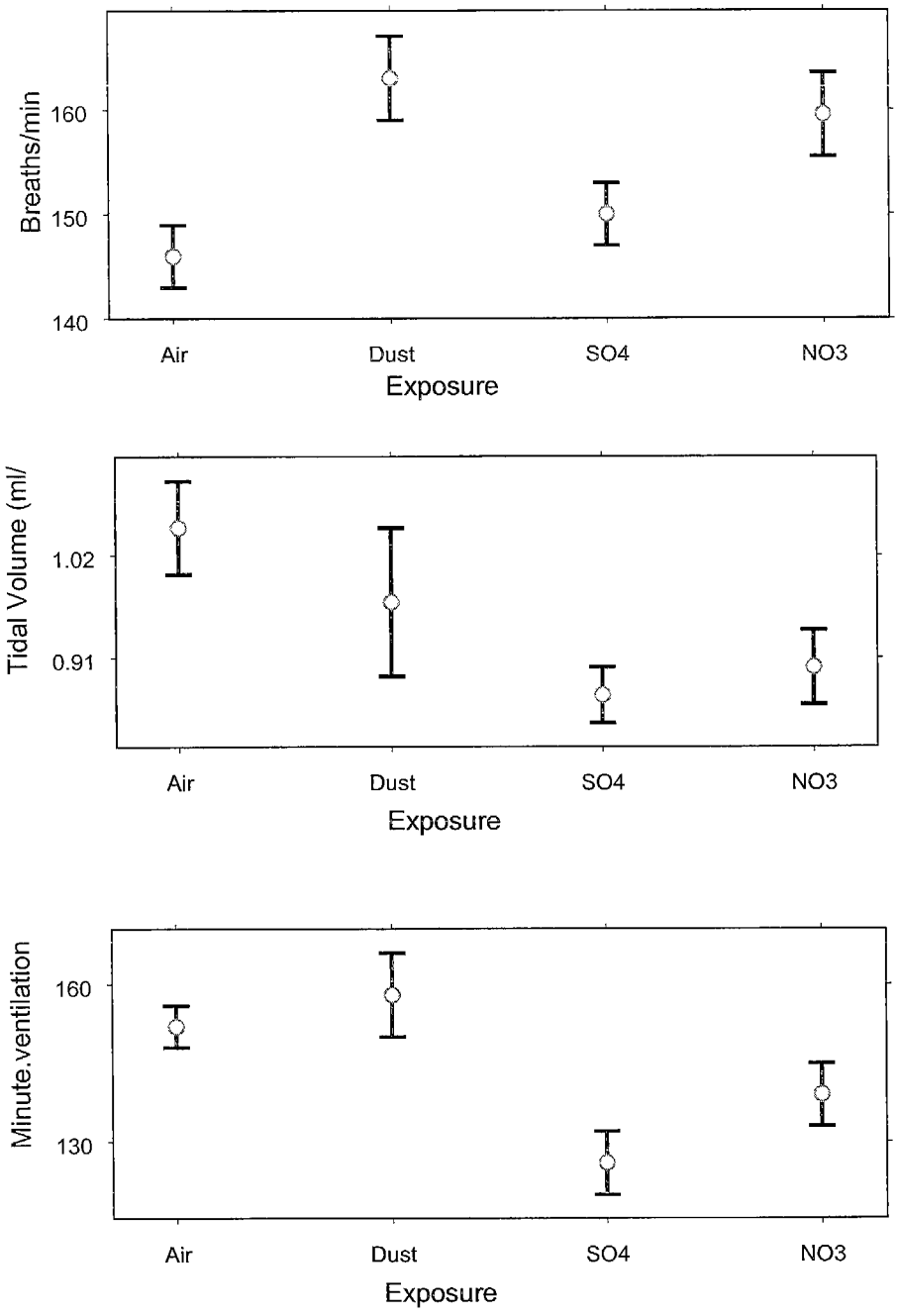
Figure 5A: Schedule of breathing pattern measurements during each 8 week experiment. Measurements were made on days marked X.



Breathing pattern (breath frequency and tidal volume) and minute ventilation data were examined for each of the four experiments. Because the rats were growing and changing body mass during the exposure period, it is possible that breathing pattern changed over the course of an experiment, independent of any exposure effects. Therefore, significant effects of the exposure could include either a main effect of exposure atmosphere on breathing pattern or an interaction between exposure atmosphere and week during the experiment. The main effect of exposure atmosphere was tested by averaging breathing pattern and minute ventilation data for each rat by hour, day and week of an experiment. A one way analysis of variance was performed, and Tukey multiple comparison testing was used to determine which pollutant atmosphere(s) produced significantly different effects.

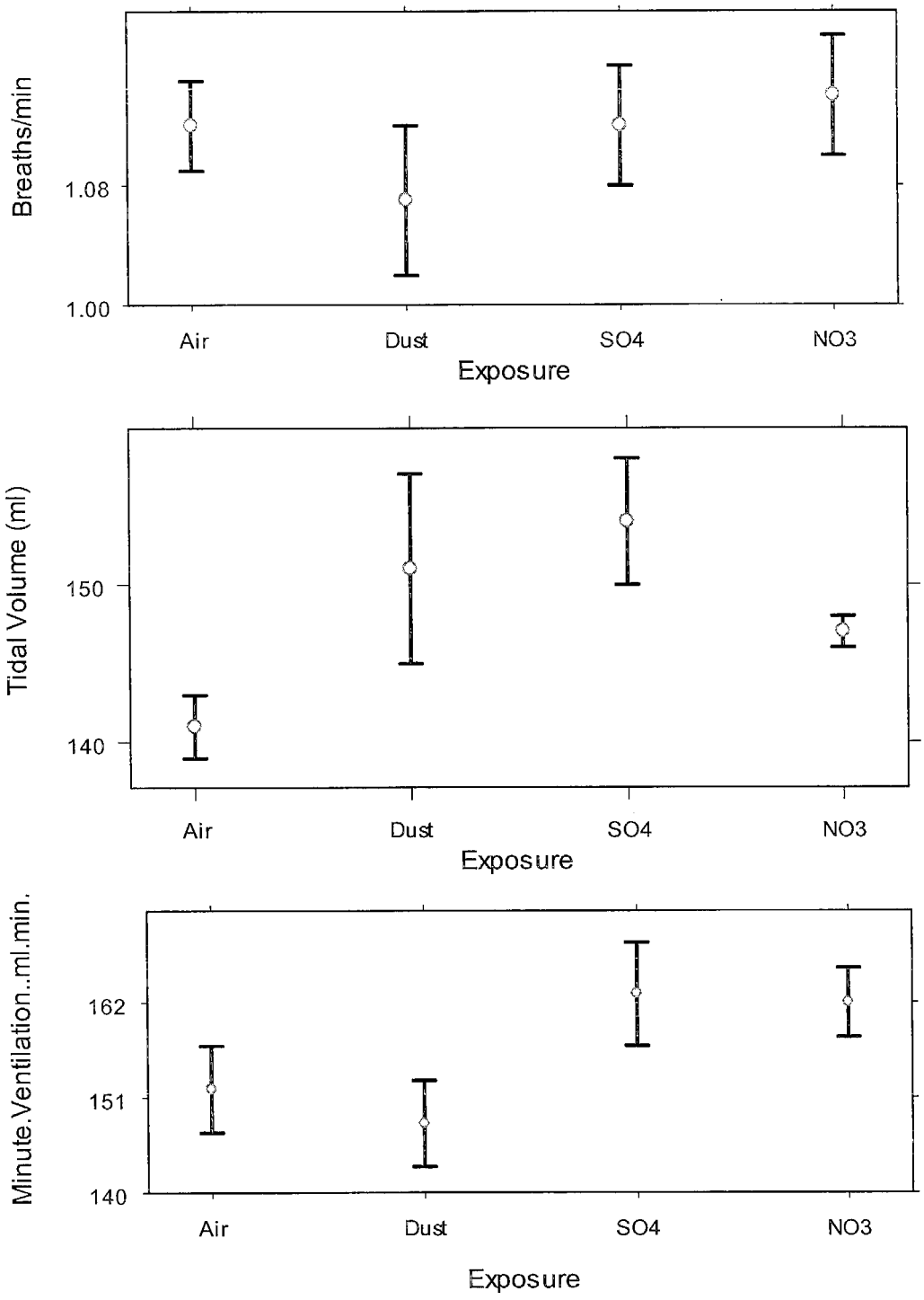
In the exposure to high concentrations of PM10 components (Figure 5B) breathing frequency and tidal volume were significantly ($P<0.05$) affected on the first day of each week, but not on the fourth day of each week. Figure 5B shows breathing pattern and minute ventilation of rats averaged over successive first days of each week. Rats exposed to the three PM10 components breathed more rapidly and shallowly (Frequency $F = 5.74$, $p<0.003$; Tidal Volume $F = 3.5$, $p<0.03$). Breathing frequency was significantly elevated over the control exposure to purified air for road dust ($p<0.01$) and nitrate ($p<0.05$), while tidal volume was significantly reduced compared to purified air exposure with exposure to sulfate ($p<0.05$). Minute ventilation was also reduced on the first day of each week ($F = 4.8$, $p<0.008$), while rats exposed to sulfate had significantly lower V_E compared to purified air control ($p<0.01$). There were no significant ($P >0.05$) effects on the fourth day of each week.

