

**Final Report**

**Health Effects of PM Components on Sensitive Animal Models**

**Principal Investigator: M.T. Kleinman, Ph.D.**

**Co-Investigators: S. George, Ph.D; M. Hallman, M.D., Ph.D., R. Sindhu, Ph.D., and D. Meacher, Ph.D.**

**University of California, Irvine**

**Collaborators: K. Pinkerton, Ph.D and D. Hyde, Ph.D.**

**University of California, Davis**

**Statistician: K. Osann, Ph.D.**

**University of California, Irvine**

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**AIR POLLUTION HEALTH EFFECTS LABORATORY**

**Department of Community and Environmental Medicine**

**University of California, Irvine**

**Irvine, CA 92697-1825**

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## Abstract

Particle-induced lung injury and heart responses were measured as a function of particle size in young adult and geriatric (senescent) rats exposed to laboratory generated particles composed of elemental carbon and ammonium nitrate. The particle compositions and concentrations used were consistent with ambient aerosols collected and characterized in California. Three particle size ranges were tested, 'Ultrafine' ( $d_p \leq 200$  nm), Fine ( $500 \leq d_p \leq 1000$  nm) and Coarse ( $2000 \leq d_p \leq 4000$  nm). The biological responses studied included measures of cell replication, which are part of normal injury-repair processes, cellular immunological measures, which are related to defense mechanisms and hemodynamic factors (changes in blood pressure, variability in heart rate and abnormal heart rhythms).

Our primary hypothesis was that particle-induced lung injury at the tissue and cellular levels, and systemic effects would be a function of particle size when composition and concentration of particles were held constant. Also, given that epidemiological studies indicated that older individuals were at greater risk of adverse particle induced health effects, we hypothesized that senescent (geriatric) rats would be a more sensitive to the effects of PM than would be healthy young adult rats.

Acute (6 hour) exposures to mixtures of particles and ozone at concentrations relevant to short-term peak ambient levels caused inflammatory responses in both old and young rats. The particle exposures also altered immunologic responses of lung macrophages, compared to responses measured in rats exposed to purified air. Particle exposures caused changes in blood pressure and heart rate, compared to measurements in rats exposed to purified air. These changes were observed in both young rats and in senescent rats, but the senescent rats responded with greater sensitivity than did the younger rats. Exposures to 'ultrafine' particles elicited greater blood pressure responses than either fine or coarse particles. However, ultrafine particles deposit more efficiently in the gas exchange (pulmonary) region of the lung than do either fine or coarse particles. Thus, the dose of 'ultrafine' particles could have been greater than the dose of particles in either of the other two size fractions, even though the exposure concentrations were the same.

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## Executive Summary

### Background

This study analyzed the effects of acute, short-term exposures to particulate matter (PM) on the cardiopulmonary system. Cardiopulmonary effects were examined because epidemiological studies established associations of increased risks of respiratory illness and mortality in people exposed to elevated levels of ambient PM. This study used laboratory-generated particles containing two components of ambient PM, ammonium nitrate (AMN) and elemental carbon (EC). AMN and EC are major constituents of fine particle ambient PM (i.e. PM<sub>2.5</sub>) in California. The concentrations of nitrate in polluted cities of the Northeastern US are much lower than in California, and very few studies had examined the potential health effects of nitrate-containing particles.

Previous laboratory (and some epidemiological) studies had demonstrated that particle mixtures with ozone (O<sub>3</sub>) were more toxic than the particles alone or ozone alone. Also, in ambient air, periods of high photochemical activity that produce O<sub>3</sub> are often periods of high PM<sub>2.5</sub> concentrations. We therefore included O<sub>3</sub> in our laboratory-generated PM atmospheres. The concentrations of each component used in this study were representative of estimated peak concentrations, based on extrapolations from ambient air data. The sizes of the particles used were relevant to sizes of inorganic aerosols in ambient air.

The overall objectives of this ARB-sponsored research were:

- To examine the health effects of atmospheric mixtures which realistically model sizes and compositions of particles in California's air.
- To examine whether or not particle-induced lung injury could initiate systemic adverse cardiopulmonary responses in young healthy rats and in a presumably sensitive model, the aged (senescent) rat; and
- To determine whether these cardiopulmonary responses were particle size-dependent.

To achieve these objectives, we examined particle-induced lung injury and heart responses as a function of particle size, and evaluated the possibility that particle-induced oxidative stress mechanisms contributed to the development of adverse responses.

Our primary hypothesis was that particle-induced lung injury at the tissue and cellular levels would be a function of particle size when composition and concentration of particles were held constant. We further hypothesized that in addition to direct effects on the lung, systemic cardiopulmonary effects could be initiated when particles deposited in the lung. Also, given that epidemiological studies indicated that older individuals were at greater risk of adverse particle induced health effects, we hypothesized that senescent (geriatric) rats would be a more sensitive to the effects of PM than would be healthy young adult rats.

### **Approach**

This study built upon the results of a previous study, performed in our laboratory, that demonstrated statistically significant and mechanistically relevant PM-induced pulmonary effects in senescent and young, healthy rats exposed to mixtures of laboratory-generated particles and ozone. That previous study examined effects following exposure to particles in a single size range and demonstrated that senescent rats were more sensitive to these effects than were young adult rats. The present study incorporated a novel inertial particle size classifier to allow us to perform exposures to particles in three different size ranges while maintaining the particle composition and concentrations. We also developed and installed new non-invasive biological endpoints to measure NO in expired breath, heart rate and systolic blood pressure in young adult and senescent Fischer 344 rats.

An important feature of the present research was that it included a direct collaboration with Dr. Pinkerton and other researchers at U. C. Davis (UCD). UCD specifically examined relationships between dose and epithelial injury in juvenile, young adult and senescent rats and UCI examined cardiopulmonary responses as a function of particle size in young adult and senescent rats. In order to provide a strong degree of comparability between the efforts at these two campuses, the composition of the aerosol tested was agreed upon and maintained throughout the study. In addition the middle range particle size used for the studies at UCI was the same as the size used for studies at UCD (approximately 700 nm mass median aerodynamic diameter; MMAD). Tissue and cell samples were also shared between the campuses so that, for a subset

of exposures at both laboratories a complementary set of endpoints were measured. This potentially allowed us to increase the amount of information obtained from these studies.

## **Methods**

This state-of-the-art inhalation study examined biological endpoints that could be associated with certain of the possible mechanisms for PM-induced toxicity using aged rats. Aged rats were used as laboratory models of older people in the human population. People older than 65 years of age, or people with pre-existing lung or heart disease have been reported to be especially susceptible to PM-induced toxic effects. The PM components that were studied were elemental carbon (EC) and ammonium nitrate (AMN), both of which are important constituents of PM in California. To perform this study a novel particle size separator was constructed and used to expose rats to purified air and three ranges of particle sizes, at the same time. The concentrations and relative composition of the particle mixtures were maintained constant, and only particle size was varied. The biological responses studied, which included measures of cell replication, are part of normal injury-repair processes, while others, such as cellular immunological measures, are more related to defense mechanisms. Several epidemiological studies have established an association of PM exposures with increased risk of cardiovascular disease and death. Changes in blood pressure, variability in heart rate and abnormal heart rhythms have been associated with PM exposures. We therefore included measures of heart function (blood pressure and heart rate) as endpoints in our study.

Rats were exposed, nose-only, to atmospheres containing particles and ozone under carefully controlled conditions. Control rats were exposed to purified air at the same time, and under the same conditions, as were the particle-exposed rats. Samples were collected from the rat's breathing zone and analyzed during the exposures to characterize the particle composition and the particle size distributions. Exposures were for single 6 hour periods. The concentrations that were used were similar to those measured in ambient air during peak periods of elevated PM levels. Rats were exposed to particles three size ranges. The particle size ranges were characterized as Ultrafine ( $d_p \leq 200$  nm), Fine ( $500 \leq d_p \leq 1000$  nm) and Coarse ( $2000 \leq d_p \leq 4000$  nm). A key in successfully performing these studies was achieving equivalent concentrations and particle compositions in each of the three size ranges.

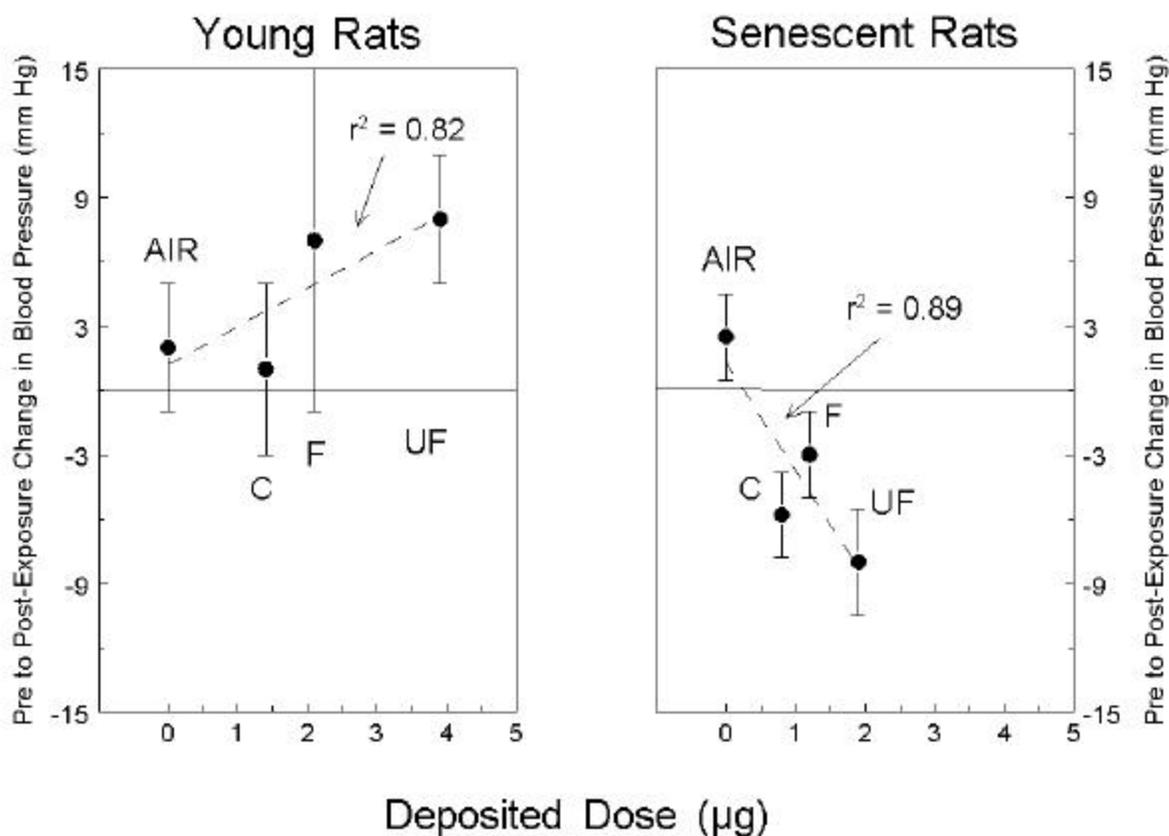
The rat's blood pressure and heart rate were measured before and after exposures. Nitric oxide was then measured in their expired breath. The rats were then euthanized, their lungs were surgically exposed and the lungs were lavaged to obtain samples for analysis of cells and proteins. The types of cells recovered were determined and anti-infection related functions of lung immune system cells (macrophages) were measured. Lung fluid proteins were assayed to determine if particle exposure increased the permeability of the lung to blood serum proteins (a measure of lung 'leakiness' related to edema).

## **Summary**

Acute exposures to mixtures of particles and ozone at concentrations relevant to short-term peak ambient levels caused inflammatory responses in both old and young rats. The exposures also altered antibody-directed and nonspecific responses of lung macrophages, compared to responses measured in rats exposed to purified air. Thus, particle exposures could be linked to increased risks of lung infections, as has been seen in epidemiological studies. The defense system in older rats were less robust than in younger rats, hence one might expect that older rats would be more susceptible to respiratory infections than younger rats, and that particle-induced depression of immunological functions could be more serious in the older rats. If the pattern of age-related reduction in immune system competency in humans parallels that in rats, this could provide a biologically plausible mechanism to explain the increased risk of respiratory infections seen in elderly people during periods of elevated ambient PM exposure.

Particle exposures caused systemic effects in exposed rats, i.e. changes in blood pressure and heart rate, compared to measurements in rats exposed to purified air. These changes were observed in both young rats and in senescent rats. A particle deposition computer model for rats was used to estimate dose of particles to the pulmonary region of the lung for the 3 size classes of particles. The changes in blood pressure are plotted as a function of pulmonary dose in the figure below. Senescent rats were more sensitive, responding at exposure levels that were  $\frac{1}{2}$  those used in the younger animals. These changes occurred even though there were only small, and statistically non-significant, inflammatory changes in the lung or changes in exhaled nitric oxide. Exposures to ultrafine particles elicited greater blood pressure responses than either fine or coarse particles. However, ultrafine particles deposit more efficiently in the critical gas exchange (pulmonary) region of the lung than do either fine or coarse particles. Therefore, in

this experiment, the dose of ultrafine particles could have been greater than the dose of particles in either of the other two size fractions, even though the exposure concentrations were the same. In the figure below, differences in systolic blood pressure measured before and after exposures were plotted as a function of pulmonary dose. The dose (in  $\mu\text{g}$  of particles deposited in the pulmonary region of the lung) was calculated using the measured mass concentrations of AMN + EC to which the rats were exposed and the fraction of the inhaled particles that would deposit in the pulmonary region of the rat's lung for particles of the stated sizes. As shown in the figure, the greater effect of ultrafine particles could have been due to the greater dose to the deep lung.



It is important to note that in ambient air, the sources and chemical composition of ultrafine particles are different from that in fine or coarse fractions of PM, in contrast to this study in which composition was relatively constant. Thus, this finding does not undercut the

need to more carefully study the toxicology and health effects of ambient ultrafine, fine and coarse particles on health.

In summary, the major findings of this study were:

- Acute exposures to mixtures of particles and ozone at concentrations relevant to short-term peak ambient levels caused inflammatory responses in both old and young rats. The exposures also altered antibody-directed and nonspecific responses of lung macrophages, compared to responses measured in rats exposed to purified air.
- Particle exposures caused systemic effects, i.e. changes in blood pressure and heart rate, compared to measurements in rats exposed to purified air. These changes were observed in young rats as well as in senescent rats. Senescent rats responded at exposure levels that were ½ those used in the younger animals. These changes occurred in the absence of significant inflammatory changes in the lung or changes in exhaled nitric oxide.
- Exposures to ultrafine particles elicited greater blood pressure responses than either fine or coarse particles. However, a considerably larger fraction of ultrafine particles deposits in the pulmonary region of the rat's lung than does either fine or coarse particles, all other exposure parameters being equal. Thus, when the blood pressure responses were plotted as a function of pulmonary dose delivered under the conditions of this experiment, linear relationships between dose and response were observed.

## **Conclusions**

We conclude that particle size is an important determinant of air pollution health effects. For particles of the same composition, particle size influences the dose of material delivered to the critical region of the lung in which gas exchange and transport of oxygen to the blood occurs. The degree of lung irritation or injury produced in this region of the lung will depend on the delivered dose and the effects of this injury or irritation can extend to other organs or body systems.

Furthermore, we conclude that older animals are more susceptible to air pollution health effects, than are young, adult animals. In this study, the dose needed to induce a significant

effect in older rats was 10 times lower than the dose needed to achieve a significant response in younger rats.



# Introduction

## **Background**

Human exposures to particulate matter (PM) have been associated with acute illness and death, especially in people older than 65 years of age or people with pre-existing lung or heart disease. Current federal and California ambient air quality standards were established for particles smaller than 10  $\mu\text{m}$  (PM10) in mass median aerodynamic diameter (MMAD), because particles in this size range are inhaled and penetrate deeply into the respiratory tract, and they are associated with adverse effects on human health. The California and federal PM10 standards were promulgated to (a) prevent excess deaths from short-term exposures and from exacerbation of symptoms in sensitive individuals with respiratory disease; and (b) prevent excess seasonal declines in pulmonary function, especially in children. The current National Ambient Air Quality Standard (NAAQS) for PM10 is 50  $\text{mg}/\text{m}^3$  (annual arithmetic mean) and 150  $\mu\text{g}/\text{m}^3$  (24 hr arithmetic mean). The State of California has set more stringent PM10 standards of 30  $\mu\text{g}/\text{m}^3$  (annual geometric mean) and 50  $\mu\text{g}/\text{m}^3$  (24 hr arithmetic mean concentration). More recently, epidemiological studies suggested that PM10-based Federal standards are not sufficiently protective and that it was also important to reduce exposures to ambient particles less than 2.5  $\mu\text{m}$  MMAD (PM2.5). PM2.5 concentrations are strongly influenced by emissions from combustion sources and atmospheric chemical reactions, whereas larger particles ( $>2.5 \mu\text{m}$ ) are primarily generated by mechanical processes such as abrasion and resuspension.<sup>1,2</sup> The chemical constituents of the larger sized particles are predominantly those characteristic of the earth's crust (e.g. iron, silica, aluminum). The PM2.5 particles are more enriched in heavy metals, elemental carbon, organic carbon, and products of atmospheric chemical and photochemical processes including sulfate and nitrate salts.<sup>3,4</sup>

Exposures to urban PM10 at levels near the State's (50  $\mu\text{g}/\text{m}^3$ , 24 hr average) and often below the PM10 national (150  $\mu\text{g}/\text{m}^3$ , 24 hr average) ambient air quality standard (NAAQS) are associated with increased hospital admissions and emergency room visits for respiratory illnesses,<sup>5,6,7,8</sup> increased incidences of asthma attacks,<sup>9,10</sup> increased asthma medication use,<sup>11</sup> reduced pulmonary function,<sup>11,12,13</sup> and increased daily mortality.<sup>14,15,16,17</sup> People whose deaths

have been attributed to PM<sub>2.5</sub> and PM<sub>10</sub> exposures tend to be older than 65 years of age, although health effects have also been reported for children and people with asthma. The study reported by Pope et al.<sup>18</sup> showed increased mortality for individuals 35 to 64 years of age associated with PM<sub>2.5</sub> exposures at or below the promulgated PM<sub>2.5</sub> NAAQS. Individuals with pre-existing cardiovascular or chronic pulmonary diseases are at even greater risk of PM-related mortality.<sup>19</sup>

Other pollutants, such as ozone (O<sub>3</sub>), sulfur dioxide (SO<sub>2</sub>), nitrogen dioxide (NO<sub>2</sub>) and carbon monoxide (CO) have been examined in conjunction with PM health effects in some studies, as have meteorological variables such as temperature, relative humidity and season. Interactions of O<sub>3</sub> or CO with PM-induced health effects have been reported and there is some contention that in some studies it is not possible to disentangle the effects of the co-pollutants from those of PM. However, in most of the reported epidemiological studies, PM has been shown to have an independent association with mortality which remains significant even when other copollutants are introduced into the statistical model, whereas the copollutants often do not.

PM<sub>10</sub> standards are mass-based and do not explicitly consider particle composition with respect to the health effects observed. It should be noted that some components of PM are separately considered as hazardous air pollutants (or toxic air pollutants), however they are regulated differently than are criteria pollutants, such as PM or O<sub>3</sub>.

The present PM NAAQS address the effects of fine particles with diameters ( $d_p$ ) < 2.5  $\mu\text{m}$  (PM<sub>2.5</sub>) and particles with diameters  $d_p$  < 10  $\mu\text{m}$  (PM<sub>10</sub>) but do not explicitly address the possible effects of coarse particles ( $2.5 \leq d_p \leq 10 \mu\text{m}$ ), except as covered within the PM<sub>10</sub> NAAQS. Some data suggest that associations between mortality and fine particle or acidic particle concentrations are stronger than those between mortality and coarse particle concentrations.<sup>20</sup> For example, exposures to respirable fine ( $\leq 2.5 \mu\text{m}$  MMAD) particles and sulfates have been associated with increased total annual mortality rates.<sup>21,22</sup> Other data suggest that ultrafine particles ( $d_p \leq 0.1 \mu\text{m}$ ) may be an especially toxic subfraction of PM. However, there are some studies that show stronger associations between health effects and exposure to coarse particles<sup>23,24,25</sup> than between health effects and fine particle exposures. Thus, at the present time, the PM<sub>10</sub> NAAQS is retained even though a PM<sub>2.5</sub> standard has been promulgated.

