Final Report

Mechanisms of Particulate Toxicity:
Effects on the Respiratory System of Sensitive Animals and Asthmatic Humans

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Disclaimer

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Abstract

The primary goal of this research project is to test the effects of particulate matter (PM) on allergic airways in the lungs of sensitive animals and human asthmatic subjects. Four experiments were designed in animals, while complementary studies were done in human asthmatic volunteers. The first two animal experiments were to determine whether the Brown Norway (BN) rat can be treated with ovalbumin (OVA) to make it suitable as a model of asthma, and sequentially to measure the physiologic, biochemical and structural effects of BN rats exposed weekly to aerosolized OVA for up to four weeks. The goal of these experiments was to optimize conditions of exposure to aerosolized OVA, while minimizing any changes in the lungs that may potentially mask the effects of subsequent exposure to particles. With knowledge gained from these first two experiments, the third and fourth experiments were designed to use this allergic airway model to allow us to test the effects of short-term exposure to PM on the structure and function of compromised lung airways and parenchyma. Physiologic, biochemical and histopathological parameters were used to assess particle-induced effects in this animal model. The effects of repeated particle exposure on immune factors to potentially alter the allergic response in the lungs were also examined. We found a model of allergic airways could be produced in the BN rat. We found short-term repeated exposure to ammonium nitrate and carbon demonstrated significant effects of particles to alter airway epithelial cells, increase airway inflammation and transiently elevate IL-4 mRNA levels in the lungs, all indicators of an adverse particle effect on the lungs. Human airway biopsies from asthmatic volunteers exposed to particles similar to those used with BN rats were analyzed using *in vitro* techniques to demonstrate detectable changes in expression for a panel of cytokines due to particulate exposure alone or in combination with ozone. For asthmatic subjects, the most significant changes noted in mRNA levels following PM exposure were increases in IL-1ß and IL-12p35. These findings in both an animal model of allergic airways disease as well as human asthmatics suggest the airway epithelium is an important target of particle-induced effects associated with inflammation and the perturbation of proinflammatory cytokines present in the lungs. We would advocate, based on these findings, a combined approach to test sensitive animals and human asthmatics to similar particles by size and composition can serve to further elucidate the impact as well as the potential mechanisms of action of airborne particles on the respiratory system in individuals with allergic airway disease.

Executive summary

Background

Numerous epidemiological studies present growing evidence of adverse health effects associated with exposure to ambient airborne particles. These health effects appear to be greatest among susceptible populations of individuals, including children and those with pre-existing cardiopulmonary disease. The mechanisms by which these adverse health effects occur with exposure to particulate matter are not clear. Asthmatic individuals could be more susceptible to airborne particulate matter due to underlying changes in the respiratory system associated with this disease process. The objectives of this project were to examine the functional and cellular responses of the respiratory system in asthmatic human volunteers and airway sensitized rats to controlled, short-term particle exposure. Through these studies we hope to gain a better understanding of the potential mechanisms by which an allergic airway condition may be impacted by exposure to airborne particles common to the state of California (nitrates and carbon).

Methods

Four animal experiments were designed to correlate with complementary studies in the human. All subjects were exposed to identical forms of airborne particles composed of ammonium nitrate and carbon. The first experiment was undertaken to determine whether the Brown Norway (BN) rat can be treated with ovalbumin (OVA) to make it suitable as a model of an allergic airway condition to mimic asthma. The second experiment was designed to measure the physiologic, biochemical and structural effects of BN rats exposed weekly to aerosolized OVA for up to four weeks. The goal in this experiment was to optimize conditions of exposure to aerosolized OVA, while at the same time minimizing any overwhelming effects of OVA in the lungs that may potentially mask the effects of subsequent exposure to particles. Therefore, a sequential aerosol challenge protocol was employed and tested in BN rats. With knowledge gained from the first two experiments, a third experiment was designed to create an allergic airway that would be different from that of an untreated rat to allow us to test the effects of short-term exposure to PM on the structure and function of the lung airways and parenchyma. Physiologic, biochemical and histopathological parameters were used to assess potential particle-induced effects in this animal model. The final experiment was designed to determine the effects of repeated particle exposure on immune factors to potentially regulate an allergic response in the lungs. Complementary studies in human asthmatic volunteers also exposed to ammonium nitrate and carbon were studied. Biopsy materials from the airways of these individuals were obtained and examined under experimental culture conditions for potential detection of gene expression against a large panel of cytokines.

Results

(1) We established the ability to create an allergic airway model using the BN rat and ovalbumin. The model was created using a sensitizing dose of ovalbumin followed two weeks later with five sequential aerosol challenges of ovalbumin delivered in three-day intervals.

(2) We determined the best conditions in the BN rat to facilitate particle studies and measurement of particle-induced changes in the lungs. A single sensitizing dose of ovalbumin delivered two weeks prior to the onset of sequential ovalbumin aerosol challenges delivered on a weekly basis for up to four weeks was studied. We found BN rats rapidly adjust to repeated aerosol challenge. However, we also noted a robust centriacinar alveolitis associated with repeated challenge we felt might obscure any potential particulate matter effect. Therefore, a single ovalbumin challenge two weeks following ovalbumin sensitization was selected for future particulate matter studies.