Figure 5B: Breathing pattern and minute ventilation of rats exposed to high concentrations of PM10 components: Road dust, 900 mg/m3; 70 mg/m3 (NH4)2SO4; NH4NO3, 350 mg/m3. Data are averages for hour 4 on exposure days 1, 9, 17, 25 and 29.



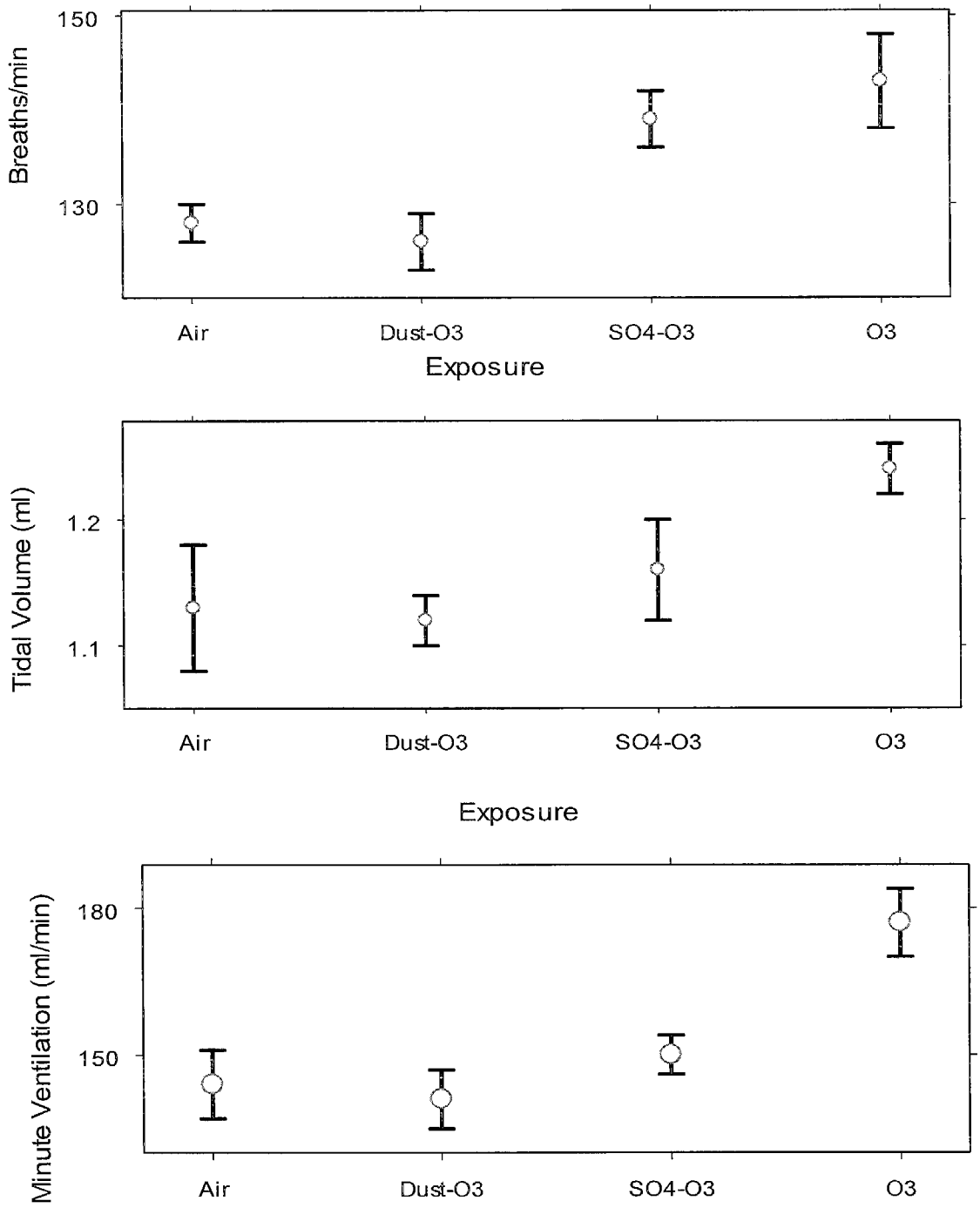
Exposure to lower concentrations of the same PM10 components (Figure 5C) showed no significant ($P<0.05$) effects for either first or fourth exposure days. There was, however, a non-significant trend for breathing frequency to be elevated (Figure 5C) in the exposure groups on the fourth day of each week ($F= 2.8$, $p<0.06$). The range of concentrations for the PM10 components studied in these 2 experiments span the threshold for detecting breathing pattern and ventilation effects in these exposures, and suggests the presence of mild pulmonary irritation.