## ***Project Objectives and Hypotheses***

### **Objectives**

It is important to note that the composition of ambient particles varies seasonally and regionally. Much of the literature cited above relates to ambient atmospheres that are different from those in polluted urban California areas. For example there have been very few studies that address the possible effects of the nitrate component of ambient particles.

The overall objectives of this ARB-sponsored research were: (1) to examine the health effects of atmospheric mixtures which realistically model sizes and compositions of particles in California air; (2) to examine whether or not particle-induced lung injury could initiate systemic adverse cardiopulmonary responses in young healthy rats and in a presumably sensitive model, the aged (senescent) rat; and (3) to determine whether these cardiopulmonary responses were particle size-dependent.

Our primary hypothesis was that particle-induced lung injury at the tissue and cellular levels would be particle size dependent when composition and concentration of particles were held constant. We further hypothesized that in addition to direct effects on the lung, systemic cardiopulmonary effects could be initiated when particles deposited in the lung and injured epithelial tissue. Also, given that epidemiological studies indicated that older individuals were at greater risk of adverse particle induced health effects, we hypothesized that senescent (geriatric) rats would be a more sensitive model than would be healthy young adult rats.

There are several possible ways in which lung injury can affect cardiopulmonary physiology and many of the proposed mechanisms involve the production of reactive oxygen species, such as superoxide, and other free radicals that can cause oxidative stress. Particle and injury-induced oxidative stress could initiate the production of several different mediators and alter the production and availability of nitric oxide (NO) which plays an important role in regulation of airway and arterial smooth muscle tone. Depletion of NO could cause bronchoconstriction and vasoconstriction, and thus increase systemic and pulmonary blood pressure. On the other hand, an increase in the production of NO could lead to a decrease in smooth muscle tone and blood pressure. Reactions between superoxide radicals and NO could produce peroxynitrite, a potent oxidant that can react with proteins and cell membranes leading

to additional cellular injury. To test our hypotheses, we examined particle-induced lung injury and heart responses as a function of particle size, and evaluated the possibility that particle-induced oxidative stress mechanisms contributed to the development of adverse responses.

## **Approach**

This study built upon the results of a previous study, performed in our laboratory, that demonstrated statistically significant and mechanistically relevant PM-induced pulmonary effects in senescent and young, healthy rats exposed to mixtures of laboratory-generated particles and ozone. That previous study examined effects following exposure to particles in a single size range and demonstrated that senescent rats were more sensitive to these effects than were young adult rats. The present study incorporated a novel inertial particle size classifier to allow us to perform exposures to particles in three different size ranges while maintaining the particle composition and concentrations. We also developed and installed new non-invasive biological endpoints to measure NO in expired breath, heart rate and systolic blood pressure in young adult and senescent Fischer 344 rats.

An important feature of the present research was that it included a direct collaboration with Dr. Pinkerton and other researchers at U. C. Davis (UCD). UCD specifically examined relationships between dose and epithelial injury in juvenile, young adult and senescent rats and UCI examined cardiopulmonary responses as a function of particle size in young adult and senescent rats. In order to provide a strong degree of comparability between the efforts at these two campuses, the composition of the aerosol tested was agreed upon and maintained throughout the study. In addition the middle range particle size used for the studies at UCI was the same as the size used for studies at UCD (approximately 0.7  $\mu\text{m}$  mass median aerodynamic diameter; MMAD). Tissue and cell samples were also shared between the campuses so that, for a subset of exposures at both laboratories a complementary set of endpoints were measured. This potentially allowed us to increase the amount of information obtained from these studies.

### **Specific Aims.**

Four exposure experiments were conducted over the three-year period of this contract to provide the data with which to test the key hypotheses. These experiments satisfied the following specific aims:

1. Determine the effects of a particle mixture in the fine particle size range containing AMN and EC in the presence and absence of O<sub>3</sub> in young adult rats to determine if the particles alone did not produce significant effects, whether the mixture of particles plus O<sub>3</sub> would produce group mean effects that were significantly different from controls.
2. Compare the effects of particle-containing atmosphere exposures on young and senescent rats to determine if senescent rats were more sensitive than were the younger rats.
3. Compare the responses of senescent and younger rats after exposure to particles in three particle size ranges (“ultrafine”  $200 \leq d_p$ ; “fine” ,  $500 \leq d_p \leq 1000$ ; and “coarse”,  $2000 \leq d_p \leq 4000$ ; where  $d_p$  represents particle mass median aerodynamic diameter (MMAD) in nm).

At the outset of the study our expectations were that:

- The size characteristics of aerosols influenced deposition and dose of particles, and that, for a given particle composition, dose differences would modulate the biological responses;
- For a given dose, ultrafine particles would be more toxic than either fine particles or coarse particles; and
- Older individuals would be more susceptible to oxidative stress and impairment of cardiopulmonary function than younger adult individuals.

## **Materials and Methods**

### ***Exposures and Atmospheres***

#### **Selection of the Atmospheric Components**

This study focused on Southern California PM because of the frequent incidences of high particulate concentrations in ambient air in this region, and because of the large population subject to potentially adverse impacts (Hall et al., 1992).

Southern California ambient particulate matter is a complex mixture with respect to both composition and particle size. Primary emission particles include combustion aerosols, particles generated by motor vehicles (tire wear, brake linings, elemental carbon from exhaust fumes), and fugitive dusts. Secondary aerosols contain nitrate and sulfate salts from atmospheric gas to particle conversion processes as well as organic carbon-containing particles.

In selecting the composition of the test atmospheres to be used in this toxicological evaluation of PM, 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. PM<sub>10</sub> was sampled in Claremont, CA, as part of the South Coast Air Quality Sampling (SCAQS) program in the summer of 1987<sup>26</sup>. Some of these data are summarized in Table 1.

<b>Table 1 Concentrations (mg/m<sup>3</sup>) of Particulate Components in Claremont, CA</b>			
	Mean ± SD	Max	% of PM10
PM10	60 ± 24	120	-
Fine Particulate Matter (d <sub>p</sub> <2.5 μm)	36 ± 17	91	60
Coarse Particulate Matter (2.5 μm< d <sub>p</sub> < 10 μm)	23 ± 10	47	38
Sulfate	6.5 ± 3.8	18	10.8
Nitrate	12.4 ± 6.5	28	20.7

As shown in Table 2, the characteristics and dominant sources of particles in different size fractions are quite diverse. The data presented are derived from various sources<sup>27,28,29</sup>; the size classifications are approximate.

<b>Table 2 Origin and Composition of Ambient Particles</b>			
Size Range ( $\mu\text{m}$ )	Particle Class	Predominant Generation Source	Environmental Composition Characteristics
$d_p \leq 0.1$	Ultrafine	gas to particle conversion, condensation	organic C, $\text{SO}_4$ , $\text{NO}_3$ , elemental C, metals
$0.1 < d_p \leq 2.5$	Fine	combustion, condensation, particle coagulation	organic C, $\text{SO}_4$ , $\text{NO}_3$ , elemental C, metals
$2.5 < d_p \leq 10.0$	Coarse	Mechanical processes	soil, resuspended matter from paved and unpaved roads, industrial materials, brake linings, tire residues, $\text{SO}_4$ , $\text{NO}_3$ , elemental C, metals

Carbon and nitrate are found in both fine and coarse fractions of PM, although they represent larger fractions of PM<sub>2.5</sub> than they do of PM<sub>10</sub>. Given this and the fact that EC and AMN represent a significant fraction of PM, it was reasonable to study mixtures that contain both EC and AMN.

Ozone ( $\text{O}_3$ ), another pollutant whose concentration frequently exceeds Federal and State standards in Southern California, is often present at high concentrations in ambient air in cities with high concentrations of ambient PM, albeit the times of peak exposures are not necessarily the same. Never the less, previous studies of inhaled pollutant mixtures showed that particle plus ozone mixtures produced significant changes in lung morphology and immunological endpoints. Therefore, this study examined combinations of PM components with ozone in a series of inhalation exposures.

The sizes of particles in the exposure atmospheres tested were selected based upon estimates of peak exposures in the South Coast Air Basin. Both EC and nitrate ( $\text{NO}_3^-$ ) particles are found in the 0.3-0.6  $\mu\text{m}$  size range. In general, nitrate particles are formed as an ammonia neutralization product of nitric acid. The nitric acid is formed by oxidation of gaseous nitrogen oxides that are emitted from combustion processes. There are also significant amounts of both particle species in ultrafine and coarse components of ambient air in the south coast air basin. Nitric acid vapors are formed in the atmosphere during periods of high photochemical activity when high concentrations of ozone ( $\text{O}_3$ ) are also formed. There is often a very strong correlation between levels of nitrate and  $\text{O}_3$ . The atmospheres selected for study therefore contained elemental carbon (EC), ammonium nitrate (AMN) in the ultrafine and fine particle size ranges and  $\text{O}_3$ .

The concentrations of particles in the exposure atmospheres tested were similar to those used in a previous PM study. In that study we used EC concentrations of 100  $\mu\text{g}/\text{m}^3$  and ammonium bisulfate concentrations of about 150  $\mu\text{g}/\text{m}^3$ . Significant responses in terms of epithelial injury and macrophage function changes were observed after episodic exposures of 4 hr per day, 3 days per week for 4 consecutive weeks. In the present study, we substituted AMN for ABS (which was more representative of the California aerosol). We also performed single 4 hr exposures. Therefore a range of concentrations that bracketed the previous level was selected, with a high concentration level 2x the previous level and a low concentration level of  $\frac{1}{2}$  the previous level.

### **Inhalation Exposures**

Exposures of rats were nose-only to a continuous stream of the test atmosphere. Nose-only exposures were performed in order to prevent artifacts due to contamination of chamber air by dander, ammonia and dried excreta. Between exposures, rats lived in purified-air barrier housing and they were given purified water and fed NIH-31 Teklad Premier Laboratory Diet (Teklad, Bartonville, IL) *ad lib*. Animals were housed in wire cages over beds of rock salt that dried feces and urine and suppressed dust and ammonia production. Personnel wore gowns, hair bonnets, masks, shoe covers and surgical gloves when handling the animals to prevent the spread

of pathogens to the rats. Exposure tubes were inspected daily and thoroughly machine-washed in hot soapy water after each use. Animal cages were washed and sterilized twice weekly, and quarters were cleaned daily.

### **Pollutant generation**

Ozone was generated by passing medical grade oxygen through two corona-discharge ozonizers (Sander Ozonizer, Type III, Osterberg, Federal Republic of Germany). The ammonium nitrate + carbon aerosol was generated by preparing a suspension of Monarch 120 carbon black particles (Cabot Corp., Boston, MA) in a dilute solution of ammonium nitrate. Monarch 120 consists of primary particles 75 nm in diameter, and contains less than 1% extractable organic compounds. The suspension was agitated ultrasonically and delivered in a continuous, metered stream to a custom-designed high output generator system designed around an array of Babbington-type nebulizers. Particles were nebulized with compressed air to produce a dense aerosol with a broad, polydisperse size distribution. The aerosol was split into 3 fractions as follows. The first fraction was passed through a BGI impactor, dried by rapid dilution, discharged to Boltzmann equilibrium using  $^{85}\text{Kr}$  sources and then introduced into a nose-only exposure chamber. This fraction produced an aerosol with a mass median aerodynamic diameter (MMAD) of approximately 0.7  $\mu\text{m}$  and a geometric standard deviation (GSD) of about 2.5. This fraction was similar in size to that used in the UCD studies. The second fraction was separated into “coarse” and “ultrafine” sizes using an inertial separator. The separator operated on the concept of a virtual impactor. The aerosol was funneled into a jet (100 Lpm) under positive pressure (40 psi). The inertial separator, which acts as a virtual impactor, has a major flow port which was located behind the point of entry (requiring the airstream to abruptly change direction) that passes 80% of the inlet air, and a minor flow port (at which the air jet was directed) that passed the remaining 20% of the inlet air. The coarse particles tend to travel in a straight line due to their inertia while the fine particles tend to follow the airstream. Thus the major flow air is enriched in fine particles and the minor flow air is enriched in larger particles. The particles were generated from the same nebulizer mixture, thus the chemical composition of the particles should be the same regardless of the size mode. Each fraction was introduced into a specific nose-only exposure unit. This system allowed us to deliver particle mixtures with the

same chemical compositions but with different particle sizes simultaneously into three separate nose-only exposure units with good control of the mass concentration of the particles.

### **Pollutant Characterization**

Aerosol particles were collected on pre-weighed and equilibrated (50% relative humidity [R.H.]) Pallflex T60A20 fluorocarbon-coated glass fiber filters (PALL Corp., Cincinnati, OH). The filters were weighed after collection to determine the total collected mass. Pallflex filters were extracted with dilute carbonate/bicarbonate buffer and the extract analyzed for  $\text{NO}_3^-$  by ion chromatography. Samples for particle size analysis were collected using an 8-stage cascade impactor (Andersen Model 208) and pre-cut and pre-weighed Pallflex substrates. The substrates and backup filter were weighed after collection to determine the total collected mass. Pallflex substrates and the backup filter were extracted with dilute carbonate/bicarbonate buffer and the extracts analyzed for  $\text{NO}_3^-$  by ion chromatography. Cumulative mass percentages were plotted versus impactor stage cutoff diameters on log-probability paper and the mass median aerodynamic diameters and geometric standard deviations were estimated.

Samples for carbon analysis were collected on acid-treated quartz fiber filters (Microquartz; Gelman, Ann Arbor, MI). Carbon was determined by combustion in oxygen in a flow-through furnace. The evolved  $\text{CO}_2$  was measured using a non-dispersive infrared absorption analyzer (Dasibi Model 3003, modified with a  $\text{CO}_2$  absorption cell). The analyzer was calibrated against known amounts of  $\text{CO}_2$  generated by the decomposition of oxalic acid.

### **Sacrifice of rats and timing of endpoints**

Nitric oxide (NO) in expired breath, blood pressure and heart rate were measured in unanaesthetized rats immediately after and 18 hr. after their final exposure. Animals were then euthanized and tissue and biological fluids were collected and stored for endpoint analyses. The 18 hr. timepoint was selected because previous studies with younger animals exposed to mixtures of  $\text{O}_3$  + EC + ammonium bisulfate (ABS) had shown that the permeability change peaked 12 to 18 hr after acute exposures, and were not detectable immediately post-exposure.<sup>30</sup> This timing was also suitable for detecting the onset of inflammatory responses, such as cell turnover, and observation of functional changes in macrophages.

## ***Measurements of Cellular and Non-Cellular Lung Fluid Constituents***

### **Bronchoalveolar Lavage**

The rats were anesthetized by intraperitoneal injection (i.p.) of sodium pentobarbital (50 mg/kg). The abdominal aorta was severed and a polyethylene catheter was placed and tied in the trachea. An incision was made in the diaphragm to allow lung expansion during the lavage. Lungs were lavaged by introduction of 28 mL/kg body weight HEPES buffered Hanks Balanced Salt Solution (HBSS) without  $\text{Ca}^{+2}$  or  $\text{Mg}^{+2}$  through the tracheal catheter followed by withdrawal of the fluid. The introduction and withdrawal of this fluid was repeated three times and the first 5 mL of lavage fluid was recovered and transferred to a 15 ml polypropylene centrifuge tube. The process of introduction and withdrawal of lavage fluid was repeated two additional times with fresh HBSS. The fluid from the last two lavages was pooled in a separate centrifuge tube. The tubes containing lavage fluid from each animal were centrifuged at 300 g for 10 min. The cell-free supernatant from the first lavage was analyzed for total protein and serum albumin. The cells from the two tubes were pooled for each animal.

### **Total Protein**

A bicinchoninic acid (BCA) procedure<sup>31</sup> was used for determination of total protein in the BAL.<sup>30</sup> Standards were prepared by dilution from a stock solution of bovine serum albumin (BSA). Protein standards, unknown samples, and diluent used for blanks (0.10 ml each) were pipetted into appropriately labeled test tubes. Each tube then received 2 ml of BCA working reagent prepared from the kit reagents to the manufacturer's specifications (Pierce BCA Protein Assay Reagent, Pierce Chemical Co., Rockford, IL). All samples were incubated at 60°C for 30 min, then cooled. Absorbance was measured at 562 nm. Absorbances of blanks were subtracted from that for standards and unknown samples. A standard curve was prepared using known concentrations of rat albumin.

### **Albumin**

ELISA procedures described by Schwerer et al.<sup>32</sup> and Macy et al.<sup>33</sup> were used as a guide for establishing an albumin assay in our laboratory.<sup>30</sup> Polystyrene non-flexible 96 well microtiter plates (Costar; Van Nuys, CA) were coated with goat anti-rat albumin antibody (Organon Teknika, Durham, NC) in carbonate buffer, pH 9.6. The plates were covered, refrigerated

overnight and then washed with carbonate buffer to remove excess material or antibody. Non-specific binding was blocked by the addition of gelatin in carbonate buffer. The plates were placed in a humid chamber at room temperature for 1 to 2 hr and then washed two times with PBS-Tween 20-gelatin. A standard curve was developed using serial dilutions of a standard (15 mg/ml) rat albumin solution (Sigma Chemical Co.). Rat lavage fluid samples in PBS-Tween 20-gelatin solution were added to each well. The plates were covered and incubated in a humid chamber for 1 to 2 hrs. After washing, 100  $\mu$ l of a 1:2000 dilution of peroxidase-conjugated rabbit anti-rat albumin antibody (5.0 mg/ml) was added to each well. The plates were incubated in a humid chamber at room temperature for 1 hr. Color was developed by the addition of 100  $\mu$ l per well of citrate-phosphate buffer (pH 5.0) containing 1 mg/ml O-phenylenediamine dihydrochloride (OPD) and 1  $\mu$ l/2 ml of 30% H<sub>2</sub>O<sub>2</sub>. The plates were covered and incubated, in the dark, at room temperature for 20 minutes. The reaction was stopped by the addition of 50  $\mu$ l per well of 2N H<sub>2</sub>SO<sub>4</sub>. The plates were read at 492 nm in a plate reader (Titertek Multiscan; Salzburg, Austria).

### ***Measurements Related to Airway Inflammation***

#### **Inflammatory Cell Infiltration (PMNs)**

Cells from lavage fluid were deposited on slides using a cytocentrifuge and stained with Diff Quik (Baxter Healthcare Corp., McGaw, IL) for differential cell counts. Cells were scored as being macrophages or monocytes, lymphocytes, PMNs or “other” cells (the other category included epithelial cells, multinuclear macrophage-derived cells or cells which were not otherwise distinguishable).

### ***Measurements Related to Acute Lung Injury and Lung Disease Processes***

In order to maximize the potential utilization of these exposure studies, in addition to the rats required to perform the UCI-related evaluations, groups of rats were exposed and lung tissue was prepared for histological evaluation. These tissues were sent to UCD for analysis.

## Cell Proliferation in Lung Tissue

### In vivo cumulative labeling index:

Two days prior to exposure, the animals were anaesthetized with Halothane ® and an Alzet minipump was implanted under the skin on the rat's upper back. The pumps were charged with 5-Bromo-2-Deoxyuridine (BrdU, 30 mg/ml in 0.01N sodium hydroxide; Sigma Chemical Corp.). The animals were allowed to recover for 24 hr. after the surgery before exposures were performed. The pump provided a constant metered dose of BrdU from the time of insertion to the time of necropsy, providing a stronger signal for our studies than previous methods in which the BrdU was injected. Cells undergoing DNA synthesis incorporate BrdU, a thymidine analog, into DNA and this incorporation is indicative of cell proliferation (a marker of epithelial repair and interstitial cell response to epithelial injury). This endpoint was used to identify sites of cell replication in epithelial and interstitial respiratory tract tissues.

The rats being assayed for histopathological endpoints were euthanized after exposure and physiological measurements were completed (18 hr. post-exposure). The rats were first anesthetized with sodium pentobarbital (50 mg/kg i.p.). Then the trachea was exposed, a tracheal cannula was inserted, the abdomen was opened, the liver reflected caudally, the diaphragm cut at the cardiac shadow and the lungs were collapsed to a minimum volume.