(3) We found the effects of airborne particles on the airways of the Brown Norway rat with an allergic airway condition result in the following conditions: (a) The presence of a systemic serum IgE OVA-specific elevation induced by exposure to ammonium nitrate and carbon black particles over a period of two days; (b) a significant PM-induced effect on the airway epithelium to induce cell proliferation; and (c) a significant PM-induced elevation in mRNA levels of interleukin (IL)-4, a pro-inflammatory cytokine involved in augmenting an allergic-based response.

(4) Exposure of human asthmatic volunteers to identical ammonium nitrate and carbon particles produced tissue changes that could be detected by culture methodology. These included (a) detectable changes in expression for a large panel of cytokines, and (b) a distinct cytokine expression profile within the lung that can be elicited following stimulation with either antigen or a non-specific activator of cytokine expression.

Discussion

Animal toxicology studies in concert with human clinical studies can be used to determine specific consequences of exposure to particulate matter. The use of ammonium nitrate and carbon to create a unique exposure condition for animals with an allergic airways condition and human volunteers with a history of asthma provides a powerful and informative approach to assess the immunomodulatory and histopathologic impact of air pollutant exposures on the lungs. From animals with allergic airways, we found short-term particle exposure was associated with a significant perturbation of epithelial cells lining the intrapulmonary airways, transient elevation in mRNA levels for IL-4 and systemic changes in OVA-specific serum lgE levels. In humans, we found exposure to particles alone or in combination with ozone was associated with significant increases in mRNA levels for IL-1ß and IL-12p35.

Conclusions

Short-term exposure of animals and humans to airborne particulate matter of an identical nature has been shown to produce alterations in the lungs of both species. These changes involve the conducting airways of the respiratory system. Animal studies have shown the epithelium of the airways undergoes cellular proliferation and potential increase in the extent and severity of airway inflammation accompanied by an increase in mRNA levels of IL-4. Human exposure to PM was also associated with changes in expression for a panel of cytokines. Based on these findings, a combined approach to test sensitive animals and human asthmatics to similar particles by size and composition can serve to further elucidate the impact as well as the potential mechanisms of action of airborne particles on the respiratory system in individuals with

allergic airway disease. These studies further suggest the airway epithelium is an important target of particle-induced effects associated with inflammation and the perturbation of proinflammatory cytokines present in the lungs. This work provides further evidence of the impact of inhaled ammonium nitrate and carbon particles on the respiratory health of susceptible individuals with underlying respiratory disease.

Introduction

Numerous epidemiological studies have presented mounting evidence of adverse health effects associated with exposure to ambient airborne particles. These health effects appear to be greatest among susceptible populations of individuals including children and those with pre-existing cardiopulmonary disease. The mechanisms by which these adverse health effects occur with exposure to particulate matter are not clear. Asthmatic individuals could be more susceptible to airborne particulate matter due to underlying changes in the respiratory system associated with this disease process. The objectives of this project are to examine the functional and cellular responses of the respiratory system in asthmatic human volunteers and airway sensitized rats to controlled, short-term particle exposure.

In collaboration with investigators at the University of California, San Francisco (UCSF), we examined airway biopsy tissues obtained from human volunteers with a history of asthma. These individuals were exposed to carbon and ammonium nitrate particles. Identical particles were used at the University of California, Davis (UCD) to expose Brown Norway rats. Pulmonary function studies were done on human volunteers to examine airway function and biopsy samples were obtained to measure cellular responses along the tracheobronchial tree following exposure to carbon and ammonium nitrate. Brown Norway rats sensitized and challenged with ovalbumin were simultaneously studied at UCD following exposure to ammonium nitrate and carbon particles. Pulmonary function studies were performed and lung tissues obtained to study cellular (epithelial and interstitial) responses along the tracheobronchial tree and parenchyma. These combined studies in humans and animals provide the opportunity to better understand the relationship of particle exposure, airway inflammation, and cellular function in individuals with asthma, as well as potential insights into the effects of particle exposure in site-specific regions of the lungs. This combined group approach to examine both animals with sensitized airways (UCD), as well as human asthmatic volunteers (UCSF) provides a powerful data set to better elucidate the potential mechanisms by which airborne particles adversely affect the respiratory system of sensitive individuals. Such information is critical for better understanding the health effects of airborne particulate matter and is useful in addressing air quality issues that benefit public health.

Background and rationale for an animal model of allergic airways

Asthma is a pulmonary disease affecting millions of children and adults in the United States. The number of asthma cases in the United States has steadily increased since 1980 to a present day estimate of 12 million (Holt 1998, Swain et al 1990). The number of asthma-related deaths has also increased. From 1990 to 1995, the estimated cost of asthma-related care in the United States increased from 6.2 billion dollars to over 10 billion dollars (Abehsira-Amar et al 1992). The dramatic increase in the number of asthma cases worldwide, but especially in the United States, has brought enormous attention to this chronic inflammatory disorder of the airways.

There is still uncertainty between the correlation of inflammatory processes of asthma to airflow obstruction/bronchial hyperresponsiveness, also called "ticklish airways." However, recent advances, including the identification of appropriate animal

models, such as the Brown Norway rat, have enabled researchers to better study inflammatory cells and mediators involved in the pathogenesis of asthma (Hsieh et al 1992). Inflammatory cells such as mast cells and eosinophils are recruited during the asthmatic process (Shirakawa 1997). Other factors involved in asthma include airway hyperreactivity, mucous cell hyper-secretion, basal lamina thickening, and inflammation with recruitment of eosinophils and mast cells into the lungs. Any combination of three of these four factors is considered to be diagnostic for asthma (Scannel 1996).