Figure 5C: Breathing pattern and minute ventilation of rats exposed to PM10 components at lower concentrations: Road dust, 300 μg/m3; $(NH_4)_2SO_4$, 20 μg/m3; NH_4NO_3, 90 μg/m3. Data are averages for hour 4 on days 4, 12, 20, 28 and 32.



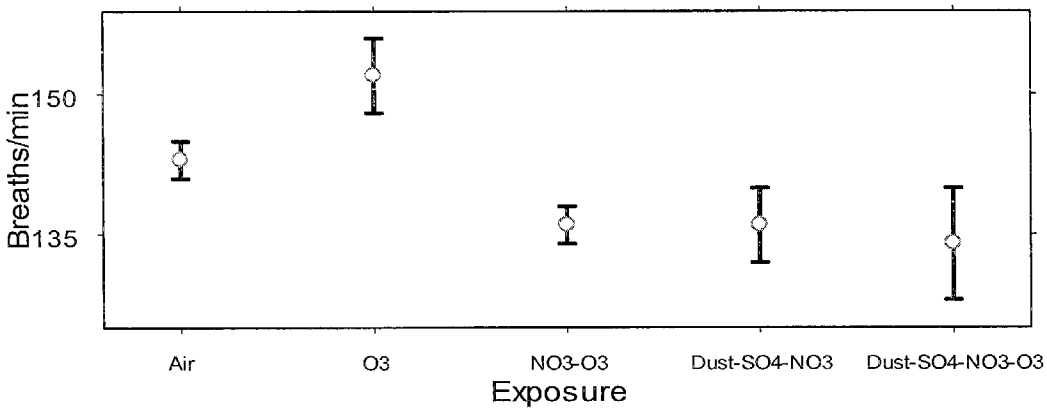
In the third experiment, 0.2 ppm O_3 was combined with the sulfate, nitrate and dust components of PM10 (Figure 5D). On the first exposure day of each week there were no significant ($P>0.05$) main effects of exposure atmosphere. There was, however, a significant interactive effect ($P<0.05$) between exposure atmosphere and first day of the exposure week on tidal volume in the first days of episode. The effect appears to result from the 0.2 ppm O_3 group. Tidal volume was initially low with exposure to 0.2 ppm O_3, but then increased in successive first days of exposure weeks. On the fourth day of each week (Figure 5D) there was a main effect of exposure frequency on frequency ($F = 6.0$, $p<0.003$) and on minute ventilation ($F = 8.1$, $p<0.0005$). Frequency was elevated in 0.2 ppm O_3 ($p<0.05$) compared to the purified air control and the combination of dust + O_3. Tidal volume was not significantly ($P>0.05$) affected by the exposures, thus minute ventilation showed the same pattern of elevation in O_3, as in purified air ($p<0.01$), and in O_3 as in dust + O_3. Breathing pattern effects, though present, were small and consistent with an effect of the O_3 component with little contribution of PM10 components.

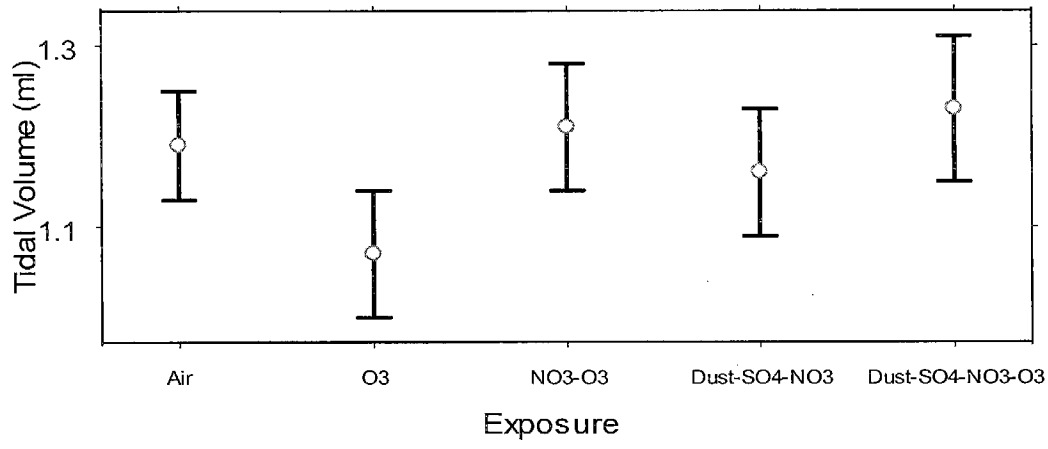
Figure 5D: Breathing pattern and minute ventilation in rats exposed to 0.2 ppm O3 alone and combined with: Road dust, 300 μg/m3 or (NH4)2SO4, 70 μg/m3. Data are averages for hour 4 on days 4, 12, 20, 28 and 32.

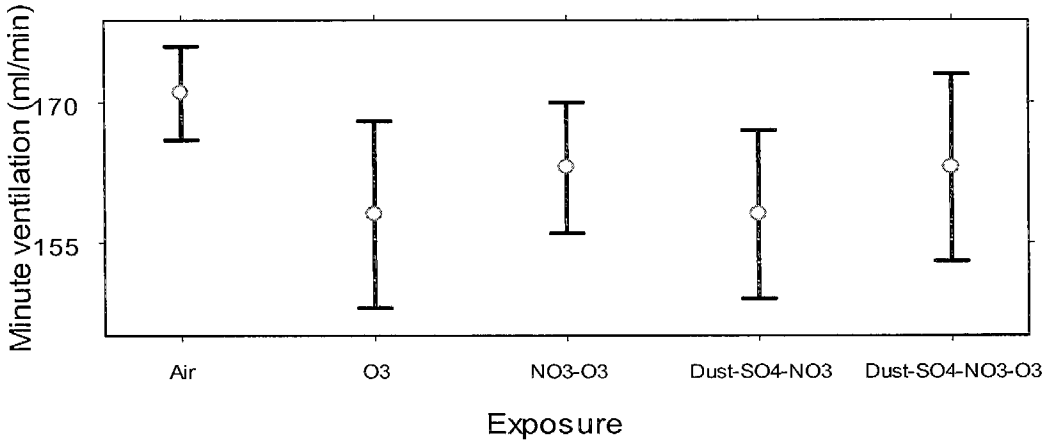


Results of the fourth experiment, which involved exposure to more complex mixtures of PM10 components and O_3 are shown in Figure 5E. On the first exposure day of weekly episodes there was a significant main effect of exposure on breath frequency ($F = 5.7$; $p<0.001$). Breath frequency was elevated in O_3 and reduced in mixtures of PM10 components both with and without O_3 ($P<0.05$; Figure 5E). While multiple comparison tests did not show significant ($P>0.05$) differences between the pollutant-containing atmospheres and the purified air control, the O_3 group was significantly elevated compared to all the combinations of PM10 constituents, and PM10 constituents + O_3 groups ($p<0.01$). There were no significant ($P>0.05$) main effects on tidal volume and minute ventilation, but there were significant ($P<0.05$) interactive effects between exposure atmosphere and successive weekly episodes. Tidal volume was reduced in O_3 at the beginning of the exposure, but recovered with successive episodes. The mixture of all PM10 components and O_3 induced tidal volume depression through most of the successive episodes, and then rose at the last episode. Minute ventilation was depressed with exposure to all mixtures of PM10 components and O_3, then rose at the last exposure of the week. Similar patterns of effects were present on the fourth days of successive episodes. Frequency was elevated in O_3 and depressed in PM10 mixtures; this pattern was weak and did not produce an exposure main effect ($P>0.05$) but there was a significant ($P<0.05$) interaction between exposure atmosphere and successive episode. Tidal volume and breath frequency were reduced in the early episodes of the exposure to O_3, but they recovered to the levels of purified air controls in the latter episodes. These results indicate that at the higher total concentrations of PM10 in these multi-component mixtures, the mild pulmonary irritant response to O_3 is modified. While the actions of these components may interact at times to produce antagonistic effects on breathing pattern, the depression of minute ventilation suggests that respiratory tract irritation is occurring from both O_3 and PM10 components with exposure to these pollutant mixtures.