### Tissue Fixation for Immunocytochemistry

After the central rib cage and sternum were removed, the lungs were removed *en bloc* and fixed at a constant pressure of 25 cm H<sub>2</sub>O pressure with a zinc formalin fixative (Z-fix; Anatech Ltd., Battle Creek, MI) for a minimum of 24 hours. Subsequently, the lungs were removed from the fixation apparatus and stored in the same fixative until they were embedded for sectioning. Samples of gut tissue were removed and immersed in fixative for later processing. Since gut epithelium is constantly undergoing cell removal and replacement processes, gut tissue could be used as a positive control for the BrdU assay.

All of the samples for histopathology assays were sent to Dr. Hyde at UCD.

Slides were prepared for determining morphometry changes, infiltration of inflammatory cells and cell proliferation rates by BrdU incorporation.

### Light microscope immunocytochemistry for BrdU

Paraffin sections (5 $\mu$ m) of lung and duodenum were affixed to untreated, cleaned glass slides. The sections were deparaffinized with xylene and rehydrated through a graded series of aqueous alcohol solutions. The avidin-biotin peroxidase method as outlined by Hsu et al.<sup>34, 35</sup> was used to detect incorporation of BrdU into the DNA of replicating cells. All the reagents, with the exception of the primary antibody, were supplied by Vectastain Laboratories in kit form (Vectastain ABC kit) (mouse, chicken or rabbit IgG). Controls included: (1) substitution of phosphate buffered saline (PBS), biotinylated equine anti-rabbit IgG or normal serum for the primary antibody; (2) series in which serial dilutions of the primary antibody were incubated with serial sections (culture media dilutions run from 1:2 to 1:10,000 in steps).

Slides were incubated with heat-inactivated normal horse serum (Sigma Chemical Co.) blocked non-specific antibody binding. Sections were then incubated overnight at 4 $^{\circ}$  C with a primary mouse anti-BrdU antibody and the slides were washed with phosphate-buffered saline (PBS). The samples were then incubated for 30 minutes at 20 $^{\circ}$ C with a secondary antibody (biotinylated rabbit anti-mouse IgG that that was treated for rat cross-reactivity by affinity chromatography), washed in PBS, incubated for 30 minutes at 20 $^{\circ}$ C with a Vectastain ABC avidin-conjugated peroxidase reagent (Vector Labs). The samples were washed in PBS, and incubated 3-5 minutes with H<sub>2</sub>O<sub>2</sub> and diaminobenzidine (DAB) as the color-forming substrate.

### Selection of airway tissue for morphometry

Unique airway dissection techniques were used to isolate a minimum of 12 terminal bronchioles from the right cranial lung lobe of each rat. In brief, the lobe was dehydrated and embedded in paraffin. Then, beginning at the lobar bronchus, the intrapulmonary airways and accompanying parenchyma were split down the long axis of the largest daughter branch or down the axial pathway of the primary airway. An attempt was made to expose as many minor daughter side-branches as possible. Sections that were 30  $\mu$ m thick were cut for BrdU staining. Twelve to twenty terminal bronchioles were visible in each section and each one was given a sequential number beginning at the hilus and ending at the pleural surface. Four terminal bronchioles were selected from each section using stratified sampling with a random start within

the first interval (i.e., if there were 16 terminal bronchioles, then the random number was selected from 1-4 for the first selection and each subsequent selection was the initial number +4). This method of sampling provided for unbiased selection of terminal bronchioles independent of size or location.

## ***Measurements Related to Respiratory Infection***

### **Fc Receptor Binding**

Macrophages ( $10^5$  cells) isolated from lavage fluid, were incubated in Tissue-Tek chambers in Hank's balanced salt solution (HBSS) with  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  for 60 min. Nonadherent cells were removed by washing with HBSS. For assay of Fc receptor binding, adherent macrophages were incubated with rat anti-sheep red blood cell antibody,<sup>36,37</sup> excess antibody was removed by washing with HBSS, and the cells were incubated with sheep red blood cells (SRBC). Excess SRBC were removed by washing with HBSS, and the macrophages were examined using an inverted phase contrast microscope. The number of rosettes, or macrophages attached to 3 or more SRBC, was scored as positive. Control preparations, using macrophages, unactivated with antibody, were scored as described above to correct the number of rosettes observed for non-specific binding.

### **Respiratory Burst Activity**

Integrated superoxide production during respiratory burst activity after stimulation with opsonized zymosan (Sigma Chemical, St. Louis, MO) were measured in the presence and absence of superoxide dismutase (SOD) as follows. Lung lavage cells ( $2 \times 10^5$ ) were added to luminometer cuvettes (LKB Pharmacia) in 1 ml HHBS. Samples were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  for 90 min and non-adherent cells were removed by gentle washing. The medium was replaced with RPMI 1640 supplemented with 10 mM glucose. SOD-inhibitable superoxide production was determined by lucigenin-amplified chemiluminescence using an LKB-Pharmacia Model 1251 Luminometer. Chemiluminescence measurements were made beginning immediately after the addition of 200 mM bis N Methylacridinium Nitrate (lucigenin, Sigma), with or without stimulating agent (50 ng/mL opsonized zymosan). Measurements were continued until readings returned to near baseline levels (typically 30 min). Duplicate cuvettes

with SOD-treated samples were used to correct readings to yield SOD-inhibitable chemiluminescence readings.

### **Macrophage NO Production**

Macrophages were isolated from BAL by adherence. Non-adherent cells were removed with gentle washing and the medium was replaced with RPMI 1640 supplemented with 10 mM glucose. Macrophages were incubated overnight at 37°C in 5% CO<sub>2</sub>. Macrophage conditioned medium (50 µL) was analyzed for NO spectrophotometrically by the Griess reaction using a commercially available kit.

### ***In vivo physiological measurements***

#### **Nitric Oxide in Expired Breath**

Rats were gently restrained in a plastic tube with their heads exposed through a dental dam membrane that sealed around their necks. A small plastic cone was sealed around their heads forming a head-only chamber. NO-free air was metered into the head-only chamber and effluent air was sampled using a fast response chemiluminescent NO analyzer (Sievers, Inc, Palo Alto, CA). The instrument response was stored and integrated using a dedicated computer.

#### **Blood Pressure and Heart Rate**

Rats were placed into a warming chamber with their tails exposed. A blood pressure cuff and pressure transducer, designed for use on rodent tails, were placed on the tail and monitored using a calibrated recorder/integrator system (Gould, Inc.) until a reproducible heart pulse was obtained. The systolic blood pressure (SBP) was determined by automatically inflating the pressure cuff to a pressure sufficient to occlude the pulse. The cuff was slowly deflated and the resulting pressure/heart rate pattern recorded. The pressure at which the pulse reappeared was taken as the systolic blood pressure. Each measurement was repeated up to 5 times and averaged or until 3 valid pressure pulse curves were recorded with SBP that agreed to within 10%.

## ***Statistical Analyses***

The morphometric analyses evaluate sample mean values to within  $\pm 10\%$  using a 95% confidence interval with the Student t distribution. For other endpoints, data were analyzed using one-way or two way analyses of variance (ANOVA). Tukey multiple comparison tests were then used to assess significant differences between exposure groups.<sup>38</sup> Data transforms or nonparametric statistical methods were used to analyze variables that were not normally distributed and for comparing groups that had unequal variances. Two-tailed tests with  $\alpha \leq 0.05$  were used to establish statistical significance.

## ***Study Design***

This study represented a collaborative effort between researchers at CARB, U.C. Davis and U.C. Irvine. The goal was to elucidate the PM effects on the cardiopulmonary system. Coordination of the project was maintained by quarterly video- or teleconferences between the CARB Project Manager and the UCI/UCD research groups. These regular meetings allowed us to examine and discuss progress and results on an ongoing basis and to develop and modify the research plan to take advantage of the results of studies on both campuses in planning subsequent exposures. Thus, critical findings from one experiment guided the development of subsequent phases of the study.

UCD developed a sequence of 5 research blocks to evaluate the dependence of biological responses on particle concentration and age of the animals. Target concentrations were designated as “high” ( $300 \mu\text{g}/\text{m}^3$  AMN,  $150 \mu\text{g}/\text{m}^3$  EC); “intermediate” ( $150 \mu\text{g}/\text{m}^3$  AMN,  $100 \mu\text{g}/\text{m}^3$  EC) and “low” ( $75 \mu\text{g}/\text{m}^3$  AMN;  $50 \mu\text{g}/\text{m}^3$  EC). (See the previous discussion of how these concentrations were selected). UCD performed studies with juvenile, young adult and senescent animals.

The studies at UCI complemented the UCD studies by evaluating the role of particle size on specific biological responses. The UCI budget was not sufficient to replicate all of the exposure groups and endpoints performed under the UCD program. Thus the total number of studies was fewer and only young adult and senescent rats were exposed and examined. An advantage, however, of the collaboration was that samples could be shared. Thus, fixed lung samples from the UCI studies were available to UCD for immunocytochemical analysis for

BrdU uptake, and cells recovered from bronchoalveolar lavage from the UCD studies with senescent and juvenile rats were sent to UCI for respiratory burst analyses.

The UCI studies targeted 3 particle size ranges, where  $d_p$  represents mass median aerodynamic particle diameter in nm: “ultrafine” ( $200 \leq d_p$ ); “fine” ( $500 \leq d_p \leq 1000$ ) and “coarse” ( $2000 \leq d_p \leq 4000$ ). Studies were performed with both young adult and senescent rats. From the outset, it was recognized that the terms ultrafine, fine and coarse, as used in this study were to be operationally defined. For the purpose of this research, the definitions of UF, F and C were modified from the conventional definitions. This was done, for the coarse particles, to take into account differences between rats and humans with respect to regional deposition of particles as a function of size. Thus, since particles larger than 3 to 4  $\mu\text{m}$  do not penetrate beyond the rat’s nose to any significant degree, it would not make sense to use the conventional definition of “coarse” (ambient particles  $2500 \leq d_p \leq 10000$  nm). For the UF particles, we were unable to hold the composition of the particles constant when we reduced the size below 200 nm. Therefore, for this study we designated ultrafine particles to be those with  $d_p \leq 200$  nm. The composition constraint was necessary so that the dependence of biological response on particle size could be examined in the absence of the influence of differences in composition or concentration.

To test the key hypotheses, four exposure experiments were conducted over the three year period of this contract. In the first two exposure experiments, the effects of a particle mixture in the fine particle size range containing AMN and EC in the presence and absence of  $\text{O}_3$  were tested in young adult and senescent (geriatric) rats, respectively. These experiments showed that even at relatively high concentrations only the mixture of particles plus  $\text{O}_3$  produced group mean effects that were significantly different from controls. We therefore, in the third experiment, exposed young adult rats to ultrafine, fine and coarse particles plus  $\text{O}_3$  to determine whether the effects of particle exposures were particle size dependent. The comparison of young and senescent rats in the first two exposures showed that senescent rats were more sensitive than were the younger rats to the effects of the particle plus  $\text{O}_3$  mixture. We therefore tested the particle size-dependence of particle plus  $\text{O}_3$  effects in senescent rats at a lower concentration in the fourth exposure. This experiment allowed us to compare the dose response of senescent and younger rats to the fine particle size range and to compare the responses of the senescent rats at

all three particle sizes. In these studies, rats were exposed at high concentration to: (a) purified air; (b) 0.2 ppm O<sub>3</sub>; PM (300 µg/m<sup>3</sup> AMN, 150 µg/m<sup>3</sup> EC); and (d) PM + O<sub>3</sub>. The low concentration exposures were to: (a) purified air; (b) ultrafine; (c) fine and (d) coarse particles at the intermediate concentration level (150 µg/m<sup>3</sup> AMN, 75 µg/m<sup>3</sup> EC, 0.2 ppm O<sub>3</sub>). Note that all of the particle atmospheres contained 0.2 ppm O<sub>3</sub>.

## **Results**

### ***Initial Considerations***

#### **Atmosphere Constituents**

Ammonium nitrate (AMN) and elemental carbon (EC) were the particle components selected for this study. These components represent substantial fractions of the ambient fine particles, but are also constituents of the coarse particle size mode. Acid nitrate compounds are formed in the atmosphere by the heterogeneous nucleation and oxidation of NO<sub>2</sub>. The resulting nitric acid can be neutralized with atmospheric ammonia to form ammonium nitrate particles or can react with basic metal oxides on the surface of larger particles to form nitrates.

Photochemical processes are important, not only for the formation of atmospheric acids, but also for formation of ozone (O<sub>3</sub>) in ambient atmospheres, especially in southern California. Thus O<sub>3</sub> concentrations are often strongly correlated with nitric acid concentrations.

#### **Integrity of Air Samples**

Preliminary studies were performed to determine whether AMN might volatilize during collection to form ammonia and nitric acid vapor and thus yield erroneous estimates of AMN concentrations in the exposure atmosphere. This phenomenon had been noted in ambient air measurements of nitrates and was especially problematical during periods of high temperature. AMN was generated under conditions identical to those under which rat exposures were planned. The sample collection system was also identical, with the following difference. A nylon (Nylasorb®) filter was placed in tandem behind the PallFlex filter. Nylon filters have a high affinity for nitric acid vapor and if AMN did volatilize during the collection, nitrate should be

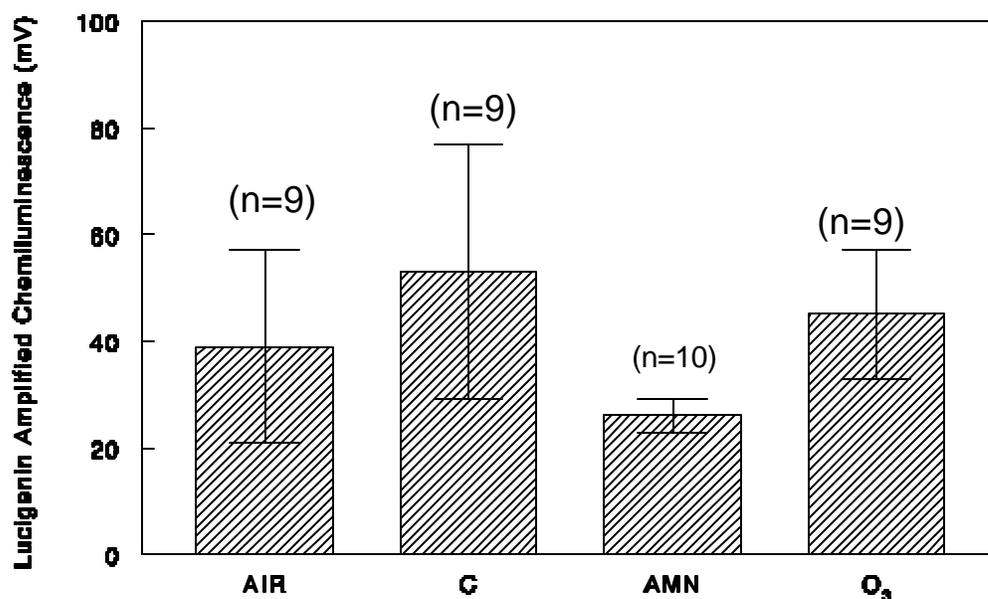
detected on the nylon filter. We ran several samples. The amounts of nitrate on the nylon backup filter were at or below the detection limit of the analysis method ( $3 \mu\text{g}/\text{m}^3$ ). Thus, we concluded that under the conditions of this study, there was no significant error due to estimating nitrate concentrations from the nitrate measured on the PallFlex filter.

The method used to generate ozone (corona discharge) has been reported to produce ultrafine particles. To minimize particle formation we generated ozone using oxygen rather than air to eliminate nitrogen and reduce the flow of gas needed to supply ozone for the experiments. We measured the numbers of ultrafine particles in a chamber with 0.2 ppm ozone to which no particles were added. There was a small addition of particles ( $\sim 34$  per liter) to the chamber air when ozone was introduced, however this is unlikely to influence our study since we deliberately added more than  $10^6$  particles per liter during the exposures.

### **Exposure Parameters**

Based on the ambient atmosphere data described earlier in this report, it was decided to initiate the studies with AMN + EC mixtures in the presence and absence of  $\text{O}_3$ . Before beginning this study, an experiment had been performed in which rats were exposed to atmospheres containing the individual components (EC,  $\text{O}_3$  and AMN). This preliminary data indicated that exposure to EC,  $\text{O}_3$  or to AMN alone did not induce significant changes in respiratory burst activity, permeability or epithelial injury. The respiratory burst activity results from that study are shown in Figure 1. In that same study mixtures of particles with ozone did evoke significant changes. Therefore, even though the exposure conditions in the prior study were not identical with those to be used in this study, we decided that the best use of our limited resources would be to focus on particle mixtures.

**Figure 1 Respiratory burst activity in macrophages from rats exposed to ammonium nitrate, carbon and ozone.**



**Experiment 1. Effects of Mixtures of Particles in the PM 2.5 size range and Ozone.**

Young adult Fischer 344 rats (8 to 10 weeks) were purchased from Simonsen Laboratories. The rats were shipped in filter-equipped boxes and were housed in laminar flow isolation units supplied with filtered air. They were allowed access to food and water *ad lib*. The rats were randomly assigned to treatment groups and nose-only exposed for 6 hours to one of four atmospheres: (1) purified air; (2) O<sub>3</sub>; (3) AMN + EC; and (4) AMN + EC + O<sub>3</sub>. The experimental design and endpoints evaluated are summarized in Table 3 and the concentrations of components and particle sizes measured during the exposures are summarized in Table 4. The atmospheres were well controlled and the size/concentration values met the stated specifications. On average the measured values were ± 20% of their targets.

Ten animals from each group were exposed for histopathology endpoints and an additional 15 from each group were exposed for bronchoalveolar lavage-related endpoints. Between exposures, rats were returned to the purified air-barrier environment and given access to clean water and dry laboratory chow *ad lib*.

**Table 3 Atmospheres and Endpoints Evaluated in Experiment 1**

Group	Atmosphere	Conc.	Exposure	Endpoints
1	Purified Air		6 hour	Inflammatory Response Epithelial Permeability  Fc Receptor Binding  Respiratory Burst Activity
2	O <sub>3</sub>	0.2 ppm	"	
3	EC +	150 µg/m <sup>3</sup>	"	
	AMN	300 µg/m <sup>3</sup>		
4	EC	150 µg/m <sup>3</sup>	"	
	+			
	AMN			
	+	300 µg/m <sup>3</sup>		
	O <sub>3</sub>			
		0.2 ppm		

**Table 4 Atmosphere Component Concentrations and Particle Sizes Measured During Experiment 1 Exposures**

Group	Atmosphere	Particle Size <sup>a</sup> (MMAD; GSD)	Actual Concentration Mean, $\mu\text{g}/\text{m}^3 \pm \text{SD}$	Relative Humidity <sup>b</sup> (%)
1	Purified Air	NA <sup>c</sup>	NA	59.1 $\pm$ 3.0
2	O <sub>3</sub>	NA	0.209 $\pm$ 0.019	
3	Carbon + AMN	0.69 $\mu\text{m}$ ; 2.5	210 $\pm$ 15 271 $\pm$ 20	
4	Carbon + AMN + O <sub>3</sub>	0.68 $\mu\text{m}$ ; 2.8	194 $\pm$ 21 285 $\pm$ 20 0.206 $\pm$ 0.030	

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<sup>a</sup> The particles size values for Carbon represent pooled averages of 3 samples each.

<sup>b</sup> Relative humidity was measured in the purified air chamber.

<sup>c</sup> NA – Not Applicable

## **Observed biological responses**

### **Inflammatory Response**

The presence of inflammatory cells (macrophages, polymorphonuclear leucocytes and lymphocytes) in the lungs of exposed rats was examined as an indicator of inflammatory response. Cells recovered from rats by bronchoalveolar lavage had, on the average, viabilities greater than 94%. There was a small reduction in the total numbers of viable cells recovered from the group exposed to the particle mixture + O<sub>3</sub>. The percentages of macrophages and lymphocytes recovered in BAL averaged  $97 \pm 1$  and  $2.2 \pm 0.5$ , respectively, and were not significantly different among the exposure groups (Table 5).