The primary goal of this research project is to test the effects of particulate matter (PM) on the asthmatic lung. However, the first step is to identify whether the Brown Norway rat can be treated with ovalbumin to make it suitable as a model of asthma. Following sensitization, the rats were exposed to aerosolized ovalbumin for up to four weeks. The dose and exposure protocol that yields the closest resemblance of the rat lung to the human asthmatic lung was studied to determine optimal conditions to test particulate matter on the rat ovalbumin allergic (asthmatic) lung model. Corollary particle inhalation studies with human asthmatic volunteers were performed at the San Francisco General Hospital (UCSF) to determine the potential consequences of particle exposure on the physiologic, biochemical and immunomodulatory condition of the lungs following short-term exposure.

To characterize this model of allergic lung symptoms and disease, specific aspects of the lung and its responses to ovalbumin need to be evaluated. These include (1) The quantity of mucus in the lungs, since mucosal inflammation may play a role in the pathogenesis of asthma, as well as chronic airflow limitation and airway hyperresponsiveness (AHR) (Gent et al 2003); (2) the severity of the inflammation in the centriacinar (BADJ) region and the perivascular space of the blood vessels of the lungs and (3) cellularity in the perivascular space and epithelial and submucosal layers of the central airways. Each of these features may be associated with an allergic condition reminiscent of the asthmatic lung.

Contribution of airborne particles to respiratory disease

Particulate matter (PM) pollution is a worrisome air contaminant problem facing the public, scientific communities and regulatory agencies. A number of epidemiological studies suggest an association between ambient particulate matter in the environment and increased morbidity and mortality in individuals with compromised pulmonary function, including asthma. Asthma affects more than 15 million Americans. The incidence of asthma in young children has increased 75% from 1980 to 1994 and is still increasing. Asthmatic individuals could be more susceptible to airborne particulate matter due to underlying changes in the respiratory system associated with this disease process. Evidence suggests that exposure to PM poses significant health risks to those individuals with pre-existing cardiopulmonary conditions, but the mechanisms and severity of these effects are unknown.

Numerous studies (many unreported) have failed to demonstrate significant effects from airborne particles. We hypothesized exposure to PM would exacerbate inflammation in allergic airways of both animals and humans. By utilizing a model of allergic airways in Brown Norway (BN) rats, we opted to examine potential changes in levels of serum IgE as well as markers of airway change and proliferation using a nucleotide analog, bromodeoxyuridine (BrdU) in rats exposed to PM compared with FA

controls. In human subjects with a known history of asthma, we examined biopsy materials obtained from these individuals to determine if exposure to PM alone or in combination with ozone caused increases in mRNA levels for cytokines known to be associated with inflammation and injury.

We generated airborne particles by aerosolization of ammonium nitrate and carbon in a size range less than 2.5 μm in diameter. Such particles are within a size range that should be easily respirable for laboratory rodents as well as humans. Ammonium nitrate and carbon are also the most common chemical forms of PM present in the atmosphere of California and have therefore been used for both our animal studies at UC Davis as well as human studies at UC San Francisco.

The development of airway inflammation and hyperreactivity in BN rats sensitized to ovalbumin (OVA) closely mimics the pathophysiology of human asthma (Allakhverdi et al, 2002; Sapienza et al, 1991). A goal of this study was that the effects of PM exposure in this animal model would provide essential data to better elucidate specific factors relating PM exposure to augment and/or exacerbate asthmatic symptoms in humans.

Brown Norway rats sensitized to OVA and challenged with OVA aerosol are a reasonable model of asthma due to their ability to mount a Th-2 response via T-cell mediated sensitization to allergens, in a similar manner observed for individuals predisposed to developing allergies and allergic diseases (Amin et al, 2000; Careau et al, 2002; Hakon et al, 1999; Hideyasu et al, 1998). However, the mechanisms by which adverse effects occur as a result of particle exposure are not clear. Therefore, we examined changes in pulmonary function and inflammatory cell profiles in sensitized BN rats to characterize potential factors responsible for exacerbating the asthmatic state. The acquisition of this information is critical to better understand potential particle-induced health effects observed in susceptible populations.

The primary goal of this research project was to test the effects of particles (PM) on allergic lung airways. The first experiment was to demonstrate whether the Brown Norway rat can be treated with ovalbumin (OVA) to make it suitable as a model of asthma. The second experiment was to demonstrate the physiologic, biochemical and structural effects of BN rats exposed weekly to aerosolized OVA for up to four weeks. In this second experiment, the goal was to optimize conditions of exposure to aerosolized OVA, while attempting to minimize any overwhelming changes in the lungs that may potentially mask the effects of subsequent exposure to particles. In Experiment 2, a sequential aerosol challenge regimen was employed in BN rats. With knowledge gained from the first two experiments, the third experiment was designed to create an allergic airway that would be different from that of an untreated rat to allow us to test the effects of short-term exposure to PM on the structure and function of the lung airways and parenchyma. Physiologic, biochemical and histopathological parameters were used to assess particle-induced effects in this animal model. The fourth and final experiment was designed to determine the effects of repeated particle exposure on immune factors to potentially regulate an allergic response in the lungs.