Figure 5E: Breathing pattern and minute ventilation of rats exposed to 0.2 pm O3, alone, and in mixtures with: Road dust, 300 μg/m3; (NH4)2SO4, 70 μg/m3; NH4NO3, 350μg/m3. Data are the average for hour 4 of exposure days 1, 9, 17, 25 and 29.







Discussion

Summary

Exposure to some of the pollutants in this study significantly impaired the lung's normal barrier function. Disruption of this epithelial barrier could allow antigens present in the environment to penetrate to sub-epithelial regions, leading to the release of histamine and induction of bronchoconstriction. Measurements of breathing patterns and ventilatory parameters in this study demonstrated that these pollutants had an irritant effect on the lung. Evaluation of macrophage functions in PM10-exposed rats showed that critical defenses against respiratory infections could be impaired. The significant effects observed in this study are summarized in the table below. The symbol "+" indicates a significant increase, while a "-" indicates a significant decrease; responses that were not statistically significant are unmarked.

Table 7. Changes in Respiratory Parameters in Response to Exposure to PM10 Components, Alone, in Mixtures, and Combined with Ozone

	PM10 COMPONENTS +O3					COMB. WITH O_3			
	SO_4^{-2}	NO_3^{-1}	Dust	Mix	O_3	SO_4^{-2}	NO_3^{-1}	Dust	Mix
Permeability - High	+	+							
- Low	+								
Goblet Cell - High									
- Low									
FcR Binding - High		-				-			
- Low	-	-			-				
Resp. Burst - High	-	-				+	+		
- Low									
Inflammatory Mediators									
Day 1 Tidal Vol. - High	-	-	-	-					
- Low					-	-*	-*	-*	-*
Day 1 Breath. Freq.- High	+	+	+	+					
- Low					+*	+*	+*	+*	+*

*Note: These changes appear to be attributable to the effects of O_3 alone.

Study Design and Atmosphere Characteristics

This study incorporated several novel features. It is the first inhalation toxicology study designed to specifically test for human disease-related effects of PM10 in a systematic manner. PM10 is a complicated mixture of particles from numerous sources, hence the chemical and physical characteristics will very from location to location and from sampling period to sampling period. The exact composition will depend upon the mix of sources and the activities of these sources relative to the location of the sampler, the time of sampling and the meteorological conditions at the time. Given this extraordinary variability, the high degree of convergence in findings of adverse health effects associated with PM10 is surprising. In general, these reports, from a wide range of localities, indicate a relatively stable relationship between PM0 exposure and human mortality or morbidity. This convergence suggests a possible commonality among PM10 from various localities that is accountable for the observed effects on health.

Recent intensive studies have provided excellent data on the chemical and physical characteristics of PM10 in Southern California. With respect to size, particles tend to cluster among three modes: a "condensation" mode (~0.2 μm); a "droplet" mode (0.7 μm); and a coarse mode (4.4 μm) (John, 1993). The relative contributions of particles in these modes to the mass of PM10 are 10%, 50% and 40% for the condensation, droplet and coarse modes, respectively. These reported values agree quite well with those used in the design of our study (see Table 3). Sulfate and elemental carbon are major contributors to the condensation mode, and nitrates and sulfates are major contributors to the droplet mode. Nitrates and sulfates were included in our study; sulfate as a representative of the condensation mode, and nitrate as a representative of the droplet mode. The coarse mode of PM10 is generally that associated with mechanical processes (grinding, abrasion, spallation). Chemically, the coarse fraction is often similar in composition to that of the earth's crust. As stated earlier, resuspension of dust from paved and unpaved roads as a major source of the material for the coarse fraction of PM10. Since our study was to test for the potential health effects of urban PM10, a road dust component derived from a paved freeway was included as representative of the PM10 c0arse fraction. To our knowledge, this is the first time that dust from paved roads was used in toxicological study.

The road dust was obtained from a relatively new, but well-traveled freeway. Care was taken to sample from a region that had had no reported spills of toxic materials. A large enough sample was taken (about 50 lbs. Over the course of a one month period) so that sufficient material could be preserved for future studies and to provide material for interested colleagues.

Permeability

Levels of total protein and serum albumin recovered in the lavage fluid were measured as indicators of permeability of the lung epithelium. The lung's epithelial cells provide a protective barrier by means of tight junctions between the cells. A disrupted barrier would allow the allergens present in the environment to penetrate between the cells and be deposited in the sub-epithelial regions. Hence, the allergens would have access to nerve endings and other cells of the lymphoid system, and toxic compounds or carcinogens could be sequested in regions with only limited ability to remove them by normal clearance mechanisms. The release of IgE and its binding to mast cells subsequent to an allergen penetrating a disrupted barrier could result in the release of histamine and other cellular mediators capable of inducing bronchoconstriction in an asthmatic response. In this study, we found altered protein concentrations ($p<0.05$) in rats exposed to high concentrations of the two fine particle fraction components (NO_3^{-1} and SO_4^{-2}) without O_3, but significant changes were not seen following exposure to these compounds, at the same concentrations in the presence of O_3. As will be discussed below, the atmospheres containing O_3 provoked changes in breathing patterns. These breathing pattern changes could have altered the dose distribution pattern and thus could have moderated the permeability effect.

Goblet Cell Density and Mucus Hypersecretion

Increased numbers of goblet cells and submucosal gland hypertrophy are well-established and moderately sensitive indices of increased mucus production (Reid, 1970). Two methods were used to detect changes in mucus secretion in this study: 1) morphometric determination of goblet cell number, and 2) measurement of mucous glycoprotein in the bronchoalveolar lavage. The data from the second method have not yet been analyzed. Other investigators have demonstrated a significant increase in submucosal gland size after three weeks of exposure to sulfur dioxide (Lamb and Reid, 1968). Thus, it was assumed that significant changes in goblet cell density would be seen with some of the exposure conditions after 30 days of exposure to PM19 components. None of the exposed groups demonstrated significant increases in goblet cell density. There was a small decrease ($p<0.05$) in bronchiolar goblet cell density in rats exposed to O_3 alone. The health significance of this finding is not clear. The measure of goblet cell density may not be an accurate indication of mucus production; these data will be put into better context when the mucin glycoprotein data are analyzed.