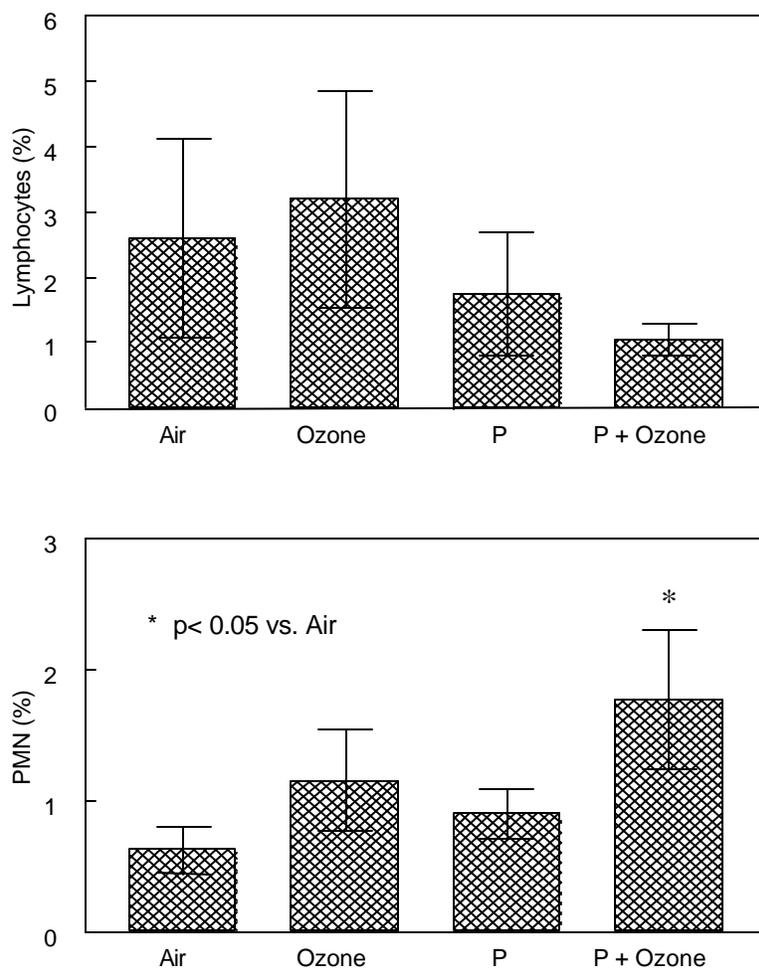
**Table 5 Inflammatory Cells in BAL of Rats Exposed to Ammonium Nitrate and Carbon Particles (P) in the Presence or Absence of Ozone (O<sub>3</sub>). N = 15 per group.**

(Number of Cells; Mean $\pm$ SE)				
Atmosphere	Total Cell Number ( $\times 10^{-6}$ )	Macrophages ( $\times 10^{-6}$ )	PMNs	Lymphocytes
<b>PURIFIED AIR</b>	$3.8 \pm 0.3$	$3.7 \pm 0.3$	$23900 \pm 1900$	$98800 \pm 8000$
O <sub>3</sub>	$3.6 \pm 0.3$	$3.5 \pm 0.3$	$41800 \pm 3500$	$115000 \pm 9600$
P	$3.7 \pm 0.2$	$3.6 \pm 0.2$	$33700 \pm 1800$	$65000 \pm 3500$
P + O <sub>3</sub>	$3.4 \pm 0.3$	$3.3 \pm 0.3$	$70400 \pm 6200$	$35700 \pm 3200$

The percent of PMN's and lymphocytes were analyzed using a 2-way ANOVA. The factors were the presence or absence of ozone and the presence or absence of particles. The data are shown graphically in Figure 2. The numbers of lymphocytes were reduced in the lavage fluid of rats exposed to particle-containing atmospheres, but the reduction only approached statistical significance ( $p=0.23$ ). There was no main effect of ozone, nor was there a significant interaction between ozone and particles. There was a nearly significant main effect of ozone on

the percent of polymorphonuclear leucocytes (PMNs) recovered from the bronchoalveolar lavage ( $p=0.06$ ). There were greater numbers of PMN's in lavage fluid from rats exposed to ozone-containing atmospheres. The percentage of PMN's was increased, compared to controls 3-fold in rats exposed to the PM + O<sub>3</sub> mixture, which was statistically significant ( $p<0.05$ ). The other group means were not significantly different from controls.

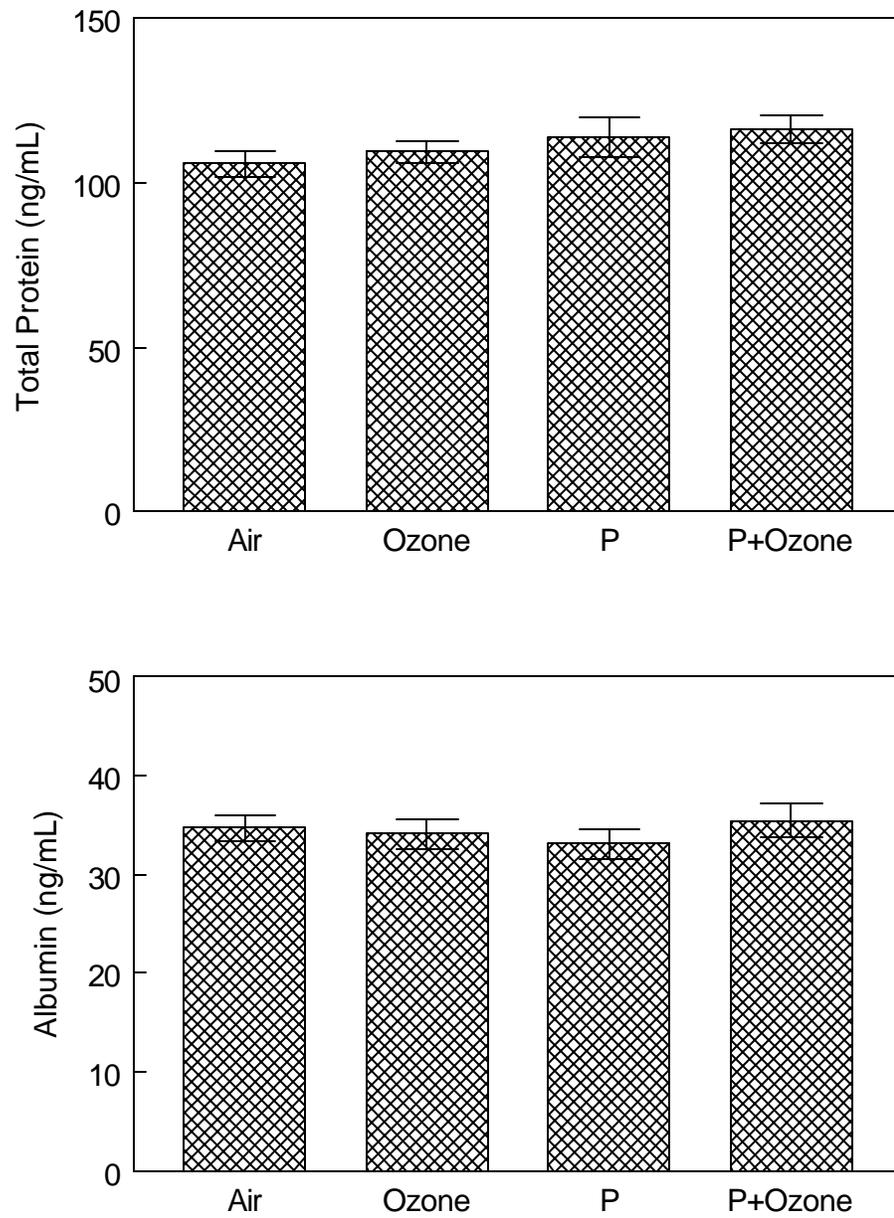
**Figure 2 Lymphocytes and Polymorphonuclear Cells Recovered from Bronchoalveolar Lavage of Rats (N = 15 per group) Exposed to Ammonium Nitrate and Carbon Particles (P) in the Presence or Absence of Ozone (O<sub>3</sub>)**



## **Epithelial Permeability**

Epithelial permeability is a measure of lung “leakiness.” When the epithelial barrier is disrupted, proteins from circulating fluids can enter the alveolar spaces and can be recovered in BAL fluids. We measured both total protein and albumin (a protein that is not elaborated in the lung but is a major blood serum constituent). Increased protein would be indicative of an adverse effect. Total protein ranged from  $106 \pm 4$  ng/mL in the control group to  $116 \pm 4$  ng/mL in the group exposed to particles + O<sub>3</sub> (P+O<sub>3</sub>). A 2-way ANOVA was performed to test the 1-tailed hypothesis of an atmosphere-related increase in protein or albumin. There was a significant ( $p=0.05$ ) main effect of particles on total protein (i.e increased protein in BAL from particle-exposed rats). The group mean values of albumin ranged from 33 to 35 ng/mL and there were no atmosphere-related significant differences. The data are shown in Figure 3.

**Figure 3 Concentrations of Total Protein and Albumin in Bronchoalveolar Lavage of Rats (N = 12 per group) Exposed to Ammonium Nitrate and Carbon Particles (P) in the presence or Absence of Ozone (O<sub>3</sub>)**



## Macrophage Function Assays

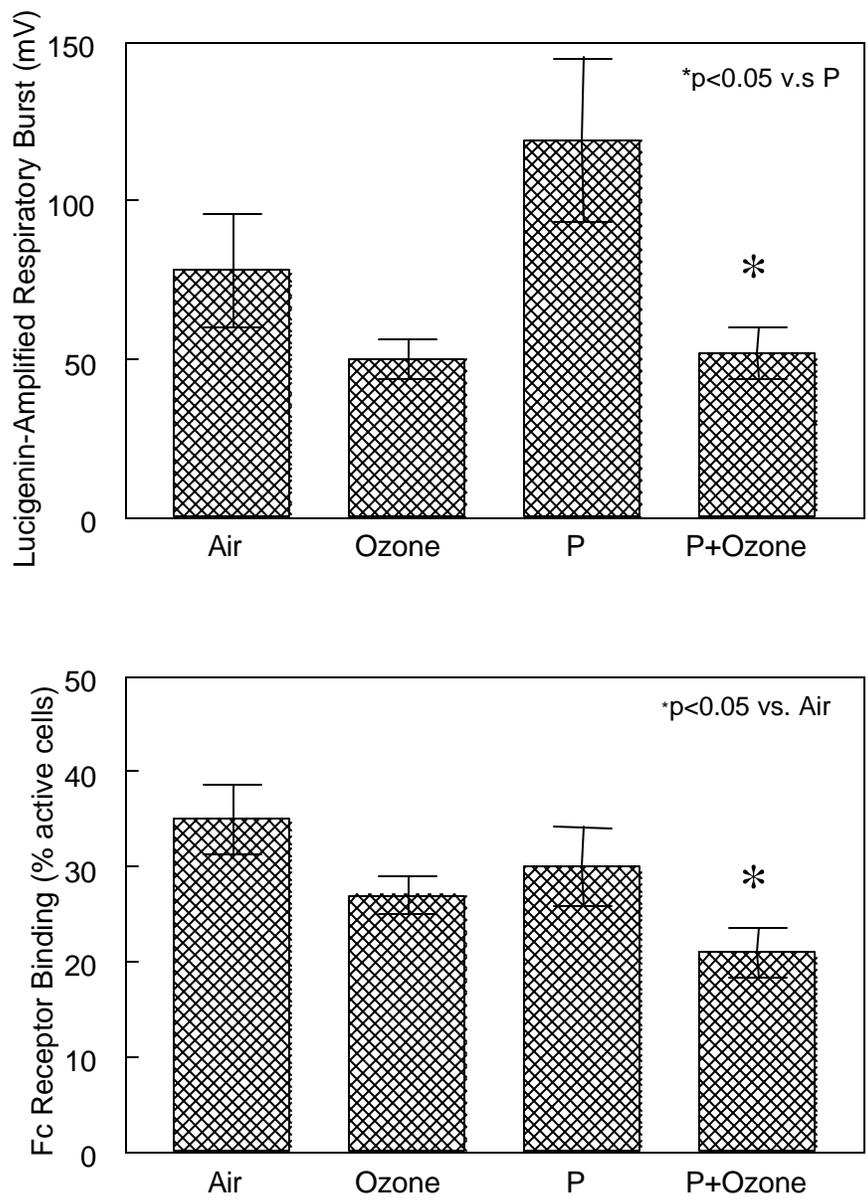
Macrophages represented between 95% and 98% of the recovered cells. Data from Respiratory Burst and Fc-Receptor binding assays on macrophages are shown in Figure 4. Macrophages respond to pathogens and particles by phagocytizing them and destroying or ‘sterilizing’ them by a non-specific mechanism known as respiratory burst in which free radicals are elaborated. Adverse effects of foreign particles could either increase or decrease this defensive response<sup>d</sup>. Respiratory burst data were therefore analyzed by 2-way analysis of variance using a two-tailed comparison for main effects of ozone and particles, as well as for an interaction effect. There was a significant main effect of ozone on respiratory burst activity. Respiratory burst activity was significantly depressed in macrophages from rats exposed to ozone-containing atmospheres ( $p = 0.009$ ). The main effect of particles was not significant ( $p > 0.2$ ) and there was no statistical interaction. Respiratory burst activity in macrophages from rats exposed to the particle + O<sub>3</sub> mixture was significantly depressed vs. macrophages from rats exposed to particles alone ( $p < 0.05$ ).

Macrophages also exhibit antibody-directed activity that facilitates their attack on antigenic or pathogenic materials. This activity is mediated by receptors (FcR) on the macrophage’s cell membrane to the Fc portion of antibodies. A decrease in Fc-binding capacity would be an indicator of an adverse effect. FcR binding data were analyzed by 2-way ANOVA and the hypothesis of atmosphere-related decreases FcR binding was evaluated as a 1-tailed test. There were significant main effects of both Particles ( $p=0.05$ ) and Ozone ( $p=0.01$ ). Macrophages from rats exposed to atmospheres containing ozone or particles had depressed FcR-binding capacity. Macrophages from rats exposed to the P + O<sub>3</sub> mixture exhibited significantly depressed ( $p < 0.05$ ) FcR binding capacity.

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<sup>d</sup> Depressed respiratory burst could imply that macrophages would be unable to kill and digest pathogens. Increased respiratory burst could lead to the local release of free radicals and cytotoxic compounds that could damage lung tissue.

**Figure 4 Respiratory Burst Activity and Fc-Receptor Binding Capacity of Macrophages in Bronchoalveolar Lavage of Rats (N = 12 per group) Exposed to Ammonium Nitrate and Carbon Particles (P) in the Presence or Absence of Ozone (O<sub>3</sub>)**



## ***Experiment 2. Comparison of Effects of PM 2.5 Particles in Young Adult and Senescent Rats***

Experiment 2 tested the second of the study hypotheses, i.e that senescent (geriatric) rats would be more susceptible to the adverse effects of inhaled particles than would be younger rats. Senescent (22 to 24 month old) Fischer 344 male rats (10) were exposed to a mixture of ozone (0.2 ppm), ammonium nitrate (AMN, 300  $\mu\text{g}/\text{m}^3$ ) and elemental carbon (EC, 200  $\mu\text{g}/\text{m}^3$ ). A second group of 10 rats were exposed to purified air (controls). The exposures were nose-only for a single 6 hour period. The exposure was coordinated with Dr. Pinkerton (UCD). Four animals from each group were euthanized and the lungs micro-dissected. An aliquot of the parenchymal tissue was frozen and analyzed at UCD for glutathione. Six animals from each group were surgically implanted with Alzet mini-osmotic pumps that released a measured amount of 5-bromodeoxyuridine (BrdU) over a three-day period. The animals were euthanized 24 hour post-exposure. The trachea was surgically isolated, the right lung was clamped and the lungs were lavaged with balanced salt solution to recover cells and proteins.

The atmospheres and endpoints evaluated are summarized in Table 6.

**Table 6 Atmospheres and Endpoints Evaluated in Experiment 2**

Group	Atmosphere	Conc.	Exposure	Endpoints
1	Purified Air		6 hour	Inflammatory Response
2	EC + AMN + O <sub>3</sub>	150 µg/m <sup>3</sup>  300 µg/m <sup>3</sup>  0.2 ppm	"	Cell Injury and Replication  Fc Receptor Binding  Respiratory Burst Activity

The atmospheres were generally well controlled and the average concentrations of the ozone and particulate species were within 10% of the target values (Table 7). The values agreed well with the concentrations achieved in Experiment 1 (also shown for the young rats in Table 7) so that, in addition to evaluating the responses of the senescent animals alone, the results could also be used to compare the responses of the old rats with those of the young adult rats discussed previously.

**Table 7 Atmosphere Component Concentrations and Particle Sizes Measured During Experiment 2 Exposures**

Group	Atmosphere	Particle Size <sup>e</sup> (MMAD; GSD)	Actual Concentration Mean, µg/m <sup>3</sup> ± SD	Relative Humidity <sup>f</sup> (%)
Senescent	Purified Air	NA <sup>g</sup>	NA	60 ± 3.0
Senescent	Carbon + AMN + O <sub>3</sub>	0.69 µm; 2.5	213 ± 7 291 ± 40 0.21 ± 0.01	
Young	Purified Air	NA	NA	59 ± 3.0
Young	Carbon + AMN + O <sub>3</sub>	0.68 µm; 2.8	194 ± 21 285 ± 20 0.21 ± 0.03	

<sup>e</sup> The particles size values for Carbon represent pooled averages of 3 samples each.

<sup>f</sup> Relative humidity was measured in the purified air chamber.

<sup>g</sup> NA – Not Applicable

## **Inflammatory Response**

The numbers of inflammatory cells (macrophages, polymorphonuclear leucocytes and lymphocytes) in the lungs of the exposed and control rats determined. The viability of cells recovered from rats by bronchoalveolar lavage ranged between 81 and 96% with a mean  $\pm$  SD of  $89 \pm 2.4\%$  for the control group and  $92 \pm 1.8$  for the exposed group. The total numbers of viable cells recovered from the senescent rats were less than that for the young rats (both the control group and the group exposed to the particle mixture + O<sub>3</sub>), however the values can not be compared because the lavage protocols were not identical (i.e. the right lung of the senescent rats was tied off and not lavaged). The percentages of inflammatory cells can, however, be compared. These are shown in Table 8 for the senescent rats exposed in Experiment 2 and the young rats exposed in Experiment 1. There were no exposure-related differences in numbers of PMN's or lymphocytes in senescent rats exposed to purified air of the P + O<sub>3</sub> mixture. There was a significant ( $p < 0.01$ ) difference in the percentage of inflammatory cells in the lavage fluid from senescent rats compared to the young adult rats. These findings may suggest that the level of chronic lung inflammation is increased as a consequence of aging. If this is paralleled in aging humans it might partially explain the greater susceptibility of older individuals to the adverse effects of air pollution.

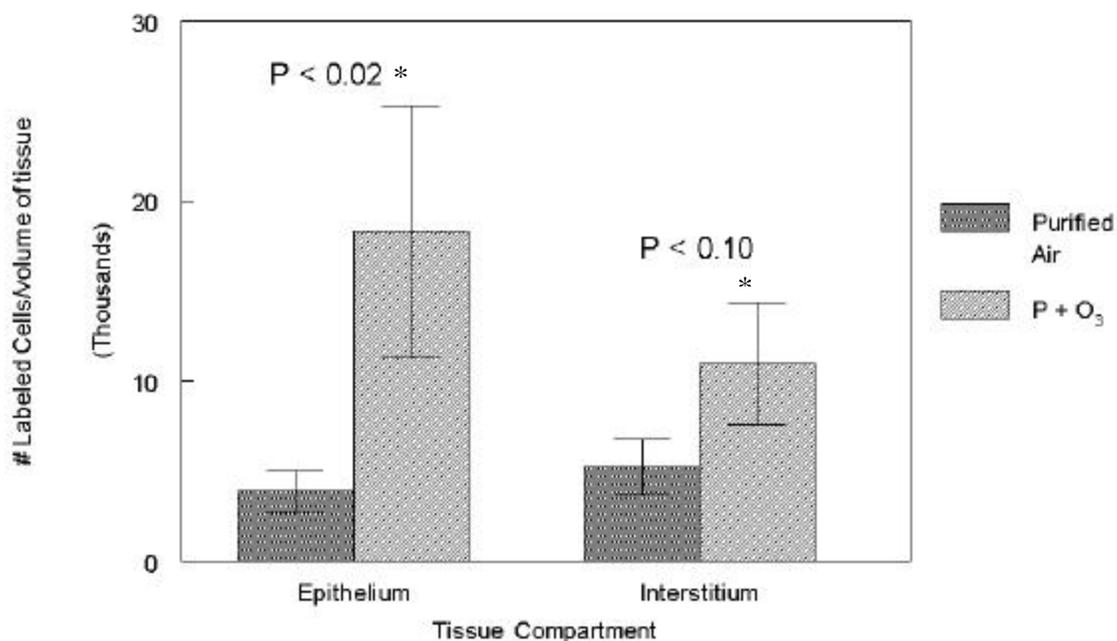
**Table 8 Inflammatory Cells in BAL of Senescent (N=6 per group) and Young Adult Rats (N=15 per group) Exposed to Ammonium Nitrate and Carbon Particles (P) in the Presence Ozone (O<sub>3</sub>).**

Group	Total Cell Number (x 10 <sup>-6</sup> )	Macrophages (%)	PMNs (%)	Lymphocytes (%)
<b>SENESCENT, PURIFIED AIR</b>	1.3 ± 0.2	94.4 ± 1.9	4.1 ± 0.9	1.4 ± 1.1
Senescent, P + O <sub>3</sub>	1.1 ± 0.1	94.6 ± 1.1	3.8 ± 1.2	1.5 ± 0.2
Young, Purified Air	3.8 ± 0.3	97 ± 1.5	0.6 ± 0.2	2.6 ± 1.5
Young, P + O <sub>3</sub>	3.4 ± 0.3	97 ± 0.2	1.8 ± 0.7	1.1 ± 0.3

### Cell Turnover and Replication

Histological sections were analyzed for incorporation of bromodeoxyuridine (BrdU) into the nuclei of cells undergoing replication. Although there is a normal background of cell turnover and replacement in both epithelial and interstitial lung tissues, tissue injury associated with particle deposition in the lung results in removal of damaged or killed cells followed by a wave of replication to replace those cells and to protect denuded basement membranes. As shown in Figure 5, exposure of senescent rats to particles resulted in significantly increased cell turnover compared to rats exposed to purified air. The data were analyzed by 2-way ANOVA and significance of group mean differences were tested using a Tukey multiple comparison test. Cell turnover in epithelial tissue was greater than that in interstitial tissue, but this difference was not statistically significant.