Materials and Methods

Animals

For all four animal experiments described, respiratory pathogen-free Brown Norway (BN/SsNHsd) rats were obtained through Harlan, Inc. (Indianapolis, IN) from barrier 218B (Prattville, AL). All animals were male. For the initial experiment to study ovalbumin aerosol-induced allergic response in BN rats, pups were four weeks of age when sensitization to ovalbumin was started. For the subsequent temporal study of the ovalbumin aerosol-induced allergic response, rats were about eight weeks of age on arrival. For the ammonium nitrate and carbon (NH_4NO_3+C) simulated particulate matter aerosol (PM) exposures, rats were about 10 to 11 weeks of age on arrival with a body weight range of 200 to 250 g. The animals were allowed to acclimate at least one week in chambers with filtered air before any experimental procedures were started.

Human Subjects

All subjects were informed of the risks of the experiment and provided informed consent prior to participation. The procedures for this experiment were approved by the University of California, San Francisco, Institutional Review Board, Committee on Human Research.

All subjects completed a medical history questionnaire, were current non-smokers, had no history of excessive smoking, and had no serious health problems. Female subjects were not pregnant throughout the project. Subjects had no respiratory-tract illness in the three weeks preceeding, or during, each session. Subjects were characterized by physical characteristics, spirometric pulmonary function, non-specific airway reactivity, and allergy skin test.

The 10 subjects had mild to moderate asthma, and were otherwise healthy. Asthma status was determined using the guidelines of the National Asthma Education Program (National Asthma Education Program Expert Panel, 1997). All subjects had non-specific airway reactivity of < 10 mg/ml methacholine. Subjects were characterized by physical, pulmonary, allergy, and medication characteristics.

Ovalbumin aerosol generation and characterization (BN rats)

Ovalbumin aerosol exposure methods were similar to those described for our procedures for house dust mite allergen in Schelegle *et al.* (2001). Grade V chicken egg albumin (Sigma-Aldrich, Inc., St. Louis, MO) 2.5% by weight was diluted in Dulbecco's phosphate buffered saline (PBS) without calcium chloride and magnesium chloride (Invitrogen Corp., Grand Island, NY). This solution was nebulized with a high-flow rate compressed air nebulizer (HEART®, Westmed, Inc., Tucson, AZ) operated at 1.69 kg/cm^2 for a flow rate of 11.9 liters/min. The resulting droplets were diluted with a 48.3 liters/min stream of dry air and conveyed upward through a 33.6 liter volume krypton-85 discharging column to reduce electrostatic charge (Teague et al, 1978). The aerosol was finally mixed with the inlet air stream of a 4.2 m^3 volume exposure chamber, producing in the chamber, an aerosol of solid particles composed of ovalbumin with salt residue. Each ovalbumin aerosol exposure was conducted for 30 minutes after allowing 18 minutes for complete chamber equilibration to 99% of the final concentration.

The ovalbumin aerosol was characterized by samples drawn from the animal breathing zone of the chamber. Total mass concentrations were measured by weighing samples collected on preweighed Teflon® coated glass fiber filters (Pallflex EMFAB, Pall Gelman Sciences, Ann Arbor, MI). The particle samples were also submitted to the UC Davis Molecular Structure Facility to measure the protein concentration. The particles collected were extracted and the protein content was determined by amino acid analysis (Ozols, 1990) (System 6300, System Gold software, Beckman Coulter, Inc., Fullerton, CA). Aerodynamic size distributions were determined from samples collected with a Mercer-type cascade impactor (Mercer et al, 1970). The content of chloride anion derived from saline residue in the particles was measured on each of the seven impactor stages and the after-filter by ion chromatography (Model DX-120, Dionex Corp., Sunnyvale, CA). A log-normal distribution was fitted to each sample set of data. The values reported are the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (σ_g) of the fitted distributions. In addition, a sample was collected on a 0.2 µm pore size Nuclepore® filter (25 mm, Whatman, Inc., Clifton, NJ) during exposure for examination by microscopy.

Inhalation Chambers (BN rats)

Exposures of rats were conducted in 4.2 m^3 volume stainless steel and glass exposure chambers that were updated from a design originally described by Hinners *et al.* (1968). This chamber design with square cross section (137 cm x 137 cm) and pyramidal top with tangential cylinder mixing inlet is perhaps the most widely used for animal inhalation exposure studies and can be appropriately termed a conventional design. These chambers have a well-documented capability of producing homogeneous distribution of aerosols and gases (Hinners et al, 1968; MacFarland, 1983). Distribution studies most representative of actual exposure conditions were those conducted by Hinners *et al.* (1968) that evaluated retention of inhaled bacterial aerosols in the lungs of exposed mice. They demonstrated that uniform concentrations were produced at all cage positions on a given level in the chamber.