Macrophage Responses

Lung defenses against infection are, in major part, dependent on alveolar macrophages, although other resources are also present (Gardner, 19185). Respiratory tract infection encompasses upper respiratory tract infections (colds), acute and chronic bronchitis, and pneumonia. Respiratory tract infections are associated with increased mortality in the elderly population and absenteeism in younger populations, exacerbation of asthma and

incidence of bronchitis in the general population. A number of ep8idemiological studies have found a correlation between particulate pollution levels and incidence of respiratory tract infection in adults and children (Pope, 1989; Dockery et al., 1989). In this study the effects of PM10 components on three measures of the ability of the macrophage to function in a defensive manner were quantified. The ability of attack and ingest, or phagocytize, pathogens depends on proper function of Fc receptors on the macrophage's membrane. Another, somewhat related, parameter of respiratory defense is the macrophage's ability to produce reactive oxygen species and other biocidal compounds. This usually occurs in conjunction with the phagocytosing of pathogenic or antigenic material. In this study, reductions ($p<0.05$) in Fc receptor mediated phagocytosis were seen in rats exposed to atmospheres containing O_3 + SO_4^{-2} (see Appendix – Table 3). Rats exposed to SO_4^{-2} alone (70 $\mu g/m^3$) had depressed ($p<0.05$) ability to mount a respiratory burst, but rats exposed to SO_4^{-2} at the same concentration in the presence of O_3 produced excess amounts of superoxide (see Appendix – Table 4). The respiratory burst can be an important parameter both in the context of lung defense, and in the context of lung injury. Depressed ability to mount a respiratory burst could reflect a depressed ability to kill phagocytosed pathogens. On the other hand, an increased respiratory burst could flood the lung with toxic chemicals including reactive oxygen species and protein-destroying enzymes. An increase in respiratory burst activity in concert with lung exposure to O_3, a compound that promotes oxidative stress, could be considered an adverse response. In context with other changes in lung defense in this study, the evaluation of macrophage functions in PM10-exposed rats show that critical defenses against infections could be impaired.

Breathing Pattern and Minute Ventilation

Exposure to high concentrations of the individual PM10 components produced significant breathing pattern and minute ventilation effects consistent with mild pulmonary irritation from all of the components: road dust, NO_3^{-1} and SO_4^{-2}. This pattern was present only on the first day of each weekly episode of 4 days of exposure. There appeared to be an adaptive response occurring between the first and fourth exposure day, however this response did not persist to the beginning of the next episode. In the second exposure experiment, a lower set of concentrations of the same PM10 components was tested, and there were no significant effects on breathing pattern and minute ventilation. The range of PM10 component concentrations tested in these two experiments thus spans the threshold for detecting breathing pattern and ventilation effects in these exposures, and the mild pulmonary irritation induced at the higher concentrations was not present at lower concentrations.

In the exposures to mixed PM10 components and O_3, breathing pattern effects, though present, were small and consistent with an effect of the O_3 component and little contribution of PM10 components. None of the PM10 components tested showed particular strength in inducing irritant breathing pattern responses, compared to the other compounds. In the final exposure experiment in which all PM10 components combined

were tested in combination with O_3, there was a modification of the response to O_3 observed as significant interactions between the effects of exposure atmosphere and sequential episodic exposure. This mixed PM10 + O_3 group was exposed to the highest combined concentration of PM10 in combination with O_3, and it is likely that the total PM10 concentration was the important factor in modifying the O_3 effect.

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APPENDIX: Tables 1-17

Table 1. Effects of PM10 Exposure on Permeability

	Atmosphere	Protein in BAL/ mg/ml ± SD	Anova P	Albumin in BAL/ mg/ml ± SD	Anova P
Experiment 1	CA	0.09 ± 0.03	0.02	0.03 ± 0.02	NS
	DUST	0.11 ± 0.02		0.06 ± 0.03	
	SO_4	0.13 ± 0.02*		0.05 ± 0.02	
	NO_3	0.13 ± 0.03*		0.07 ± 0.04	
Experiment 2	CA	0.15 ± 0.01	0.001	0.06 ± 0.03	NS
	DUST	0.16 ± 0.01		0.08 ± 0.06	
	SO_4	0.17 ± 0.01*		0.07 ± 0.02	
	NO_3	0.15 ± 0.02		0.06 ± 0.02	
Experiment 3	CA	0.17 ± 0.01	0.02	0.14 ± 0.05	0.03
	O_3	0.17 ± 0.01		0.17 ± 0.08	
	$SO_4 + O_3$	0.17 ± 0.01		0.15 ± 0.04	
	DUST + O_3	0.18 ± 0.02		0.12 ± 0.03	
Experiment 4	CA	0.18 ± 0.02	NS	0.04 ± 0.01	NS
	O_3	0.19 ± 0.02		0.04 ± 0.01	
	$NO_3 + O_3$	0.18 ± 0.02		0.04 ± 0.01	
	DUST + $NO_3 + SO_4$	0.18 ± 0.03		0.04 ± 0.01	
	DUST + NO_3 + SO_4 + O_3	0.19 ± 0.03		0.04 ± 0.01	

* $P < 0.05$ vs. control (Tukey Multiple Comparison Test)

Table 2. Effects of PM10 Exposures on Goblet Cell Numbers (Fraction of Epithelial Cells), Mean ± S.D.

Atmosphere	Trachea/Anova	Lobar Bronchia/Anova	Bronchiole/Anova
Experiment 1[+]			
AIR	1.66 ± 0.60/NS	No Data	No Data
DUST	1.77 ± 0.66		
SO_4	2.35 ± 0.86		
NO_3	2.49 ± 1.18		
Experiment 2			
AIR	0.71 ± 0.04/NS	0.50 ± 0.08/NS	0.37 ± 0.06/NS
DUST	0.74 ± 0.05	0.48 ± 0.11	0.35 ± 0.08
SO_4	0.75 ± 0.05	0.52 ± 0.07	0.39 ± 0.05
NO_3	0.70 ± 0.06	0.51 ± 0.10	0.37 ± 0.08
Experiment 3			
AIR	0.76 ± 0.04/NS	0.53 ± 0.07/NS	0.48 ± 0.05/NS
O_3	0.79 ± 0.07	0.61 ± 0.09	0.40 ± 0.08
DUST + O_3	0.78 ± 0.04	0.52 ± 0.11	0.41 ± 0.08
SO_4 + O_3	0.76 ± 0.05	0.54 ± 0.09	0.42 ± 0.09
Experiment 4			
AIR	0.81 ± 0.06/NS	0.66 ± 0.03/0.01	0.46 ± 0.05/0.003
O_3	0.76 ± 0.06	0.57 ± 0.06	0.37 ± 0.05*
NO_3 + O_3	0.81 ± 0.10	0.70 ± 0.19	0.38 ± 0.09
DUST + NO_3 + SO_4	0.77 ± 0.10	0.56 ± 0.06	0.42 ± 0.07
DUST + O_3 + NO_3 + SO_4	0.78 ± 0.06	0.62 ± 0.06	0.53 ± 0.08