**Figure 5 Cell Turnover in Epithelial and Interstitial Lung Tissue**



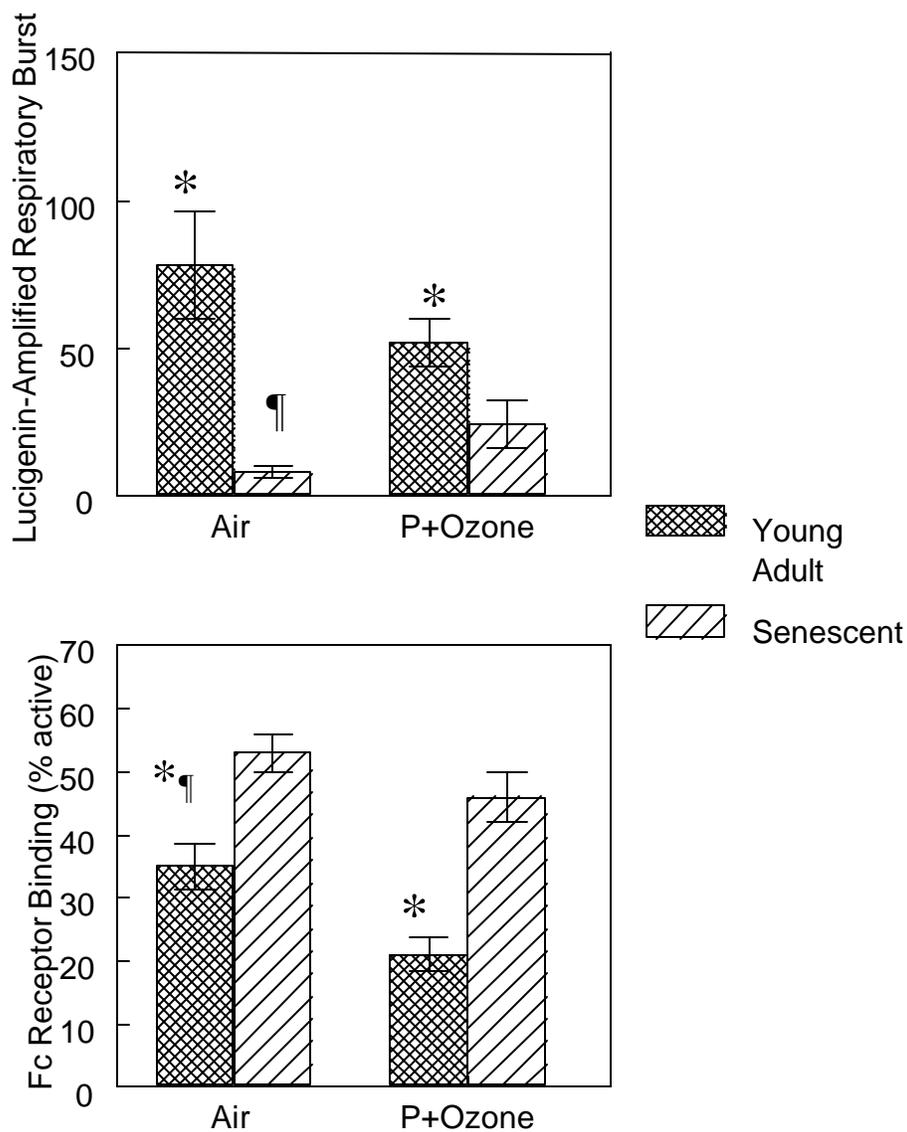
### **Macrophage Function Assays**

Data from respiratory burst and Fc receptor binding assays on macrophages from senescent rats exposed to purified air or the particle + ozone mixture are shown in Figure 6. As discussed earlier, respiratory burst activity represents a non-specific mechanism of maintaining lung sterility. Respiratory burst data for senescent rats were analyzed by a 2-tailed, one-way analysis of variance. Respiratory burst activity was increased in macrophages from senescent rats exposed to the particle + ozone atmosphere ( $p = 0.08$ ). In contrast, respiratory burst activity in macrophages from young adult rats exposed to the particle + O<sub>3</sub> mixture was depressed vs. macrophages from rats exposed to purified air. A two-way ANOVA contrasting the factors of age and exposure confirmed that the macrophages from senescent rats were less capable of producing an oxidative burst. In addition,

senescent rat's macrophage increased the production of free radicals when challenged with the particle + ozone atmosphere while younger rat's macrophages were depressed in their free radical generating capacity. This increase in activity might be consistent with the increased level of inflammation found in the lungs of the senescent rats, as indicated by the larger percentage of PMN's in BAL fluid.

FcR binding data for senescent and young adult rats were analyzed by 2-way ANOVA and the hypothesis of atmosphere-related decreases FcR binding was evaluated as a 1-tailed test. Macrophages from rats exposed to atmospheres containing ozone and particles had depressed FcR-binding capacity (main effect of exposure,  $p=0.01$ ). Macrophages from senescent rats exhibited significantly greater ( $p<0.001$ ) FcR binding capacity than did the younger adult rats. This could suggest that macrophages from senescent rats were in a more activated state than were macrophages from younger rats.

**Figure 6 Respiratory Burst Activity and Fc Receptor Binding Capacity of Macrophages in Bronchoalveolar Lavage of Senescent (N = 6 per group) and Young Adult (N=15) Rats Exposed to Either Purified Air or Ammonium Nitrate and Carbon Particles (P) in the Presence of Ozone (O<sub>3</sub>) (\* p $\leq$ 0.05 Young vs. Senescent; ¶ p $\leq$  0.05 Air vs. P+Ozone)**



### **Experiment 3. Dependence of Toxicity on Particle Size in Young Adult Rats**

The objective of this experiment was to test the hypothesis that there exists a relationship between particle size and biological responses. To test this hypothesis an inertial particle size separator was constructed and tested. The design parameters were set to enable us to perform simultaneous exposures to purified air and particles in three distinct size ranges. It was important that the relative concentrations of the particle components (AMN and EC) remained constant across the exposure groups. It was also important that the total PM concentration was maintained constant across the exposure groups. The development and testing of this system required a major fraction of the effort expended during the second year of this research.

Experiment 1 had confirmed that the EC+ABN+O<sub>3</sub> mixture was generally more biologically active than PM alone or O<sub>3</sub> alone. This mixture is representative of ambient fine particle exposures in California (Chow et al., 1992), and during episodes people are exposed to EC, AMN and O<sub>3</sub> at, or near, the same time. This mixture was therefore used in the experiments described below.

In parallel with the effort to build and test the particle generating system, we also developed two physiologically based endpoints. With the help of Dr. Steve George, we evaluated a method for measuring NO in the expired breath of rats. NO has been suggested as a useful biomarker for lung inflammation. As indicated by exposure-induced increased numbers of inflammatory cells in BAL, inflammatory processes and free radical production could be important contributors to PM-induced lung injury. Measurements of NO in expired breath had been performed with human volunteers, but scaling the system down to match the breathing parameters of a rat was challenging.

Epidemiological studies had suggested that PM strongly affected individuals with pre-existing cardiac disease. We hypothesized that particle-induced inflammation could lead to the

production of elevated levels of reactive oxygen species that could have systemic effects, and that these systemic effects could be manifested by changes in hemodynamic parameters. We therefore undertook to determine heart rate, blood pressure and double product in unanesthetized rats before and after exposure to purified air or particle plus ozone mixtures. The study design and endpoints assessed are shown in Table 9.

**Table 9 Atmospheres and Endpoints Tested in Experiment 3**

Group	Atmosphere	Conc.	Exposure	UCI Endpoints
1	Purified Air		6 hour	Macrophage Fc Receptor Binding,  Respiratory Burst Activity,  Permeability  Blood Pressure  Heart Rate  Expired NO
2	“Ultrafine” Carbon + AMN + Ozone	0.2 $\mu\text{m} \leq d_p$  200 $\mu\text{g}/\text{m}^3$  300 $\mu\text{g}/\text{m}^3$ + 0.2 ppm	“	
3	“Fine” Carbon + AMN + O <sub>3</sub>	0.5 $\leq d_p \leq 1.0$  200 $\mu\text{g}/\text{m}^3$  300 $\mu\text{g}/\text{m}^3$  0.2 ppm	“	
4	“Coarse” Carbon + AMN + O <sub>3</sub>	1.5 $\leq d_p \leq 2.5$  200 $\mu\text{g}/\text{m}^3$  300 $\mu\text{g}/\text{m}^3$  0.2 ppm	“	

### **Exposure Parameters**

Healthy, young adult rats (8 to 10 week) were studied in experiment 3. Young adult rats were obtained from Simonsen Laboratories. Rats were shipped to our laboratory in filter-equipped boxes. The rats were housed in laminar flow isolation units supplied with filtered air, and allowed access to food and water *ad lib*. The rats were randomly assigned to treatment groups. The rats were nose-only exposed for 6 hours to one of four atmospheres: (1) purified air; (2) “ultrafine” PM + O<sub>3</sub>; (3) “Fine” PM + O<sub>3</sub>; and (4) “Coarse” PM + O<sub>3</sub>. In these experiments, the concentrations of the particulate species were comparable to the levels used in Experiments 1 and 2.

Ten animals from each group were processed for histopathology endpoints (tissue sent to UCD) and 12 from each group were assessed for macrophage and permeability-related endpoints. NO, heart rate and blood pressure were measured using the 10 histology animals immediately before euthanasia (18 hr post exposure). Rats were returned to the purified air-barrier environment after exposure and given access to clean water and dry laboratory chow *ad lib*. The concentrations of atmospheric constituents and particle size characteristics are summarized in Table 10. The atmospheres were well controlled and well matched to the target size and concentration values.

**Table 10 Concentrations and Particle Sizes of Experiment 3 Atmospheres****(Mean  $\pm$  SE)**

Group	Atmosphere	Particle Size MMAD ( $\mu\text{m}$ ); GSD	Actual Concentration	Relative Humidity
1	Purified Air	NA <sup>a</sup>	NA	59.88 $\pm$ 0.84
2	“ultrafine” Carbon + AMN + O <sub>3</sub>	0.21 $\mu\text{m}$ ; 3.0	253 $\pm$ 7 276 $\pm$ 25 0.21 $\pm$ 0.03	
3	“Fine” Carbon + AMN + O <sub>3</sub>	0.68 $\mu\text{m}$ ; 2.8	172 $\pm$ 14 288 $\pm$ 49 0.19 $\pm$ 0.03	
4	“Coarse” Carbon + AMN + O <sub>3</sub>	1.7 $\mu\text{m}$ ; 3.0	189 $\pm$ 26 263 $\pm$ 30 0.22 $\pm$ 0.02	

<sup>a</sup>Not Applicable

## **Exposure-related Biological Responses**

### **Cell Replication/BrdU Labeling**

Labeling of DNA in lung epithelial and interstitial cells undergoing replication at the end of the exposure was evaluated qualitatively at UCD. There were no apparent exposure-related differences and quantitative analysis was not pursued.

### **Macrophage Function**

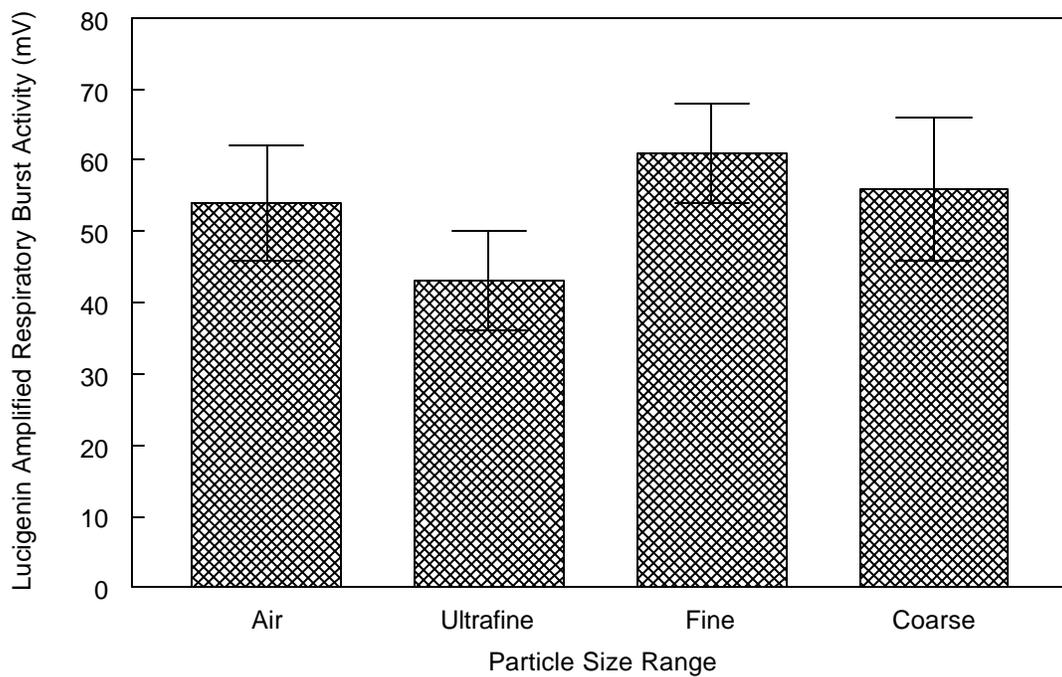
Greater than 90% of the cells recovered from these healthy adult rats by bronchoalveolar lavage were viable. Airway inflammation was evaluated as changes in the percentages of PMN's and lymphocytes recovered from BAL. There were no statistically significant exposure-related increases in numbers or percentages of lymphocytes or polymorphonuclear (PMN) cells and macrophages represented between 95% and 100% of the recovered cells. The data were analyzed by 1-tailed, one-way ANOVA. Although %PMN in BAL from rats exposed to ultrafine PM were more than 2x that in BAL from air-exposed rats, there were no statistically significant ( $p > 0.2$ ) particle exposure-related effects on inflammatory (PMN) cell counts (Table 11).

**Table 11 Cells Recovered in Bronchoalveolar Lavage Fluid From Young Adult Rats Exposed to Particles in the Ultrafine, Fine and Coarse Size Ranges.**

Group	Percent $\pm$ SE		
	Macrophages	PMNs	Lymphocytes
Air	95.6 $\pm$ 1.0	1.7 $\pm$ 1.3	2.5 $\pm$ 0.8
Ultrafine	92.0 $\pm$ 1.6	3.8 $\pm$ 1.2	3.1 $\pm$ 1.0
Fine	94.6 $\pm$ 0.4	0.14 $\pm$ 0.03	4.2 $\pm$ 1.4
Coarse	93.1 $\pm$ 0.4	0.09 $\pm$ 0.01	4.1 $\pm$ 1.0

The production of superoxide during respiratory burst activity following stimulation with PMA was measured using lucigenin-amplified luminometry. As can be seen in Figure 7, there were no significant group mean differences in respiratory burst activity from macrophages exposed to the particle plus ozone mixture compared to the group exposed to purified air. One-way ANOVA confirmed that there were no significant ( $p>0.5$ ) differences in respiratory burst activity.

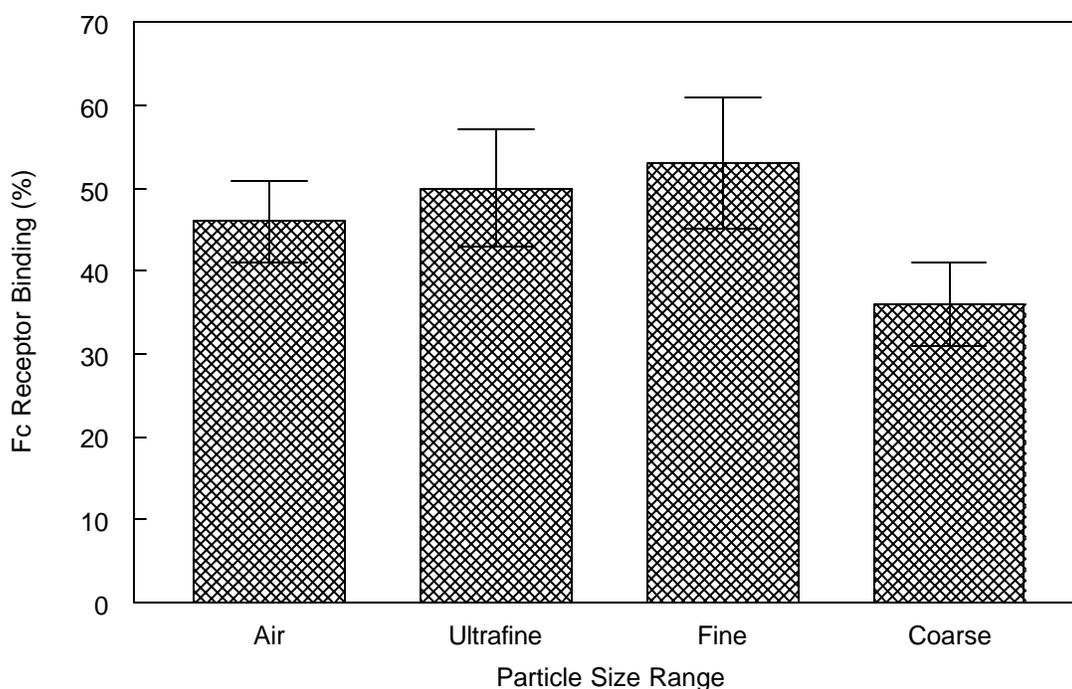
**Figure 7 Respiratory Burst Activity of Macrophages Recovered in BAL from Young Adult Rats After Exposures to Particle Plus Ozone Mixtures in Three Size Ranges**



## Fc Receptor Binding

The percentage of macrophages that exhibited Fc Receptor binding capacity were measured using the rosette method. The data were analyzed using a one-way, one-tailed ANOVA. Although macrophages from the group exposed to coarse particles had a lower percentage of Fc binding than those from macrophages exposed to other atmospheres, the differences did not achieve statistical significance ( $p=0.12$ ). The data are plotted in Figure 8.