In our facility the chambers are connected to a common air handling system in which the air supplied passed through two pre-filters, a high efficiency particulate air (HEPA) filter and finally, an activated charcoal adsorber to remove most air pollutants. Each chamber was operated at an airflow rate of 2.1 m^3/min. The high rate of ventilation at 30 air changes per hour causes rapid chamber atmosphere equilibration and lowers the level of airborne contaminants from the animals housed within. However, the chamber used for ovalbumin aerosol exposure was operated at 1.05 m^3/min for 15 air changes per hour to permit generating higher aerosol concentrations. For this series of exposures, chamber relative humidity was maintained at 44.0 ± 11.9% at a temperature of 24.7 ± 0.5° C (mean ± SD for all exposures).

Animals were held in a single level array of specially fabricated open mesh, stainless steel cages that are not reactive to ozone and permit unrestricted atmosphere exchange between the interior of the cages and the exposure chamber. Food and water were provided *ad libitum*. The feed was LabDiet® 5001 Rodent Diet (PMI® Nutrition International, LLC, Brentwood, MO). Micro filtered deionized water was provided via automatic watering systems. A small animal load relative to the chamber volume was used. Waste was flushed daily from the chambers. Animal care complied

fully with the guidelines established by the Institute of Laboratory Animal Resources (1996). One chamber was used for ovalbumin aerosol exposure, another contained the NH_4NO_3+C or PM aerosol, and a third chamber contained only filtered air and was used as a control atmosphere. The two chambers used for aerosol exposure were each fitted with an aerosol discharger and conditioning column as well as other items necessary for precisely controlled aerosol generation and characterization.

Inhalation Chambers (Human)

The exposure sessions were conducted in a custom-built steel and glass exposure chamber (Nor-Lake Inc., Model No. W00327-3R), which is 2.5 m x 2.5 m x 2.4 m in size, and has an average airflow rate of 300 ft^3 min. The chamber air supply is sourced from ambient air, which is filtered by passing through purifying (Purafil Model No. 6239), and high efficiency particle (Aeropac Model No.53 HEPA 95) filters. The filtered air is dehumidified by passing through a dryer (Cargocaire Engineering Corp.). HC-575), and the air temperature is decreased with a chilled-water coil. Subsequently, temperature and humidity are increased with steam (Nortec Model No. NHMC-050), to attain the pre-set temperature (20 ^{O}C) and relative humidity (50%) conditions in the chamber. The temperature and relative humidity inside the chamber are monitored (LabView) and controlled throughout the exposures (Johnson Controls, Model No. DSC 8500).

Generation and Characterization of PM (BN rats)

PM aerosol exposure methods were adapted from those described in Kleinman *et al.* (2000). Elemental carbon as carbon black called "Monarch® 120" was obtained from the Cabot Corporation (Billerica, MA). We selected this material for our studies due to its particle size, purity and ease in aerosolization. This carbon black consists of primary particles with a mean diameter of 75 nm in clusters with a smallest dispersible aggregate diameter of 150 to 200 nm (Cambrey, 1997). The carbon used for these studies was derived from a single manufacturing batch. Dr. Barbara Zielinska of the Desert Research Institute, Reno, NV, completed a comprehensive gas chromatography-mass spectrometry analysis of this material. Samples were taken from two 1 kg containers randomly selected out of 11 total containers. Analysis was performed to detect the presence and measure a variety of polycyclic aromatic hydrocarbons and other contaminants that might compromise assessment of the contribution of the elemental carbon to toxicity during our exposure studies. Analysis revealed very low contaminant concentrations. Fluoranthene at 0.003% and pyrene at 0.011% were the major contaminants. Results from each of the two containers from the same manufacturing batch were within 1% agreement. We, therefore, concluded that this carbon was suitable for our studies. A copy of the detailed results of this analysis is included in the final report for a previous California Air Resources Board (CARB) contract (Pinkerton et al, 2000).

The carbon was weighed and dispersed in dilute NH_4NO_3 (Analytical Reagent, Mallinckrodt Chemical, Inc., Paris, KY) solutions with an ultrasonic probe to form a slurry that was stirred for two to four days before use. This mixture was nebulized by using a modified compressed air nebulizer operated at approximately 4 liters/min. The nebulized droplets were diluted with the introduction of an equal flow rate of dry air in a

radial dilutor and conveyed upward through a vertical conditioning column 198 cm long and 14.7 cm in diameter. The column contained a ^{85}Kr source to ionize the air and therefore, reduce charge on the particles to near Boltzman equilibrium (Teague et al, 1978). The conditioned aerosol was introduced into the mixing inlet of the exposure chamber where the PM was further diluted in a chamber flow rate of 2.1 m^3/min. NH_4NO_3 deliquesces at a relative humidity of 61.2% at 25° C (Mercer et al, 1970; Kleinman et al, 2000). Since the chamber relative humidity during these studies (44.0 ± 11.9% at a temperature of 24.7 ± 0.5° C, mean ± SD) was below this deliquescence point, the PM phase was solid particles composed of NH_4NO_3 salt residue and carbon. Slurry concentrations of NH_4NO_3 and carbon were selected to ultimately produce in the chamber an aerosol of the desired mass concentrations with a mass median aerodynamic diameter (MMAD) of about 1 µm. Compressed air flow through the nebulizer was also adjusted slightly during exposure as a fine control of total mass concentration in each chamber containing the PM.