[+] Experiment 1 Goblet cell data as number per field at 600 X
* = $P<0.05$ vs. control (Tukey Multiple Comparison Test)

Table 3. Effects of PM10 Exposure on Macrophage Fc-Mediated Phagocytosis

	Atmosphere	% Cells Forming Rosettes	Anova (P)
Experiment 1	CA	55.90 ± 10.77	NS
	DUST	52.54 ± 6.58	
	SO_4	51.95 ± 12.43	
	NO_3	50.47 ± 8.38*	
Experiment 2	CA	49.07 ± 6.27	NS
	DUST	46.56 ± 8.51	
	SO_4	43.79 ± 7.33	
	NO_3	49.45 ± 6.52	
Experiment 3	CA	61.99 ± 5.55	0.01
	O_3	53.38 ± 8.62	
	$SO_4 + O_3$	51.97 ± 6.32*	
	DUST + O_3	53.14 ± 8.96*	
Experiment 4	CA	50.74 ± 8.33	0.009
	O_3	44.51 ± 7.02	
	$NO_3 + O_3$	50.29 ± 7.48	
	DUST + NO_3 + SO_4	46.33 ± 7.15	
	DUST + NO_3 + SO_4 + O_3	40.39 ± 4.63*	

* $P<0.05$ vs. Control (Tukey Multiple Comparison Test)

Table 4. Effects of PM10 Exposure on Macrophage Respiratory Burst Activity by Cytochrome C. Reduction. (Mean ± S.D.)

Atmosphere	ng/mg Prot./45 min	Anova (P)
Experiment 1		
AIR	32 ± 4	0.027
DUST	22 ± 2*	
SO_4	18 ± 4*	
NO_3	27 ± 6	
Experiment 2		
AIR	30 ± 9	NS
DUST	43 ± 20	
SO_4	33 ± 8	
NO_3	42 ± 11	
Experiment 3		
AIR	36 ± 2.6	0.003
O_3	34 ± 3.2	
DUST + O_3	32 ± 2.9	
SO_4 + O_3	48 ± 2.6*	
Experiment 4		
AIR	24 ± 2.2	0.015
O_3	30 ± 2.4	
NO_3O_3	30 ± 2.9	
DUST + NO_3 + SO_4	28 ± 2.9	
DUST + O_3 + NO_3 + SO_4	27 ± 2.3	

Table 5. Effects of PM10 Exposures on Inflammatory Mediators (Mean ± S.D.)

	Atmosphere	LTB_4 nMol/mg Protein		PGE_2 nMol/mg Protein	
Experiment 1	CA[1]	400 ± 188	NS	8.6 ± 1.6	NS
	DUST	620 ± 238		9.3 ± 1.6	
	NO_3	460 ± 180		13.9 ± 3.3	
	SO_4	420 ± 239		13.0 ± 3.1	
Experiment 2	CA	400 ± 90	NS	8.6 ± 0.6	NS
	DUST	370 ± 60		9.1 ± 0.7	
	NO_3	420 ± 71		9.7 ± 0.4	
	SO_4	400 ± 67		10.6 ± 0.9	
Experiment 3	CA	400 ± 54	NS	8.6 ± 0.5	NS
	O_3	430 ± 55		9.8 ± 1.1	
	DUST + O_3	390 ± 42		9.3 ± 0.8	
	SO_4 + O_3	410 ± 55		10.1 ± 1.2	
Experiment 4	CA	400 ± 22	NS	8.6 ± 0.6	NS
	O_3	420 ± 12		10.5 ± 1.0	
	NO_3 + O_3	422 ± 12		9.5 ± 0.8	
	MIX[2]	430 ± 10		8.9 ± 0.9	
	MIX + O_3	434 ± 10		8.9 ± 0.8	

[1] LTB_4 data normalized to average control value of 400 nMol/mg Protein/45 min and PGE_2 data normalized to average control value of 8.6 nMol/mg Protein/45 min.

[2] MIX = Road Dust + NO_3 + SO_4

Table 6. Breath frequency (min^{-1}) of rats exposed to high concentrations of PM10 components during hour 4 of the first and fourth days of successive alternate weeks of exposure. N = 8 animals per group.

Exposure Day and Hour	CLEAN AIR	DUST	SO4	NO3
	Group Mean Values			
D1H4	140	156	150	154
D9H4	138	164	146	148
D17H4	156	153	139	170
D25H4	159	174	153	162
D29H4	138	170	162	165
Grand Mean	147	164	150	160
	Group Standard Deviation Values			
D1H4	12	17	13	18
D9H4	8	23	15	17
D17H4	22	11	22	37
D25H4	15	21	14	17
D29H4	16	20	15	14

Main Effect $F = 5.74$, $p < 0.003$

Exposure Day and Hour				
	Group Mean Values			
D4H4	145	159	148	155
D12H4	144	157	151	154
D20H4	156	163	157	165
D28H4	166	169	160	166
D32H4	158	160	154	149
Grand Mean	154	162	155	158
	Group Standard Deviation Values			
D4H4	16	23	17	23
D12H4	25	25	22	27
D20H4	26	24	31	35
D28H4	44	16	17	22
D32H4	30	19	16	24

Not Significant

Table 7. Tidal volume (ml) of rats exposed to high concentrations of PM10 components during hour 4 of the first and fourth days of successive alternate weeks of exposure. N = 8 animals per group.

Exposure Day and Hour	CLEAN AIR	DUST	SO4	NO3
	Group Mean Values			
D1H4	1.00	0.91	0.86	0.83
D9H4	1.09	0.94	0.92	1.00
D17H4	1.02	1.11	0.89	0.76
D25H4	0.98	1.00	0.88	0.93
D29H4	1.09	0.90	0.72	0.92
Grand Mean	1.04	0.98	0.86	0.89
	Group Standard Deviation Values			
D1H4	0.18	0.29	0.17	0.15
D9H4	0.12	0.21	0.19	0.14
D17H4	0.16	0.12	0.22	0.22
D25H4	0.27	0.17	0.20	0.25
D29H4	0.22	0.20	0.26	0.25

Main Effect $F = 3.52$, $p < 0.03$

Exposure Day and Hour				
	Group Mean Values			
D4H4	0.80	0.85	0.92	0.91
D12H4	0.98	1.05	0.97	0.97
D20H4	0.97	1.10	0.86	0.80
D28H4	0.90	0.86	0.82	0.94
D32H4	0.95	0.99	0.91	0.98
Grand Mean	0.93	0.97	0.90	0.93
	Group Standard Deviation Values			
D4H4	0.16	0.20	0.19	0.29
D12H4	0.21	0.15	0.24	0.17
D20H4	0.18	0.22	0.15	0.17
D28H4	0.16	0.21	0.28	0.32
D32H4	0.35	0.25	0.12	0.21

Not Significant

Table 8. Minute ventilation (ml/min) of rats exposed to high concentrations of PM10 components during hour 4 of the first and fourth days of successive alternate weeks of exposure. N = 8 animals per group.