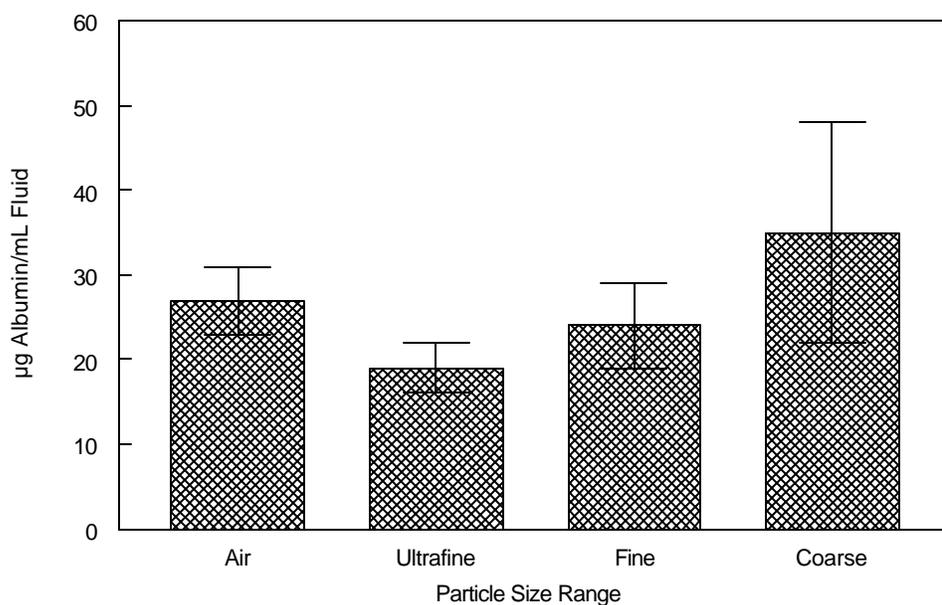
**Figure 8 Fc Receptor Binding in Macrophages Recovered in BAL from Young Adult Rats After Exposures to Particle Plus Ozone Mixtures in Three Size Ranges**



## Permeability

Bronchoalveolar lavage fluid (BAL) collected 12 hr post-exposure was analyzed for albumin as an indicator of epithelial disruption and increased mucosal permeability. The results, shown in Figure 9, exhibit no significant PM-related increases in BAL albumin concentrations.

**Figure 9 Albumin Concentrations in BAL from Young Adult Rats After Exposures to Particle Plus Ozone Mixtures in Three Size Ranges**

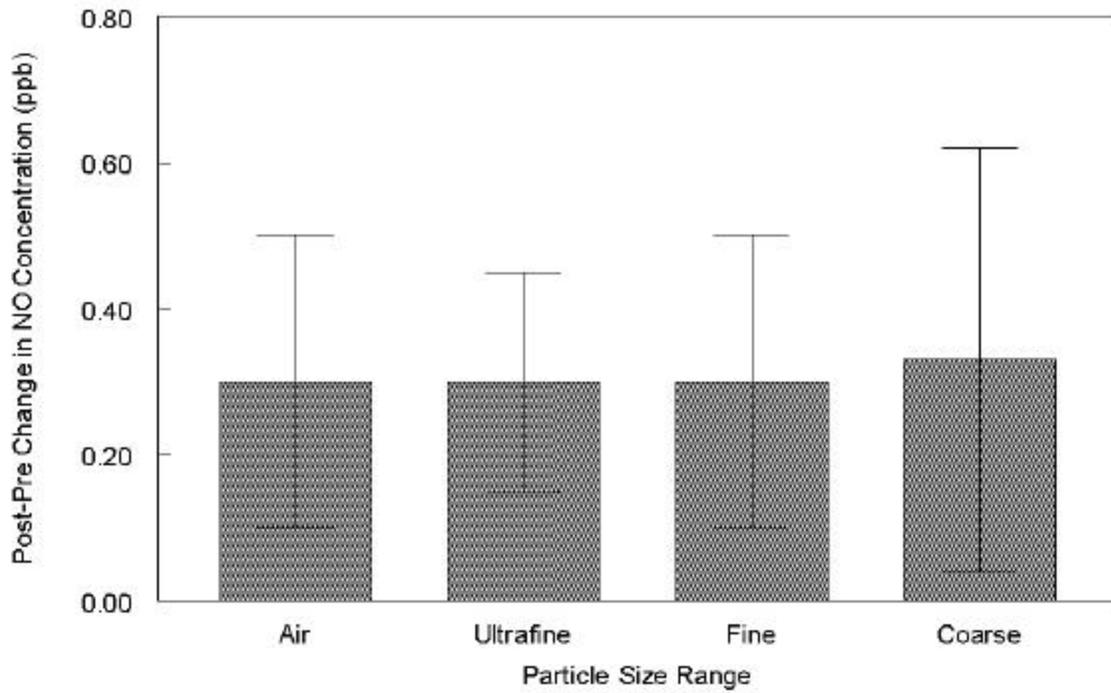


## Physiological Endpoints

Measurements of NO in expired breath, blood pressure and heart rate were made in these rats immediately after exposure and 24 hours after exposure. The normal fluctuations of NO in room air significantly increased the noise level of the measurements. Most of the measurements were not distinguishable above the background. For those measurements that were determinable, the group mean pre-exposure to post-exposure differences were not significantly different.

(Figure 10). The average concentration of NO in expired breath was  $3 \pm 2$  ppb.

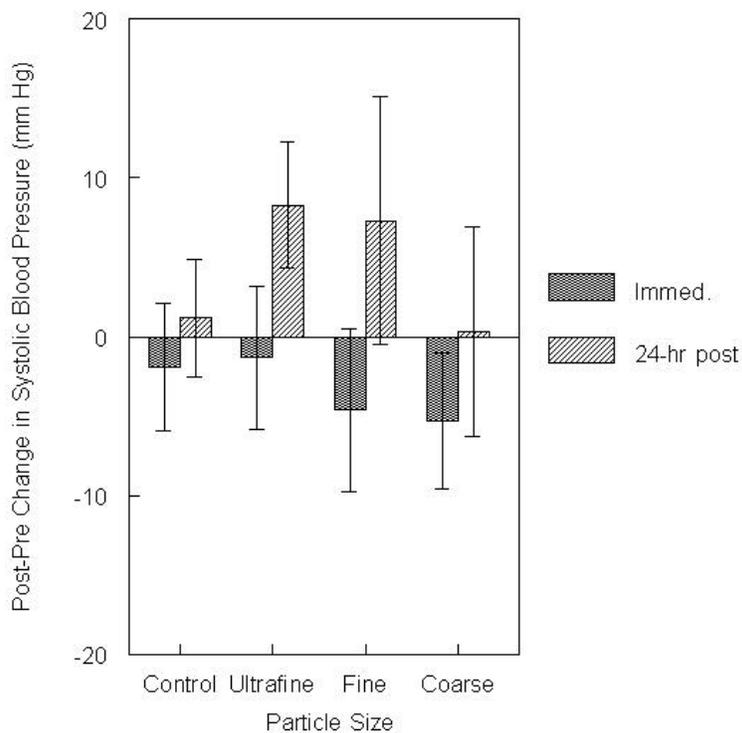
**Figure 10 Nitric Oxide in the Expired Breath of Adult Rats Exposed to Ultrafine, Fine and Coarse Particles Measured 24-hr After Exposure. The values shown are the post-exposure – pre-exposure differences (Mean  $\pm$  SE)**



## Blood Pressure

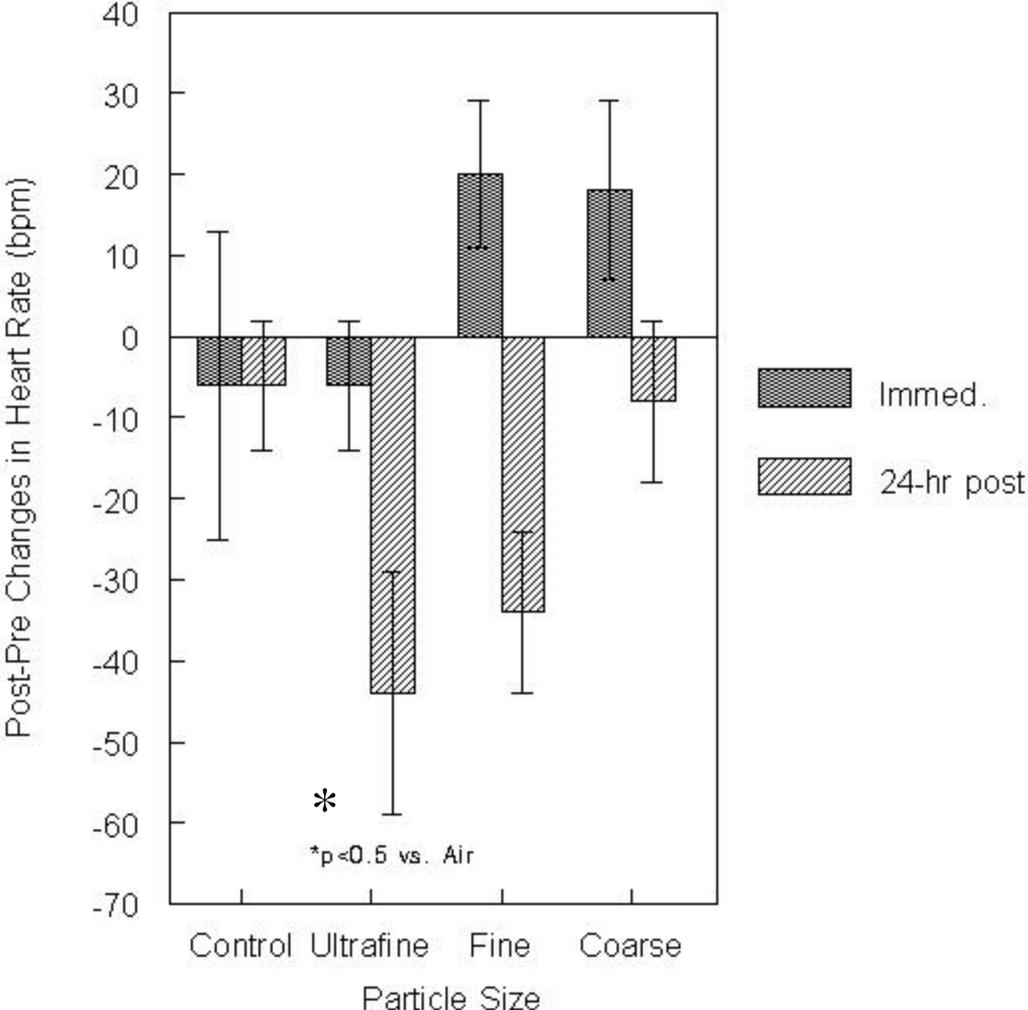
Systolic blood pressure was scored as the difference in blood pressure measured before exposure (baseline) and that measured immediately after exposure. As shown in Figure 11, blood pressure tended to decrease immediately post particle-exposure. Blood pressure was increased (but not significantly) 24-hour post-exposure in rats exposed to ultrafine or fine particles.

**Figure 11 Systolic Blood Pressure in Young Adult Rats Immediately and 24-hours Post Exposure**



Heart rate was measured immediately and 24-hr post exposure. These data are shown in Figure 12. There were no significant ( $p = 0.24$ ) differences in heart rate immediately post-exposure. However rats exposed to ultrafine or fine particles had significant decreases ( $p < 0.05$ ) in heart rate 24-hr post exposure.

Figure 12 Heart Rate in Young Adult Rats Immediately and 24-hours Post Exposure



#### **Experiment 4. Dependence of Toxicity on Particle Size in Senescent Rats**

Senescent (22 to 24 months) were studied in Experiment 4. The aged rats were barrier reared and maintained F344N-NIA rats and were obtained from colonies managed under contract to the National Institute on Aging (Bethesda, MD).

Rats were shipped to our laboratory in filter-equipped boxes. The rats were housed in laminar flow isolation units supplied with filtered air, and allowed access to food and water *ad lib*. The rats were randomly assigned to treatment groups. The rats were nose-only exposed for 6 hours to one of four atmospheres: (1) purified air; (2) “ultrafine” PM + O<sub>3</sub>; (3) “Fine” PM + O<sub>3</sub>; and (4) Coarse” PM + O<sub>3</sub>. In this experiment, the concentrations of the particulate species were about ½ the levels used in experiments 1 to 3. The experimental design and endpoints addressed are shown in Table 12.

This design was arrived at for several reasons. First, the ‘fine’ particle size range was comparable to the atmosphere used in the previous studies. Thus, a dose-response comparison could be made between senescent rats in Experiment 4 with the senescent rats exposed in Experiment 2. Second, UCD planned 3-day exposures at this concentration and there was an opportunity to compare macrophage endpoints for rats exposed at the ‘fine’ particle size (comparable to the particle size in the UCD exposures) between 1-day and 3-day exposures. Third, UCD data suggested that biological responses at the lower concentration were more effective for some endpoints than the higher concentration. This modification was implemented after extensive discussions among the investigators from UCI, UCD and CARB.

**Table 12 Atmospheres and Endpoints Tested in Experiment 4**

Group	Atmosphere	Conc.	Exposure	Endpoints
1	Purified Air		6 hour	
2	“Ultrafine” Carbon + AMN + Ozone	0.2 $\mu\text{m} \leq d_p$ 100 $\mu\text{g}/\text{m}^3$  150 $\mu\text{g}/\text{m}^3$ + 0.2 ppm	”	Macrophage Respiratory Burst Activity,  Blood Pressure
3	“Fine” Carbon + AMN + O <sub>3</sub>	0.5 $\leq d_p \leq 1.0$ 100 $\mu\text{g}/\text{m}^3$  150 $\mu\text{g}/\text{m}^3$  0.2 ppm	“	Heart Rate  Expired NO
4	“Coarse” Carbon + AMN + O <sub>3</sub>	1.5 $\leq d_p \leq 2.5$ 100 $\mu\text{g}/\text{m}^3$  150 $\mu\text{g}/\text{m}^3$  0.2 ppm	”	

Ten animals from each group were processed for histopathology endpoints and tissues were sent to UCD. Twelve rats from each group were assessed for macrophage and permeability-related endpoints. NO, heart rate and blood pressure were measured using the 10 histology animals immediately before euthanasia (18 hr post exposure). Rats were returned to the purified air-barrier

environment after exposure and given access to clean water and dry laboratory chow *ad lib*. The concentrations of atmospheric constituents and particle size characteristics are summarized in Table 13. The atmospheres were considerably more difficult to control. The average levels achieved agreed with the target size and concentration values within the limits of experimental error, however as can be seen in Table 13 the SE's were higher, relative to the mean concentration than had been seen in previous Experiments.

**Table 13 Concentrations and Particle Sizes of Experiment 4 Atmospheres (Mean ± SE)**

Group	Atmosphere	Particle Size MMAD (µm); GSD	Actual Concentration	Relative Humidity
1	Purified Air	NA <sup>a</sup>	NA	59.88 ± 0.84
2	“ultrafine” Carbon + AMN + O <sub>3</sub>	0.21 µm; 2.8	87.8 ± 19 166 ± 28 0.198 ± 0.004	
3	“Fine” Carbon + AMN + O <sub>3</sub>	0.68 µm; 2.5	91.35 ± 12.15 176.25 ± 38.36 0.194 ± 0.004	
4	“Coarse” Carbon + AMN + O <sub>3</sub>	1.8 µm; 2.3	92.35 ± 18.51 136.29 ± 27.61 0.197 ± 0.003	

<sup>a</sup>Not Applicable

Airway inflammation was examined. There were no particle-related effects on cell viability or cell yield. There were no exposure-related increases in numbers or percentages of lymphocytes or polymorphonuclear (PMN) cells and macrophages represented between 95% and 100% of the recovered cells (Table 14).

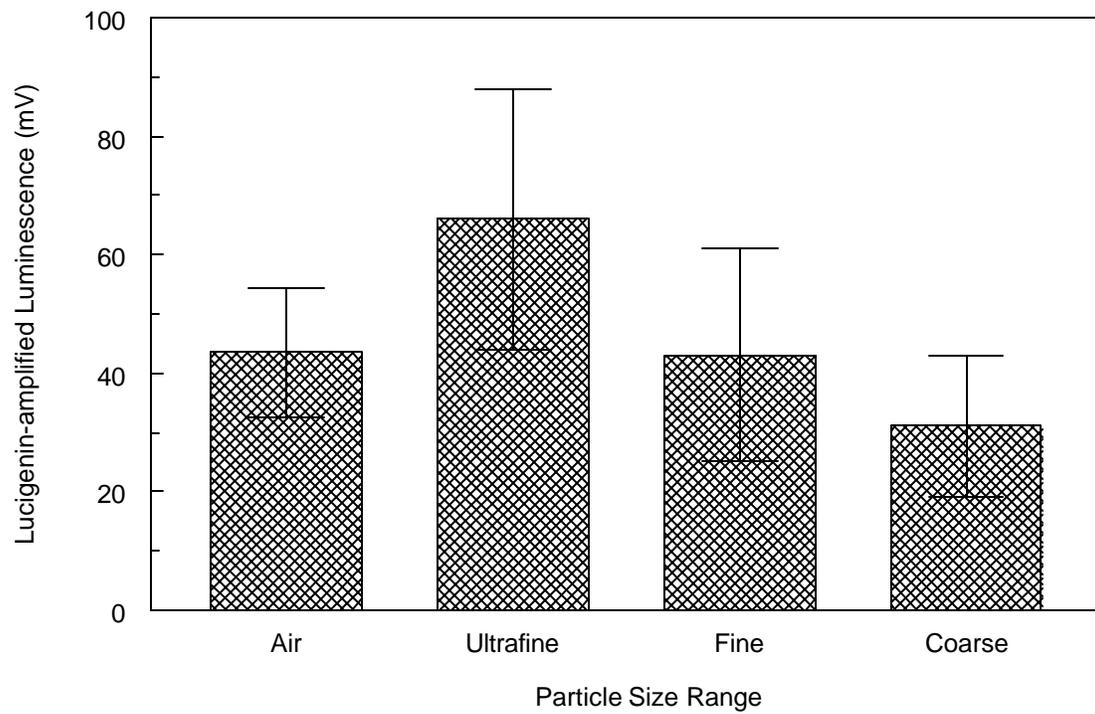
**Table 14 Cells Recovered in Bronchoalveolar Lavage Fluid From Senescent Rats Exposed to Particles in the Ultrafine, Fine and Coarse Size Ranges.**

Group	Percent $\pm$ SE			
	Macrophages	PMNs	Lymphocytes	Other
Air	93.4 $\pm$ 0.3	0	6.2 $\pm$ 0.3	0.31 $\pm$ 0.04
Ultrafine	93.7 $\pm$ 0.4	0.10 $\pm$ 0.02	5.3 $\pm$ 0.4	0.88 $\pm$ 0.07
Fine	93.1 $\pm$ 0.4	0.09 $\pm$ 0.01	6.1 $\pm$ 0.3	0.73 $\pm$ 0.05
Coarse	94.6 $\pm$ 0.4	0.14 $\pm$ 0.03	5.0 $\pm$ 0.4	0.42 $\pm$ 0.04

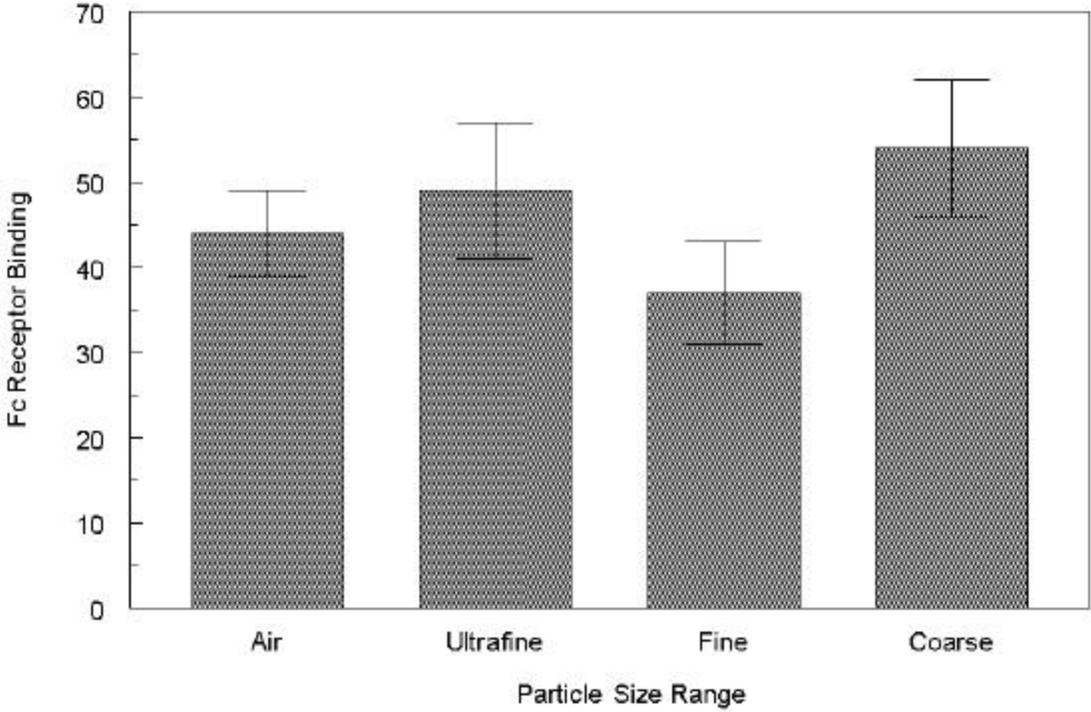
### Macrophage Function

Greater than 90% of the cells recovered from these senescent rats by bronchoalveolar lavage were viable. The production of superoxide during respiratory burst activity following stimulation with PMA (Figure 13) was slightly, but not significantly depressed by exposures to coarse particles. Fc Receptor binding was slightly but not significantly reduced in macrophages from senescent rats exposed to fine particles (Figure 14).