The carbon black particles are very hydrophobic and are difficult to keep suspended in an aqueous solution. A major problem was preventing the loss or stratification of carbon from the slurry during a six-hour exposure interval. The carbon particles tend to agglomerate rapidly, deposit on and adhere to the wetted surfaces of nebulizers, pumps, reservoirs, tubing and other components in the system. The carbon adhered less to glass and PFA Teflon® in our system, and these materials were given preference. Obviously, losses from the slurry cause lower carbon content in the aerosol, and the build up of deposits can cause poor nebulizer performance. Therefore, the nebulizer was modified to permit a continuous flow of slurry through it, minimizing recirculation within. The slurry was pumped to the nebulizer from a glass reservoir containing 3.5 liters, more than sufficient for a six-hour period. The slurry was constantly stirred to prevent the carbon from stratifying and accumulating at the bottom of the reservoir. Fresh liquid was provided to the nebulizer in excess of about 40 times the amount being nebulized. Excess slurry was continually pumped from the nebulizer to a waste vessel. Components were arranged so that tubing lengths were minimized. For supply a small internal tubing diameter about 1.6 mm was selected to maximize the velocity of the pumped liquid, and, therefore, reduce the contact time of the slurry with the pump and tubing.

Measurements of exposure concentrations of NH_4NO_3 and carbon were by standard methods used in ambient air monitoring. This ensures that the exposure data are fully comparable. Ion chromatography (Model DX-120 with PeakNet software, Dionex Corp., Sunnyvale, CA) was used to analyze NH_4NO_3 sampled on filters from the chambers, the same method used by CARB (1992). Dr. Kochy Fung of Atmospheric Assessment Associates, Inc., Calabasas, CA determined carbon content of the particle samples by selective thermal oxidation and subsequent flame ionization detection (Fung 1990).

Detailed characterization and monitoring were performed during each exposure. All air samples were drawn from the animal breathing zone of the chamber. Air sampling devices with probes were inserted through specially designed ports located on each side of the chamber in the animal holding volume. Known volumes of air were drawn at constant flow rates through filters to determine mass concentrations and a Mercer-type cascade impactor (Mercer et al, 1970) to determine aerodynamic size.

During 6-hour exposure intervals, two pairs of samples for NH_4NO_3 analysis were collected on modified polysulfone membrane filters (Supor®-800, 47 mm diameter, Pall Gelman Sciences, Ann Arbor, MI) during each three-hour segment of the interval. These samples were drawn for 60 min. at a flow rate of 21 liters/min. Within one hour after the sample was collected, deposited PM was extracted by adding aliquots of water and sonicating the filters for 60 min. Then nitrate was analyzed by ion chromatography. Simultaneously with the nitrate samples, two pairs of samples for carbon analysis were collected on quartz fiber filters (QM-A, 47 mm diameter, Whatman, Inc., Clifton, NJ). These samples were drawn for 60 min. at a flow rate of 22 liters/min. The carbon samples were sent to Dr. Fung for analysis (Fung 1990).

Samples for total mass concentration and aerodynamic size with cascade impactors required longer sampling periods to collect sufficient amounts for analysis. Therefore, one pair of total mass concentration determinations was made for each daily interval. The PM was collected on preweighed Teflon® coated glass fiber filters (Pallflex™, EMFAB, 47 mm diameter, Pall Gelman Sciences, Ann Arbor, MI). These samples were collected at 22 liters/min., and the filters were weighed after the sampling period of approximately five hours. For aerodynamic size determinations one cascade impactor sample was drawn for each daily six-hour exposure. Air was drawn through the impactor at 0.6 liters/min. Glass coverslips were used on each of the seven cascade impactor stages and a Supor™ membrane was used for the after filter. Each stage and after filter were analyzed by ion chromatography for the mass of nitrate collected. The resulting data were fitted with a log normal distribution to derive the mass median aerodynamic diameter (MMAD) and geometric standard deviation (s_g) of the PM size distribution. A piezobalance aerosol mass monitor (Model 3511, Kanomax Japan, Inc., Osaka, Japan) was also used during these exposures. Hourly measurements of the PM aerosol were made with this mass monitor. This instrument permitted a determination of total mass concentration after a two-minute sampling period. It was used for adjustment of chamber mass concentrations during exposures and allowed rapid detection of problems with aerosol generation. Also, a PM sample was collected on a 0.2 µm pore size Nuclepore® filter (25 mm, Whatman, Inc., Clifton, NJ) during the study for examination by microscopy to see the general appearance of the particles.

Generation and Characterization of PM (Humans)

Particle Generation and Measurement:

The carbon and ammonium nitrate particles were generated using a solution of 2% carbon and 2% ammonium nitrate and series of five nebulizers (McGraw Respiratory Therapy), using compressed medical grade air. The outlet from the nebulizers went directly into the inlet duct of the exposure chamber.
The total particle concentration was measured at the subjects breathing zone using a filter (Pallflex; 0.22 µm), sampling at 14 l min. The filter mass was determined pre- and post-sampling (Micro-systems). Particle concentration samples were collected for the complete 30 min of each exposure.

Study Design: BN rat Experiment 1

Three groups of Brown Norway rats were studied in this experiment (Table 1).

Table 1. Research design of Brown Norway rat asthma model.

Group 1 (n=8)	Group 2 (n=8)	Group 3 (n=8)
sham sensitization + filtered air	sensitization + filtered air	sensitization + ovalbumin challenges

All animals were placed on a pulverized rat chow diet, given water *ad libitum*, and maintained on a 12 hour light, 12 hour dark cycle. By random selection all animals were assigned to one of three groups identified in Table 2. Exposure to ovalbumin was done following the activity time-line shown in Figure 1 and explained below.