Exposure Day and Hour	CLEAN AIR	DUST	SO4	NO3
	Group Mean Values			
D1H4	140	143	128	127
D9H4	151	152	134	148
D17H4	158	169	122	124
D25H4	155	173	136	151
D29H4	152	155	114	150
Grand Mean	152	159	127	140
	Group Standard Deviation Values			
D1H4	23	44	26	18
D9H4	12	29	29	24
D17H4	27	20	29	21
D25H4	34	24	33	41
D29H4	34	45	37	33

Main Effect F = 4.82, p < 0.008

Exposure Day and Hour				
	Group Mean Values			
D4H4	117	132	136	140
D12H4	140	173	146	149
D20H4	150	178	136	128
D28H4	148	146	131	155
D32H4	142	156	141	146
Grand Mean	140	157	139	144
	Group Standard Deviation Values			
D4H4	25	20	27	44
D12H4	32	40	31	31
D20H4	20	32	36	11
D28H4	32	38	50	48
D32H4	42	34	16	31

Not Significant

Table 9. Breath frequency (min^{-1}) of rats exposed to low concentrations of PM10 components during hour 4 of the first and fourth days of successive alternate weeks of exposure. N = 8 animals per group.

Exposure Day and Hour	CLEANAIR	DUST	SO4	NO3
	Group Mean Values			
D1H4	125	138	146	143
D9H4	136	139	143	133
D17H4	132	142	152	144
D25H4	133	131	137	143
D29H4	145	146	156	147
Grand Mean	134	140	147	143
	Group Standard Deviation Values			
D1H4	10	23	25	22
D9H4	13	12	19	17
D17H4	14	11	17	11
D25H4	14	14	15	13
D29H4	22	12	13	19

Not Significant

Exposure Day and Hour				
	Group Mean Values			
D4H4	139	160	168	148
D12H4	124	142	146	148
D20H4	137	163	155	147
D28H4	149	131	154	144
D32H4	152	161	148	155
Grand Mean	141	152	155	149
	Group Standard Deviation Values			
D4H4	15	20	27	18
D12H4	17	20	21	17
D20H4	12	22	11	20
D28H4	13	20	21	16
D32H4	9	14	22	13

Not Significant

Table 10. Tidal volume (ml) of rats exposed to low concentrations of PM10 components during hour 4 of the first and fourth days of successive alternate weeks of exposure. N = 8 animals per group.

Exposure Day and Hour	CLEANAIR	DUST	SO4	NO3
	Group Mean Values			
D1H4	1.15	0.90	1.07	1.02
D9H4	0.99	0.93	1.02	1.10
D17H4	1.01	1.08	1.09	1.08
D25H4	1.23	1.25	1.27	1.24
D29H4	1.21	1.13	1.13	1.23
Grand Mean	1.12	1.06	1.12	1.14
	Group Standard Deviation Values			
D1H4	0.10	0.26	0.19	0.19
D9H4	0.15	0.14	0.20	0.13
D17H4	0.11	0.16	0.22	0.22
D25H4	0.10	0.17	0.27	0.17
D29H4	0.23	0.13	0.11	0.09

Not Significant

Exposure Day and Hour				
	Group Mean Values			
D4H4	0.91	0.83	0.87	0.94
D12H4	0.99	1.07	1.08	1.09
D20H4	1.13	1.11	1.06	1.04
D28H4	1.21	1.24	1.23	1.19
D32H4	1.18	1.10	1.14	1.19
Grand Mean	1.09	1.07	1.08	1.10
	Group Standard Deviation Values			
D4H4	0.09	0.16	0.12	0.16
D12H4	0.12	0.12	0.14	0.15
D20H4	0.11	0.18	0.13	0.18
D28H4	0.21	0.22	0.21	0.30
D32H4	0.14	0.23	0.17	0.15

Not Significant

Table 11. Minute ventilation (ml/min) of rats exposed to low concentrations of PM10 components during hour 4 of the first and fourth days of successive alternate weeks of exposure. N = 8 animals per group.

Exposure Day and Hour	CLEANAIR	DUST	SO4	NO3
	Group Mean Values			
D1H4	144	125	153	146
D9H4	134	129	144	147
D7H4	134	154	164	155
D25H4	164	162	176	178
D29H4	178	165	176	181
Grand Mean	151	147	163	162
	Group Standard Deviation Values			
D1H4	18	43	15	29
D9H4	19	16	21	27
D17H4	13	21	24	28
D25H4	17	17	43	25
D29H4	62	20	23	27

Not Significant

Exposure Day and Hour				
	Group Mean Values			
D4H4	127	132	146	138
D12H4	124	152	158	161
D20H4	154	178	165	153
D28H4	181	161	191	169
D32H4	179	175	171	185
Grand Mean	153	160	167	162
	Group Standard Deviation Values			
D4H4	15	26	24	12
D12H4	25	23	25	11
D20H4	12	15	24	31
D28H4	36	31	50	34
D32H4	13	29	37	27

Not Significant

Table 12. Breath frequency (min^{-1}) of rats exposed to PM10 components in combination with 0.2 ppm O_3 during hour 4 of the first and fourth days of successive alternate weeks of exposure. N = 8 animals per group.

Exposure Day and Hour	CLEAN AIR	DUST+O_3	SO_4+O_3	O_3
	Group Mean Values			
D1H4	123	137	132	119
D9H4	140	136	124	122
D17H4	133	146	137	134
D25H4	134	127	129	121
D29H4	137	131	142	136
Grand Mean	134	136	133	127
	Group Standard Deviation Values			
D1H4	19	13	7	14
D9H4	6	10	8	18
D17H4	9	24	21	16
D25H4	14	7	11	10
D29H4	14	21	14	19

Not Significant

Exposure Day and Hour				
	Group Mean Values			
D4H4	132	135	149	148
D12H4	126	122	132	136
D20H4	128	123	142	144
D28H4	124	123	138	147
D32H4	126	126	135	138
Grand Mean	128	126	140	143
	Group Standard Deviation Values			
D4H4	9	20	15	25
D12H4	10	17	10	17
D20H4	15	13	15	20
D28H4	13	14	12	16
D32H4	14	18	17	22

Main Effect $F = 6.02$, $p < 0.003$

Table 13. Tidal volume (ml) of rats exposed to PM10 components in combination with 0.2 ppm O_3 during hour 4 of the first and fourth days of successive alternate weeks of exposure. N = 8 animals per group.