**Figure 13 Respiratory Burst Activity of Macrophages From Senescent Rats After Exposure to Particles in the Ultrafine, Fine and Coarse Size Ranges**



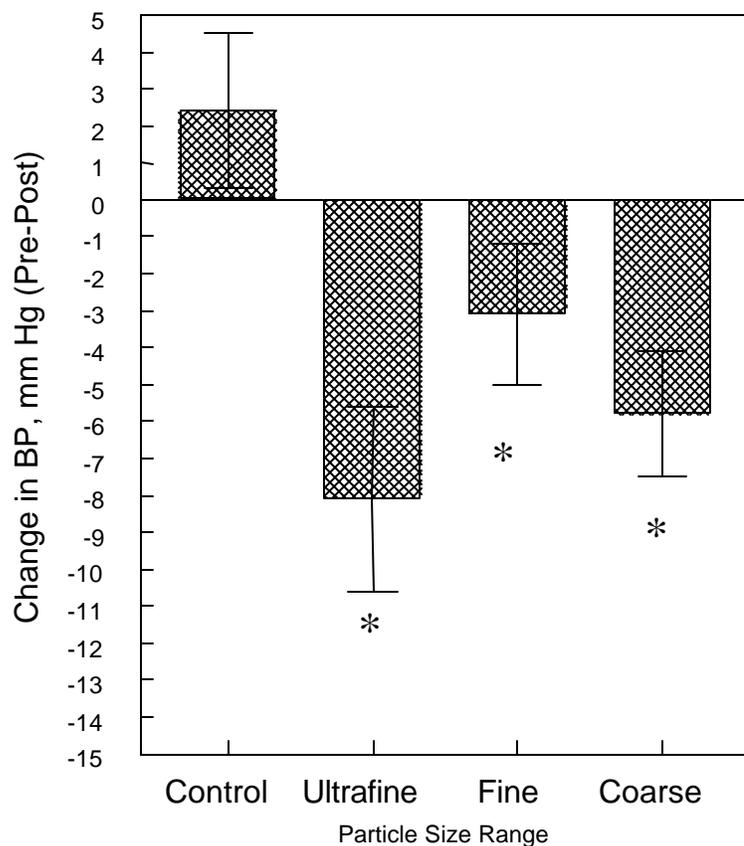
**Figure 14 Fc Receptor Binding Capacity of Macrophages From Senescent Rats After Exposure to Particles in the Ultrafine, Fine and Coarse Size Ranges**



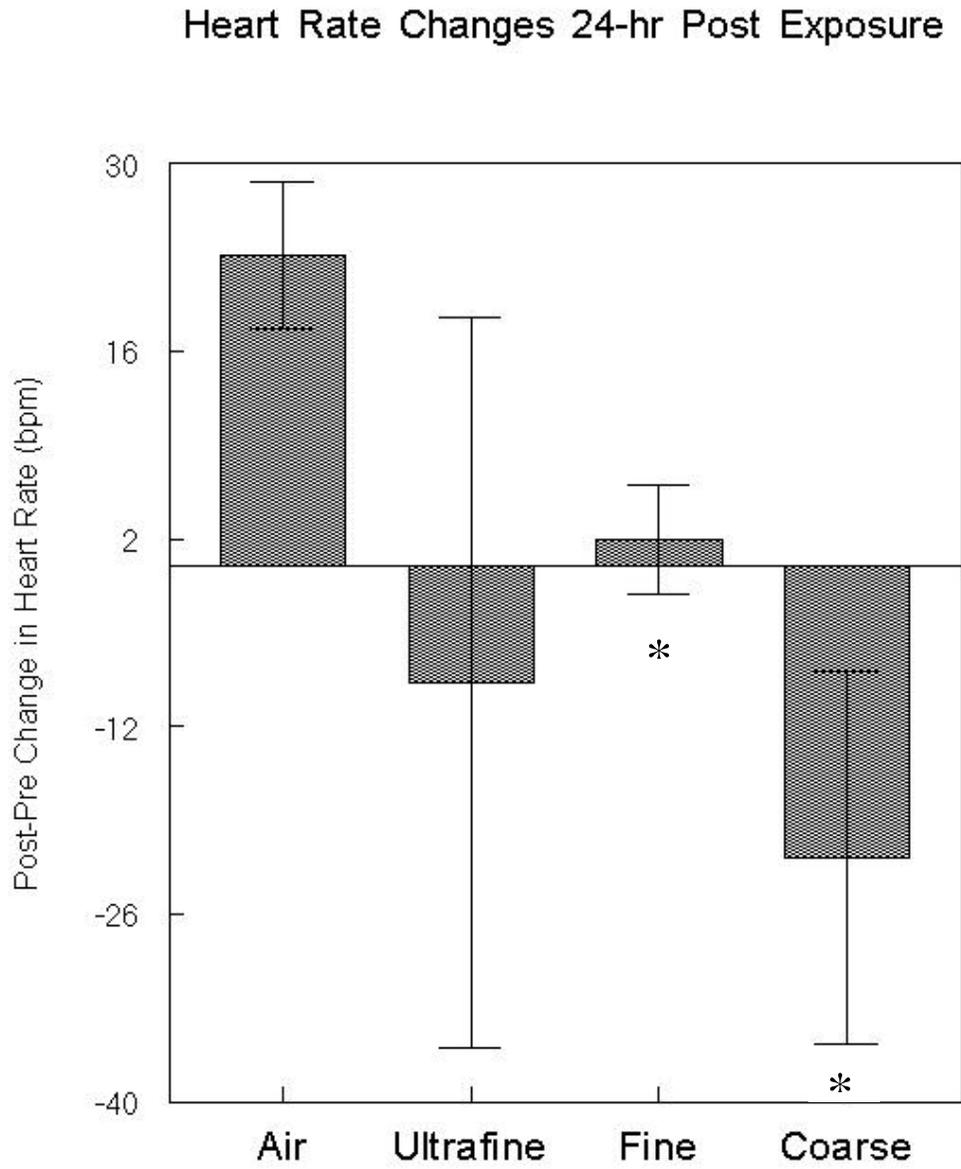
## Physiological Endpoints

Systolic blood pressure and heart rates were measured immediately and 24-hr post-exposure. The blood pressure x heart rate product was also determined. Particle exposures caused significant reductions in blood pressure (Figure 15), reduction in heart rate (Figure 16) and double product (Figure 17) which is the product of diastolic blood pressure and heart rate. Double product is a surrogate measure for cardiac work capability.

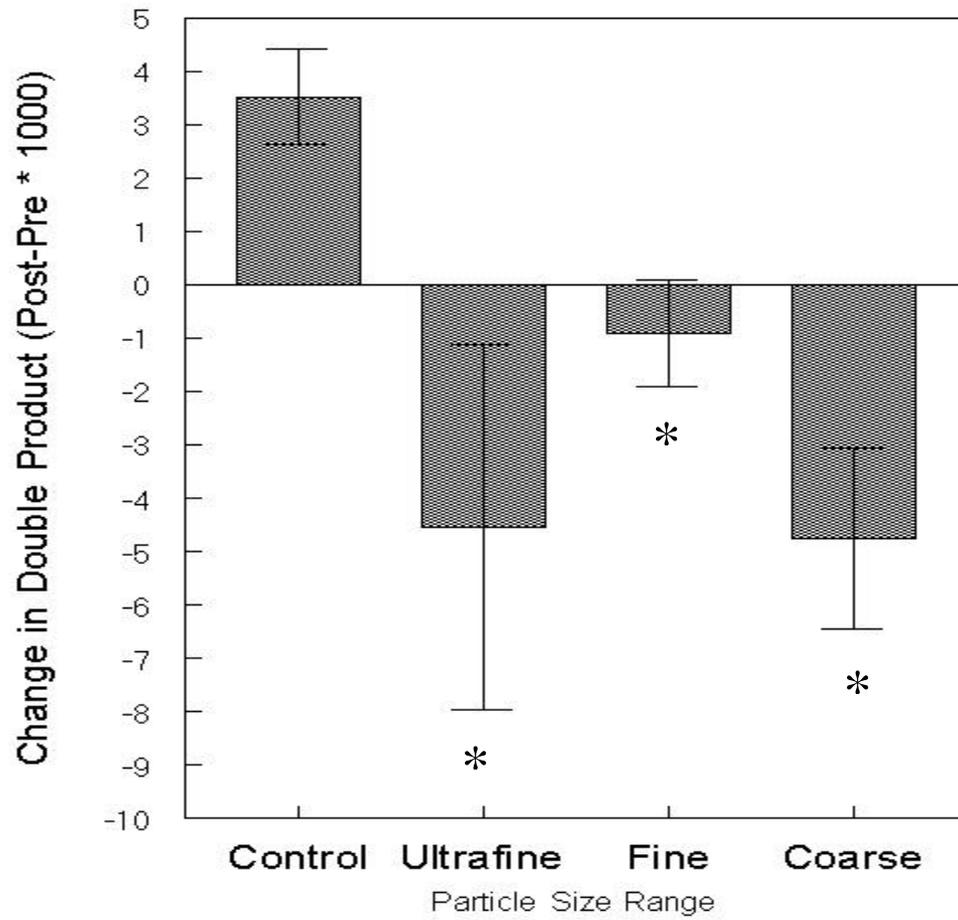
**Figure 15 Systolic Blood Pressure of Senescent Rats Measured 24 hr After Exposure to Particles in the Ultrafine, Fine and Coarse Size Ranges (\* p  $\leq$  0.05 vs. Control)**



**Figure 16 Heart Rate of Senescent Rats Measured 24 hr After Exposure to Particles in the Ultrafine, Fine and Coarse Size Ranges (\* p£ 0.05 vs. Control)**

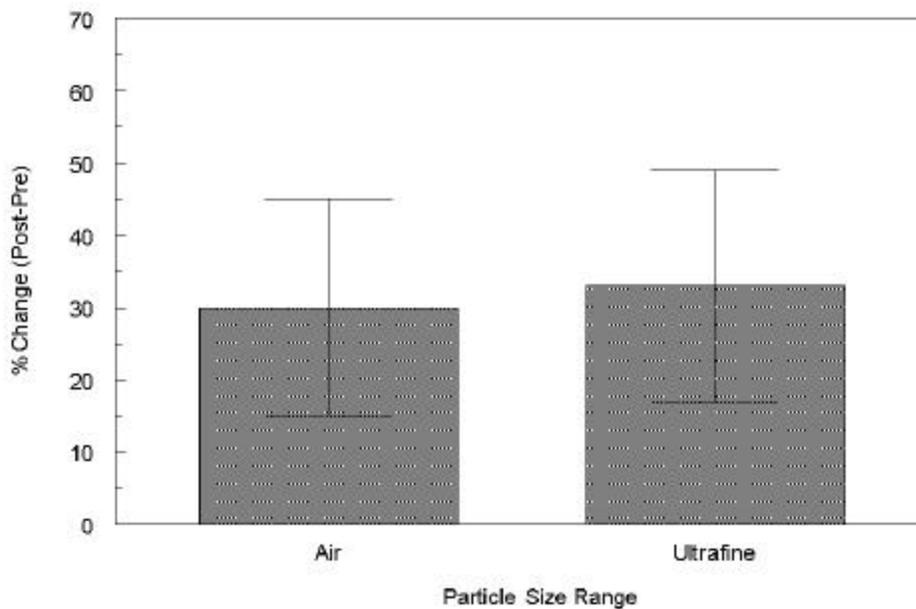


**Figure 17 Double Product of Senescent Rats Measured 24 hr After Exposure to Particles in the Ultrafine, Fine and Coarse Size Ranges (\* p  $\leq$  0.05 vs. Control)**



Nitric oxide in expired breath was measured in a subset of the rats using a rapid response nitrogen oxides analyzer. The rats were exposed to either purified air or ultrafine particles. To reduce interference from NO in room air, a NO scrubber was used. The scrubber metered O<sub>3</sub> into a stream of dry compressed air. The O<sub>3</sub> rapidly and completely oxidized the NO to NO<sub>2</sub>. The O<sub>3</sub> and NO<sub>2</sub> were removed with oxidant scrubber/activated carbon cartridges and then delivered to the NO measurement system. The mean ± SE concentration of NO in the expired breath of senescent rats was 4 ± 0.7 ppb, which is not significantly different from that in the expired breath of younger adult rats. As shown in Figure 18, the pre-exposure-post-exposure differences in expired NO were not significantly different between rats exposed to ultrafine particles and those exposed to purified air.

**Figure 18 Expired Nitric Oxide Following Exposures to Ultrafine Particles was not Different From That Observed in Rats Exposed to Purified Air**



## Discussion

The relationship between environmental PM exposure and human health effects is of continuing concern. Currently, much attention is focused on the fraction of PM<sub>10</sub> below 2.5 μm in diameter, because these particles can penetrate to the deep lung and because current epidemiological evidence suggests that human morbidity and mortality is more strongly associated with the fine particle fraction of PM<sub>10</sub> than the coarse particle fraction. Also, the chemical composition of this fine particle fraction is less variable, across geographic regions, than is the composition of the large particle fraction, and previous laboratory work has demonstrated toxic effects associated with specific components of the fine particle fraction; notably acidic sulfates and combustion-related carbonaceous aerosols. The present study analyzed the effects of two PM components, ammonium nitrate (AMN) and elemental carbon (EC), which were selected because they represent important fractions of ambient PM in California. The initially selected concentrations of each component were representative of estimated peak concentrations, based on extrapolations from ambient air data, and the sizes of the particles used were chosen based upon reported sizes of inorganic aerosols in ambient air.<sup>39</sup> Exposures were performed in the presence of ozone (O<sub>3</sub>; 0.2 ppm) because O<sub>3</sub> is often present in particle-contaminated atmospheres in California. Several biological endpoints were measured, each of which could be related to cardiopulmonary health effects. For example, changes in airway permeability and infiltration of the lung by inflammatory cells were measured to examine mechanisms related to lung inflammation. Macrophage function changes, which are related to host defenses (macrophage Fc receptor binding and Respiratory Burst activity, which entails release of biocidal reactive oxygen species, e.g. superoxide anion) were measured because impairment of these functions can relate to increased risk of lung infection.

The objectives of this study were: (1) to examine the health effects of atmospheric mixtures which realistically model sizes and compositions of particles in California air; (2) to examine whether or not particle-induced lung injury could initiate systemic adverse cardiopulmonary responses in young healthy rats and in a presumably sensitive model, the aged (senescent) rat; and (3) to determine whether these cardiopulmonary responses were particle size-dependent. To achieve these objectives, a series of four exposure experiments were performed. The main findings of these experiments are summarized in Table 17.

**Table 15 Summary of Biological Responses observed in UCI Exposure Experiments**

Exp.	Subj. <sup>h</sup>	Atmos. <sup>i</sup>	Inflam. <sup>j</sup>	Perm. <sup>k</sup>	FcR <sup>l</sup>	RB <sup>m</sup>	BP <sup>n</sup>	HR <sup>o</sup>	DP <sup>p</sup>	NO <sup>q</sup>
1	Y	O <sub>3</sub>	NS	NS	NS	NS				
		High, F	NS	NS	NS	NS				
		High, F+O <sub>3</sub>	↑ p≤0.05	NS	↓ p≤0.05	↓ p≤0.05				
2	O  O vs Y	High, F+O <sub>3</sub>	NS	NS	↓ p≤0.01	↑ p=0.08				
		Air	↑ p≤0.05	NS	↑ p≤0.05	↓ p≤0.05				
		High, F+O <sub>3</sub>	↑ p≤0.05	NS	↑ p≤0.05	↓ p≤0.05				
3	Y	High, U	NS	NS	NS	NS	↑ p=0.2	↓ p≤0.05	NS	NS
		High, F	NS	NS	NS	NS	↑ p≥.20	↓ p≤0.05	NS	NS
		High, C	NS	NS	NS	NS	NS	NS	NS	NS
4	O	Low, U	NS		NS	NS	↓ p≤0.01	↓ p≤0.01	↓ p≤0.20	NS
		Low, F	NS		NS	NS	↓ p≤0.05	↓ p≤0.05	↑ p≤0.06	
		Low, C	NS		NS	NS	↓ p≤0.01	↓ p≤0.01	↓ p≤0.05	

In Experiment 1, young (8 to 10 week) rats were exposed to purified air, O<sub>3</sub> alone (0.2 ppm), fine particles alone (F; 0.7 μm mass median aerodynamic diameter [MMAD], Carbon [EC]; 200 μg/m<sup>3</sup>, ammonium nitrate [AMN]; 300 μg/m<sup>3</sup>), or F + O<sub>3</sub>. Exposure to O<sub>3</sub> alone or to particles alone did not produce any measured responses that were significantly different from those of rats exposed to purified air. However, the rats exposed to F + O<sub>3</sub> exhibited significant

<sup>h</sup> Subjects – Young (Y; 8 to 10 weeks); Old (O; 22 to 24 months)

<sup>i</sup> Atmospheres – Ultrafine (U), Fine (F), Coarse (C); High (Carbon=200 μg/m<sup>3</sup>, AMN=300μg/m<sup>3</sup>, O<sub>3</sub>=0.2ppm), Low (Carbon=100 μg/m<sup>3</sup>, AMN=150μg/m<sup>3</sup>, O<sub>3</sub>=0.2ppm)

<sup>j</sup> PMNS in Bronchoalveolar Lavage Fluid (BAL)

<sup>k</sup> Permeability measured as protein in BAL

<sup>l</sup> Fc Receptor Binding by Macrophages from BAL

<sup>m</sup> Respiratory Burst activity of Macrophages from BAL

<sup>n</sup> Systolic Blood Pressure measured 24-hr post-exposure

<sup>o</sup> Heart Rate measured 24-hr post-exposure

<sup>p</sup> Double Product (HR x BP) measured 24-hr post-exposure

<sup>q</sup> Nitric Oxide in expired breath

responses. There were significantly greater numbers of inflammatory cells ( $\uparrow$  PMNs) in bronchoalveolar lavage fluid (BAL). Macrophages recovered from the BAL had reduced ability to bind antibodies to Fc receptors on their cell membranes ( $\downarrow$ FcR). The macrophages also had reduced ability to generate superoxide radicals when stimulated with phorbol myristate acetate, a chemical that elicits macrophage respiratory burst activity via a non-specific mechanism ( $\downarrow$ RB). Thus high concentration exposures to a particle plus O<sub>3</sub> mixture caused lung inflammation and also suppressed macrophage-mediated specific and non-specific<sup>r</sup> immunological functions. In-vitro studies have demonstrated that modification of alveolar macrophage-regulated inflammatory responses by exposure to PM10 can depress macrophage defenses against viral infection<sup>40</sup>

The particle concentrations used in Experiment 1 were high, relative to 24-hr average ambient PM levels. However, short-term peak concentrations of PM10 and PM2.5 can be quite high in some California communities. For example, in rural Sacramento during agricultural burning periods, peak 1-hr levels of 250  $\mu\text{g}/\text{m}^3$  were measured, and concentrations remained elevated for more than 6 hours (CARB). The source profile of Sacramento aerosol during the winter (averaged from 1991 –1996) indicates that nitrates, wood smoke and motor vehicle exhaust emissions comprise 37%, 18% and 25%, respectively, of the PM2.5. Both wood smoke and motor vehicle exhaust particles are important sources of elemental and organic carbon aerosols. Thus although the concentrations tested in this experiment were high, the biological responses that were observed are relevant to California exposures both with respect to composition of the particles used, and to the concentrations used.