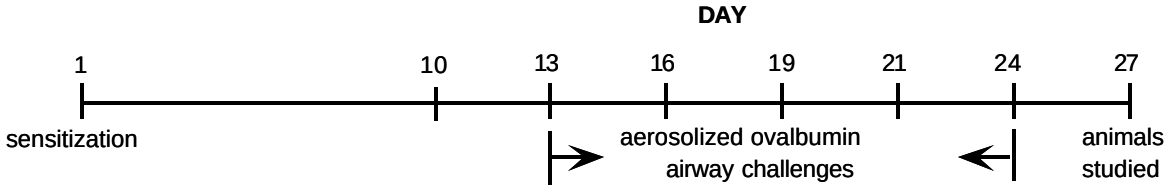


Figure 1. Activity time-line.

Sensitization to antigen and antigen challenge:

Following weaning (21-28 days of age) Brown Norway rats were anesthetized with 5% methoxyflurane. Each animal received a subcutaneous injection of 0.5 ml of a sterile suspension of 0.5 mg ovalbumin (OA) and 100 mg of aluminum hydroxide in 0.9% saline. At the same time, 0.5 ml of Boretella pertussis vaccine containing 6×10^9 heat-killed bacilli was given intraperitoneally as an adjuvant. Fourteen days following antigen sensitization (35-42 days following birth) the rats were exposed to aerosolized ovalbumin (suspended in phosphate buffered saline) introduced into a stainless steel exposure chamber (14 inches x 9.5 inches x 6 inches) for an average of 49 minutes and repeated five times at three-day intervals.

Measurement of bronchial responsiveness:

All animals were studied three days after the last airway challenge. Animals were anesthetized with alpha-chloralose/urethane (0.1 g/kg alpha-chloralose, 10 g/kg urethane at 4 ml/kg IP) and intubated with a 14 gauge catheter. Changes in airway

resistance to increasing doses of methacholine were examined by delivering aerosolized methacholine for one minute and measuring the change in airway resistance for the next three minutes. The animal was allowed to recover for five minutes and a second methacholine challenge was initiated. A starting dose of 0.125 mg/ml methacholine was used and continued in doubling doses until airway resistance had doubled or a concentration of 64 mg/ml methacholine had been reached. The concentration of methacholine required to double lung resistance (EC200RL) was obtained by linear interpolation between the two concentrations bounding the point at which lung resistance reached 200% of control.

Methods for quantitative assessment of lung histology:

Immediately following bronchial responsiveness evaluation, the lungs were removed and fixed at a volume of 30 cm H_2O using zinc-formalin (Z-fix, Anatech Battle Creek, MI.) for one hour. The lungs and mediastinal contents *in situ* were removed and placed in fixative. Tissue sections were prepared by cutting transverse lung slices immediately cranial and caudal to the hilum of the left lobe. Each tissue slice was embedded in paraffin and sectioned using a Microm HM 355 rotary microtome (Zeiss, Thornwood, NY). Four distinct anatomical regions from the lungs of each animal were examined: 1) the main axial airway path of the left caudal lobe, 2) the general pulmonary vasculature, 3) the terminal bronchiole and 4) the lung parenchyma. All sections were cut 5 μm thick. Serial tissue sections were stained with hematoxylin and eosin (H&E) to observe general pulmonary structures, alcian blue/periodic acid Schiff (AB/PAS) for epithelial distribution of mucin, sirius red for collagen and basement membrane features, combined eosinophil/mast cell (CEM) stain for visualization of eosinophils and mast cells, and Masson's trichrome (MT) for the distribution and abundance of smooth muscle.

To define the general features of the central airways in the Brown Norway rat lung, the main axial airway pathway of the left lung lobe was examined. This airway was examined in cross-section at the level of the third to sixth generation to confirm that the same general airway site would be described for all animals studied. In contrast, using a process of random field generation for each tissue section (Weibel, 1980; Pinkerton and Crapo, 1985), a total of 10 blood vessels, five terminal bronchioles and five parenchymal regions immediately arising from terminal bronchioles were examined to ensure an unbiased analysis of these anatomic features in the Brown Norway rat. Blood vessels selected appear in cross-section and 75 μm or greater in diameter. Both arteries and veins were combined in the analysis. Terminal bronchioles were identified in tissue sections as airways directly opening into alveolar-lined ducts.

Airway mucin was measured for all airways. Weakly acidic sulfated mucosubstances stained turquoise, while mucosubstances containing glycol groups stained magenta (Luna, 1968). To determine the volume of mucin present in the epithelium of each airway generation examined, single fields from four quadrants of each airway cross-section were captured using an Olympus BH2 microscope at 400X magnification. All images were captured using a Dage camera system interfaced to a MacIntosh 8100 computer. Each image was oriented with the basal lamina underlying the epithelium in the horizontal plane. Sections stained with alcian blue/periodic acid Schiff stain were used to identify intracellular mucosubstances of the epithelium. The

area of intracellular mucin present in each field was determined using the density gradient function of the stereology NIH Image program to highlight stained mucosubstances. The length of basal lamina present in each field was also measured using NIH Image program. The area of mucin within the epithelium was expressed per length of basal lamina for all airways.