Exposure Day and Hour	CLEAN AIR	DUST+O_3	SO_4+O_3	O_3
	Group Mean Values			
D1H4	1.12	1.11	1.20	1.07
D9H4	1.30	1.18	1.19	1.15
D17H4	1.20	1.23	1.14	1.18
D25H4	0.93	1.29	1.07	1.06
D29H4	1.18	0.96	1.38	1.26
Grand Mean	1.25	1.16	1.20	1.15
	Group Standard Deviation Values			
D1H4	0.16	0.23	0.19	0.23
D9H4	0.16	0.12	0.10	0.15
D17H4	0.18	0.21	0.15	0.17
D25H4	0.32	0.09	0.24	0.23
D29H4	0.14	0.32	0.08	0.12

Exposure X Day Interaction $F = 3.61$, $p < 0.0001$

Exposure Day and Hour				
	Group Mean Values			
D4H4	1.06	1.09	1.08	1.26
D12H4	1.22	1.17	1.13	1.17
D20H4	1.09	1.10	1.06	1.15
D28H4	1.10	1.04	1.19	1.36
D32H4	1.16	1.19	1.31	1.21
Grand Mean	1.13	1.12	1.16	1.24
	Group Standard Deviation Values			
D4H4	0.27	0.14	0.31	0.15
D12H4	0.26	0.13	0.09	0.20
D20H4	0.24	0.18	0.12	0.20
D28H4	0.17	0.33	0.14	0.24
D32H4	0.20	0.35	0.24	0.22

Not Significant

Table 14. Minute ventilation (ml/min) of rats exposed to PM10 components in combination with 0.2 ppm O_3 during hour 4 of the first and fourth days of successive alternate weeks of exposure. N = 8 animals per group.

Exposure Day and Hour	CLEAN AIR	DUST+O_3	SO_4+O_3	O_3
	Group Mean Values			
D1H4	139	152	159	128
D9H4	183	161	148	140
D17H4	159	180	157	159
D25H4	124	164	139	130
D29H4	162	126	197	170
Grand Mean	154	157	161	154
	Group Standard Deviation Values			
Y				
D1H4	26	32	27	28
D9H4	24	12	12	29
D17H4	21	46	31	26
D25H4	42	14	33	30
D29H4	26	44	26	14

Not Significant

Exposure Day and Hour				
	Group Mean Values			
D4H4	140	149	162	191
D12H4	156	143	149	157
D20H4	139	137	151	165
D28H4	137	127	164	202
D32H4	147	148	178	170
Grand Mean	144	141	161	177
	Group Standard Deviation Values			
D4H4	30	30	47	48
D12H4	38	29	13	27
D20H4	29	31	22	27
D28H4	23	41	21	44
D32H4	32	40	35	48

Main Effect $F = 8.07$, $p < 0.0005$

Table 15. Breath frequency (min^{-1}) of rats exposed to PM10 components with and without 0.2 ppm O_3 during hour 4 of the first and fourth days of successive alternate weeks of exposure. N = 8 animals per group.

Exposure Day and Hour	CLEAN AIR	O_3	NO_3+O_3	DUST+SO_4 +NO_3	DUST+SO_4 +NO_3+O_3
	Group Mean Values				
D1H4	144	190	133	149	136
D9H4	147	132	131	134	133
D17H4	144	154	140	126	126
D25H4	140	132	135	128	130
D29H4	138	143	133	136	139
Grand Mean	143	151	135	135	134
	Group Standard Deviation Values				
D1H4	10	22	8	16	13
D9H4	7	12	10	19	17
D17H4	14	30	5	14	23
D25H4	16	15	18	10	14
D29H4	9	19	7	30	25

Main Effect F = 5.71, $p < 0.001$

Exposure Day and Hour					
	Group Mean Values				
D4H4	147	146	149	134	145
D12H4	142	133	128	139	131
D20H4	154	144	132	137	137
D28H4	142	163	137	140	134
D32H4	137	165	147	142	147
Grand Mean	145	151	139	139	139
	Group Standard Deviation Values				
D4H4	9	25	19	12	25
D12H4	15	12	12	19	18
D20H4	14	12	16	18	16
D28H4	11	29	11	29	24
D32H4	10	13	15	18	24

Exposure X Day Interaction F = 1.87, $p < 0.028$

Table 16. Tidal volume (ml) of rats exposed to PM10 components with and without 0.2 ppm O_3 during hour 4 of the first and fourth days of successive alternate weeks of exposure. N = 8 animals per group.

Exposure Day and Hour	CLEAN AIR	O_3	NO_3+O_3	DUST+SO_4 +NO_3	DUST+SO_4 +NO_3+O_3
	Group Mean Values				
D1H4	1.11	0.80	1.08	1.25	1.02
D9H4	1.15	1.10	1.23	1.18	1.23
D17H4	1.18	0.95	1.30	0.94	1.05
D25H4	1.31	1.13	1.13	1.15	1.04
D29H4	1.20	1.32	1.27	1.25	1.79
Grand Mean	1.19	1.07	1.21	1.16	1.23
	Group Standard Deviation Values				
D1H4	0.25	0.14	0.24	0.24	0.12
D9H4	0.13	0.19	0.17	0.21	0.16
D17H4	0.16	0.24	0.16	0.21	0.30
D25H4	0.18	0.29	0.32	0.20	0.38
D29H4	0.24	0.32	0.21	0.23	0.76

Exposure X Day Interaction $F = 3.64$, $p < 0.0001$

Exposure Day and Hour					
	Group Mean Values				
D4H4	1.12	1.11	1.14	1.20	1.10
D12H4	1.23	0.99	1.19	1.23	1.26
D20H4	1.25	0.96	1.19	1.07	1.32
D28H4	1.28	1.04	1.27	1.01	1.22
D32H4	1.03	1.10	1.12	1.25	1.10
Grand Mean	1.18	1.04	1.19	1.16	1.20
	Group Standard Deviation Values				
D4H4	0.25	0.22	0.24	0.19	0.26
D12H4	0.24	0.18	0.18	0.22	0.29
D20H4	0.14	0.25	0.12	0.24	0.24
D28H4	0.18	0.21	0.27	0.21	0.16
D32H4	0.21	0.24	0.22	0.12	0.23

Exposure X Day Interaction $F = 1.89$, $p < 0.026$

Table 17. Minute ventilation (ml/min) of rats exposed to PM10 components with and without 0.2 ppm O_3 during hour 4 of the first and fourth days of successive alternate weeks of exposure. N = 8 animals per group.

Exposure Day and Hour	CLEAN AIR	O_3	NO_3+O_3	DUST+SO_4 +NO_3	DUST+SO_4 +NO_3+O_3
	Group Mean Values				
D1H4	161	151	144	185	139
D9H4	170	147	163	158	164
D17H4	171	143	183	119	130
D25H4	182	153	155	148	139
D29H4	166	193	169	172	242
Grand Mean	171	158	163	157	164
	Group Standard Deviation Values				
D1H4	41	16	29	31	21
D9H4	21	30	27	31	30
D17H4	35	32	22	32	39
D25H4	17	51	56	31	62
D29H4	30	67	28	52	99

Exposure X Day Interaction F = 3.67, p < 0.0001

Exposure Day and Hour					
	Group Mean Values				
D4H4	167	162	171	160	158
D12H4	177	132	155	169	165
D20H4	192	138	158	148	183
D28H4	182	170	175	138	166
D32H4	141	181	165	177	161
Grand Mean	172	157	165	159	167
	Group Standard Deviation Values				
D4H4	39	41	40	27	40
D12H4	45	18	36	20	49
D20H4	20	35	35	46	47
D28H4	23	50	39	29	43
D32H4	21	37	35	22	38

Exposure X Day Interaction F = 7.21, p < 0.007