In Experiment 2, old, or senescent, (22 to 24 month) rats were exposed to purified air or the F + O<sub>3</sub> mixture under the same conditions, and at the same concentrations, used in Experiment 1. The senescent rats exposed to the F + O<sub>3</sub> mixture showed significant impairment of antibody-directed immunological function ( $\downarrow$ FcR) compared to senescent rats exposed to purified air. Particle exposures increased respiratory burst activity ( $\uparrow$  RB) in the macrophages of the senescent rats compared to macrophages from senescent rats exposed to purified, albeit at

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<sup>r</sup> In this context, specific refers to immunological functions that are antibody-directed while non-specific refers to cell-mediated, non-antibody-directed activity.

a lesser level of significance ( $p=0.08$ ). Compared to the young adult rats studied in Experiment 1, the senescent rats had greater percentages of inflammatory cells (PMNs) in BAL. The total numbers of cells recovered by lavage from the older animals (purified air and particle – exposed) were only about 1/3 of the number recovered from the lavage of young rats. Overall, the lungs of the senescent rats might be less capable of mounting host defenses than are those of younger rats, and reduced immunological function caused by particle exposures could therefore take on additional significance. Thus, since there were fewer immunocompetent cells in the lungs of the senescent rats at the outset, and particle exposures reduced the competence of those that were present., and thus could plausibly explain why the susceptibility of elderly people to respiratory infections is increased following ambient episodes of elevated PM, as reported in some epidemiological studies.<sup>41,42</sup>

Experiment 3 tested the hypothesis that particle-induced lung injury at the tissue and cellular levels and systemic effects would be particle size dependent when composition and concentration of particles were held constant. In this experiment groups of rats were exposed to particles in the ultrafine (UF), fine (F) and coarse (C) size ranges. There are conventional definitions of the boundaries of these size ranges: UF particles are those with diameters ( $d_p$ )  $\leq$  100 nm median aerodynamic diameter (MMD); F particles are those in the range  $100 \text{ nm} \leq d_p \leq 2500 \text{ nm}$ ; and C particles are those in the range  $2500 \text{ nm} \leq d_p \leq 10000 \text{ nm}$ . However, for the purpose of this research, the definitions of UF, F and C were modified. This was done, for the coarse particles, to take into account differences between rats and humans with respect to regional deposition of particles as a function of size. For the UF particles, we were unable to hold the composition of the particles constant when we reduced the size below 200 nm. The composition constraint was necessary so that the dependence of biological response on particle size could be examined in the absence of the influence of differences in composition or concentration.

Ambient coarse PM has an MMAD of about 4  $\mu\text{m}$ , ambient PM<sub>2.5</sub> has an MMAD of about 1  $\mu\text{m}$  and conventionally UF PM are particles smaller than 0.1  $\mu\text{m}$  MMD. In this experiment we attempted to produce ‘coarse’ particles with an MMAD of 2  $\mu\text{m}$  because these particles would have a similar deposition pattern in rat airways as would 4  $\mu\text{m}$  particles in human airways. We were successful in generating fine particles that had an MMAD of about 1  $\mu\text{m}$ . We

were able to attain EC to AMN ratios in coarse and fine particles that were comparable to those used in Experiments 1 and 2. The UF particles, however, has greater EC/AMN ratios and the AMN fraction became more reduced as particle sizes became smaller. Therefore the UF aerosol we used had an MMAD of about 0.2  $\mu\text{m}$ .

In Experiment 3 we did not observe significant inflammatory or immunological responses in the rats exposed to the F+O<sub>3</sub> atmosphere, even though significant changes had been observed in Experiment 1. This might be attributable to a minor change in the experimental protocol. In Experiment 1 the rats were lavaged about 12 hours post exposure. In Experiment 3, the rats were lavaged about 24 hours post exposure and prior to lavage the rats had been subjected to measurements of NO in expired breath and measurements of blood pressure. It is possible that the time difference, the possible stresses attendant on the physiological measurements, or a combination of factors blunted the inflammatory and immunological responses. The major finding of this study was that UF and F particles caused significant decreases in heart rate which were accompanied by small increases in blood pressure, compared to rats exposed to purified air. The rats exposed to EC particles did not exhibit significant responses. The inverse relationship between heart rate and blood pressure might be a normal form of physiological compensation. The product of heart rate and systolic blood pressure, which is called the double product (DP) is an index of cardiac work. These young rats were apparently able to compensate for the hemodynamic changes induced by particle exposures. On the other hand, senescent rats exposed to the same three size ranges of particles, at particle concentrations about 50% lower than those used for the young adult rats, also exhibited significant hemodynamic effects due to particle exposures, but in this instance they did not compensate. Thus, both heart rate and blood pressure were reduced 24 hours after exposure and the DP was reduced as well, indicating impairment of cardiac output. This pattern of response is what one might expect to see in instances of systemic shock. At the concentrations tested, there was no evidence of inflammatory responses and there were no significant changes in RB or FcR binding. Thus short-term acute exposures to elevated levels of PM can cause systemic changes and alter cardiac and hemodynamic physiology. It has been suggested that PM can cause imbalances between sympathetic and parasympathetic autonomic nervous systems that regulate cardiac activity, and PM exposures have been associated with increased risk of cardiovascular mortality, morbidity and arrhythmias. This laboratory finding strongly supports the

epidemiological findings of increased cardiovascular morbidity following short-term episodes of elevated PM. For example, Peters et al.<sup>43</sup> reported significant associations between onset of symptoms of myocardial infarction and acute (within 2 hours) and sub-acute (24-hr average PM 2.5 on the previous day) exposures to ambient PM 2.5. The ability of young, healthy animals, and the inability of senescent animals, to adequately compensate for hemodynamic changes may be an important factor in determining why epidemiological studies consistently identify elderly people as being at elevated risk of adverse effects of PM.

A major hypothesis of this study was that biological responses would be particle size-dependent. The study demonstrated that in a sensitive animal model, the senescent rat, particle exposure could cause a significant depression in blood pressure and cardiac work capacity, with the greatest effects seen for ultrafine and coarse particle sizes and the least change with fine particles. Blood pressure was also affected in young adult rats; coarse particles had a lesser effect than either ultrafine or fine particles. These changes in blood pressure could be an important indicator that short-term particle exposures can have systemic effects and may provide a toxicological rationale for the association between human cardiovascular mortality and acute exposures to elevated levels of PM. The magnitude and direction of the effects varied as functions of both age and particle size.

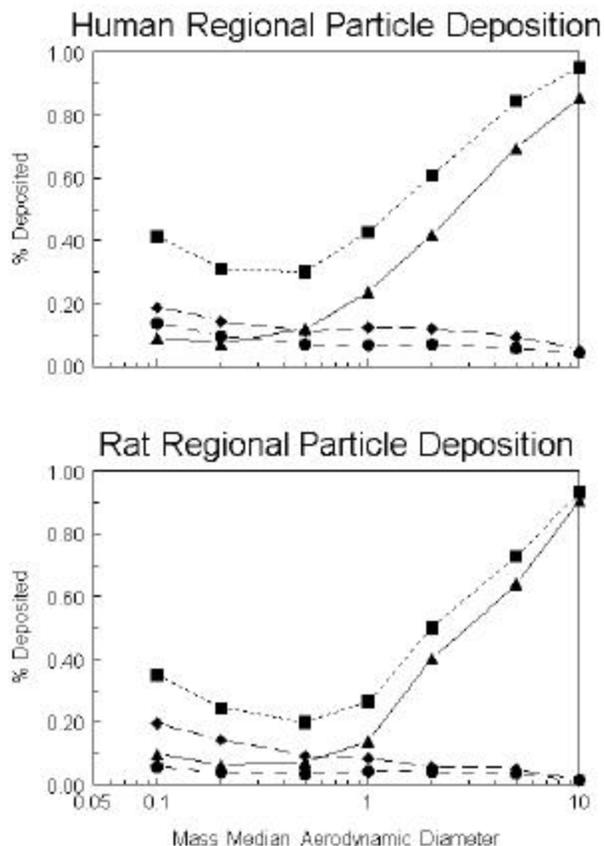
The dependence of health effects on particle size is a key issue in determining control strategies for improving air quality. The major sources of ultrafine, fine and coarse particles are different, although there are some overlaps. The differences in source characteristics lead to differences both in sizes of particles generated, and chemical compositions of those particles in ambient air. Atmospheric processes that lead to changes in composition and particle size distributions downwind of the emission site further complicate the issue. There are epidemiological and toxicological studies that suggest that ultrafine particles are more toxic than fine or coarse particles, and that fine particles are more toxic than coarse particles. However, there are epidemiological studies that indicate greater effects for particles in the PM<sub>2.5</sub> to PM<sub>10</sub> size range than for particles smaller than PM<sub>2.5</sub>.

This study provided an opportunity to further examine the question of particle size dependence of biological responses in a system where chemical composition, particle concentration and exposure durations were relatively constant. We explored this matter by using the MPDEP lung deposition model<sup>44</sup> to estimate the fractions of particles that would deposit in the nasal, tracheobroncheal and

pulmonary regions of the lungs of adult and senescent rats exposed in Experiments 3 and 4, respectively. MPDEP is a computer program that uses multiple-path models to estimate particle deposition in the lung. The software can calculate the regional dose ratio and flux of particles in human and rat airways.

As shown in Figure 19, regional deposition of particles in the respiratory tract is strongly dependent on particle size. The mass of polydisperse aerosol particles deposited during the exposures were computed for young adult rats, using the measured particle MMADs, GSDs and exposure concentrations of each of the three aerosols used in Experiment 3. The same model was used for the senescent rats in Experiment 4. The computations assumed a breathing rate of  $102 \text{ min}^{-1}$  and a tidal volume of 2.1 mL.

**Figure 19 Modeled Deposition of Particles in Human and Rat Airways (□ = Total, △ = Nasal, ○ = Tracheobronchial and ● = Pulmonary Deposition)**

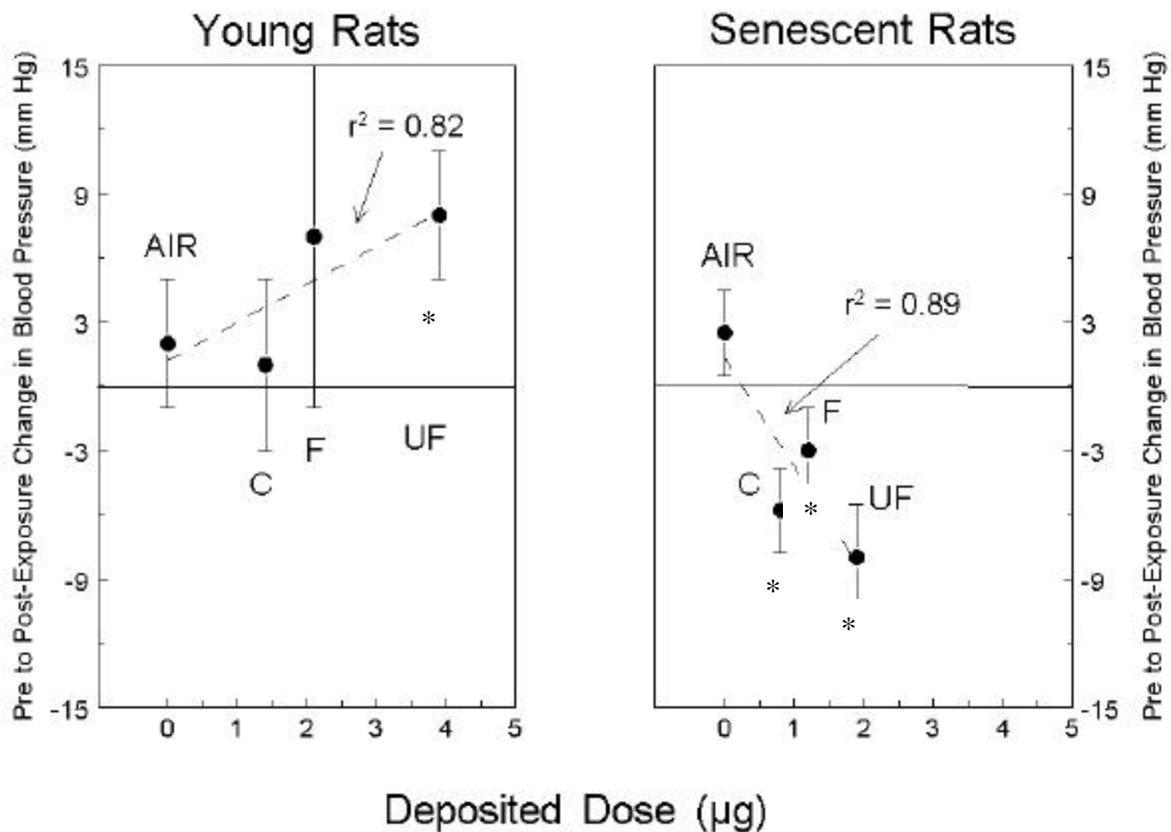


In Figure 20 below, differences in systolic blood pressure measured before and after exposures were plotted as a function of pulmonary dose. The dose (in  $\mu\text{g}$  of particles deposited in the pulmonary region of the lung) was calculated using the measured mass concentrations of AMN + EC to which the rats were exposed and the fraction of the inhaled particles that would deposit in the pulmonary region of the rat's lung for particles of the stated sizes. As shown in the figure, the greater effect of ultrafine particles could have been due to the greater dose to the deep lung.

We plotted blood pressure pre- to post-exposure changes as a function of deposited mass of AMN + EC particles in the pulmonary region of the lung over the course of the single acute

exposure. The data for both adult and senescent rats are shown in Figure 20. As mentioned earlier young rats exhibited an increase in blood pressure. As shown in Figure 20, the dose of coarse particles to the pulmonary region of the lung was less than that for the F or UF particles and the dose for UF particles was greater than that for either C or F particles. There was a linear dependence of increased blood pressure with increasing dose ( $r^2 = 0.82$ ;  $p \leq 0.05$ ). The senescent rats exhibited significant hypotension following exposures and this decrease in blood pressure was also linear with dose ( $r^2 = 0.89$ ;  $p \leq 0.05$ ). Thus, although there could be some particle size dependent effect of AMN + EC particles on blood pressure, the data from this study strongly suggest that the dose of particles depositing in the pulmonary region of the lung can explain most, if not all of the observed effects.

**Figure 20 Dose Dependence of Blood Pressure Changes After Exposures to UF, F and C AMN + EC Aerosols (\* p  $\leq$  0.05 vs. AIR)**



Under the conditions of this study, UF particles caused greater changes in blood pressure than either coarse or fine particles, but this was possibly related to the greater efficiency of deposition of UF particles in the gas exchange, or pulmonary, region of the lung. It is important to note that in ambient air the composition of UF particles is different from that in F or C fractions of PM, in contrast to this study in which composition was relatively constant. Thus, this finding does not rule out the possibility that in ambient air, the UF fraction may be more toxic than either the F or C fractions, and the fact that UF particles deposit in the critical pulmonary region of the lung more efficiently than F or C particles highlights the need to study the toxicology and effects of ambient UF particles on health.

## **Summary and Conclusions**

### ***Summary***

This study analyzed the effects of two PM components, ammonium nitrate (AMN) and elemental carbon (EC) on the cardiopulmonary system. AMN and EC were selected because they represent important fractions of ambient PM in California. The concentrations of each component used in this study were representative of estimated peak concentrations, based on extrapolations from ambient air data. The sizes of the particles used were chosen based upon reported sizes of inorganic aerosols in ambient air. Because California air pollution atmospheric chemistry is dominated by photochemical reactions, these particles are often in the presence of ozone (O<sub>3</sub>). We therefore included O<sub>3</sub> in the test atmospheres.

The objectives of this study were: (1) to examine the health effects of atmospheric mixtures which realistically model sizes and compositions of particles in California air; (2) to examine whether or not particle-induced lung injury could initiate systemic adverse cardiopulmonary responses in young healthy rats and in a presumably sensitive model, the aged (senescent) rat; and (3) to determine whether these cardiopulmonary responses were particle size-dependent. To achieve these objectives we:

1. Built and tested a particle generator system that permitted us to separate a polydisperse aerosol into three different size fractions while maintaining the relative concentrations of the AMN and EC components and then delivering these fraction into three separate

exposure chambers so that rats could be exposed simultaneously at the same concentration. The particle size ranges were characterized as Ultrafine ( $d_p \leq 200$  nm), Fine ( $500 \leq d_p \leq 1000$  nm) and Coarse ( $2000 \leq d_p \leq 4000$  nm).

2. Developed and installed a non-invasive method for assessing blood pressure and heart rate in rats to examine cardiac endpoints that were relevant to systemic responses.
3. Developed a method for measuring nitric oxide in expired breath as a non-invasive biomarker of lung inflammation and oxidative stress that could trigger systemic responses.
4. Exposed groups of healthy young rats and senescent rats to UF, F and C particles and measured blood pressure and heart rate, expired nitric oxide, protein in bronchoalveolar lavage fluid, and performed functional assays with macrophages isolated from bronchoalveolar lavage samples.

This project was complemented by a parallel effort at UCD. UCD used aerosols of the same composition, and with particles similar in size to the F aerosols used in this study. UCD examined mechanisms related to oxidative stress using endpoints other than those used at UCI and performed extensive measurements of epithelial injury using morphometric methods. UCD focused their studies on examining the concentration dependence of health effects, and also examined the effects of subject age on responses to particles. The studies at UCI focused on examining the importance of particle size in eliciting responses in young adult and old (senescent) Fischer 344 rats. The UCI study endpoints addressed immunological and physiological responses that could relate to epidemiological associations between acute PM exposures and increased risks of respiratory infections and cardiopulmonary mortality.

The major findings were:

1. Acute exposures to mixtures of particles and ozone at concentrations relevant to short-term peak ambient levels caused inflammatory responses in both old and young rats. The exposures also altered antibody-directed and nonspecific responses of lung macrophages, compared to responses measured in rats exposed to purified air.
2. Particle exposures caused systemic effects, i.e. changes in blood pressure and heart rate, compared to measurements in rats exposed to purified air. These changes were observed in

young rats as well as in senescent rats. Senescent rats responded at exposure levels that were  $\frac{1}{2}$  those used in the younger animals. These changes occurred in the absence of significant inflammatory changes in the lung or changes in exhaled nitric oxide.

3. Exposures to ultrafine particles elicited greater blood pressure responses than either fine or coarse particles. However, a considerably larger fraction of ultrafine particles deposits in the pulmonary region of the rat's lung than does either fine or coarse particles, all other exposure parameters being equal. Thus, when the blood pressure responses were plotted as a function of pulmonary dose delivered under the conditions of this experiment, linear relationships between dose and response were observed.

## **Conclusions**

We conclude that particle size is an important determinant of air pollution health effects. For particles of the same composition, particle size influences the dose of material delivered to the critical region of the lung in which gas exchange and transport of oxygen to the blood occurs. The degree of lung irritation or injury produced in this region of the lung will depend on the delivered dose and the effects of this injury or irritation can extend to other organs or body systems.

Furthermore, we conclude that older animals are more susceptible to air pollution health effects, than are young, adult animals. In this study, the dose needed to induce a significant effect in older rats was 10 times lower than the dose needed to achieve a significant response in younger rats.

## Glossary

AMN	Ammonium Nitrate
Arrhythmia	Abnormal heart rhythm.
C	Coarse Particles ( $2000 \leq \text{particle diameter} \leq 4000 \text{ nm}$ )
EC	Elemental Carbon
F	Fine Particles ( $500 \leq \text{particle diameter} \leq 1000 \text{ nm}$ ).
Hypotension	Low blood pressure.
O <sub>3</sub>	Ozone, a photochemical oxidant formed by atmospheric chemical reactions between sunlight, organic vapors and nitrogen oxides.
PM <sub>2.5</sub>	Particulate matter less than 2.5 $\mu\text{m}$ mass median aerodynamic diameter.
PM <sub>10</sub>	Particulate matter less than 10 $\mu\text{m}$ mass median aerodynamic diameter.
PMN	Polymorphonuclear leucocyte, a white blood cell with a multilobular nucleus.
Senescent rat	Rat older than 22 months.
Young, adult rat	Rat aged 8 to 10 weeks.
UCD	University of California, Davis
UCI	University of California, Irvine
UF	'Ultrafine' particles (particle diameter $\leq 200 \text{ nm}$ ).

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