The relative abundance of eosinophils and mast cells within the walls of airways and blood vessels were measured as the number of cell profiles per basal lamina length. For airways, both cells present within the epithelial layer as well as within the interstitial wall were combined. Collagen and basal lamina volume was measured for each airway using a 550 nm filter to enhance visualization of sirius red (excitation maximum wavelength approximates 550 nm) stained substances. These measurements were expressed per basal lamina surface area of each airway.

The relative cellularity of the blood vessel wall as well as the centriacinar regions of the lungs defined as those areas of parenchyma immediately arising from terminal bronchioles was based on a semi-quantitative scoring system. Normal structures with no influx of cells was defined as "0", while a mild influx of cells "+", moderate influx "++" and marked or severe influx of cells as "+++".

Statistical analysis:

Bronchial responsiveness data and morphometric measures of cell mucin content, epithelial volume, and collagen were analyzed using a one-way ANOVA (Statview, SAS institute, Cary, NC). Post hoc analysis was done using the Scheffe Test. Significance was set at $p < 0.05$. The cellularity scores of the the centriacinar regions as well as the walls of blood vessels and airways were analyzed using nonparametric ranking.

Study Design: BN rat Experiment 2

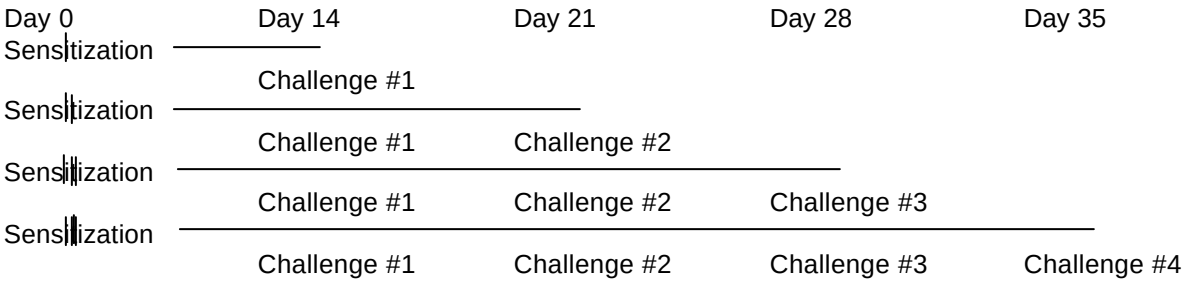
Experimental Protocol: Animal sensitization and challenge with ovalbumin

Brown Norway rats, nine to 12 weeks old, were anesthetized with 5% methoxyflurane. They subsequently received a subcutaneous injection of 0.5 ml sterile suspension of 0.5 mg ovalbumin (OVA) along with 100 mg of aluminum hydroxide in 0.9% saline. An intraperitoneal injection of 0.5 ml *Bordetella pertussis* vaccine with $6x10^9$ heat-killed bacilli was also given as an adjuvant. Two weeks following sensitization, the rats were challenged with inhalation of aerosolized ovalbumin. The solution was 25 mg/ml Grade V ovalbumin in phosphate buffered saline (PBS). Aerosol challenge with ovalbumin occurred in an exposure chamber (14 inches x 9.5 inches x 6 inches; [ovalbumin], [filtered air]) for a period of 48 minutes, once per week. There was an initial period of 18 minutes for chamber equilibration, where the concentration gradually reached its desired level using a large column ^{85}Kr discharger with a high flow rate nebulizer. This was followed by a full exposure period of 30 minutes. The first set of animals (n=7) were necropsied 48 hours following aerosol challenge to examine their lungs. The remaining rats were serially challenged once per week with ovalbumin for the following three weeks. Forty-eight hours following each aerosol challenge, rats (n=7) were necropsied. In this manner, each group of rats at necropsy received from one to four serial ovalbumin aerosol challenges (Table 2).

Table 2

Exposure regimen for sensitization and challenge of Brown Norway rats

Animals used are approximately 9-12 weeks old at commencement of exposure regimen



Necropsy two days following final ovalbumin aerosol challenge

Pulmonary function testing

Three days following OVA challenge, two rats from the filtered air control and six antigen-challenged rats were anesthetized with an IP injection of 0.4ml/100gm body weight alpha-chloralose/urethane in saline. A calibrated cannula was surgically placed midtrachea and the rat placed on a respirator (94-98 resp./min) in a whole body plethysmograph for pulmonary function testing. The plethysmograph measures changes in flow. An injection of parcuronium IP was given to prevent reflex respiration.

A fluid filled catheter was placed in the thoracic esophagus. Transpulmonary pressure was measured electronically (Validyne DP 15-26 transducer) by subtracting tracheal pressure from esophageal pressure. Tidal volume was at a constant value and based on body weight. Resistance was determined by dividing the change in pressure by the change in flow. Bronchial responsiveness measured as a function of an increase in pulmonary resistance was determined by serial exposures to increasing concentrations of methylcholine from 0.0625mg/ml to 64mg/ml using saline as a control. Each challenge was for one minute followed by three minutes of recovery before the next challenge. Testing was suspended when resistance had doubled from the saline control.

Tissue preparation/morphometric tissue analysis

Once exposures were complete, the rats were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital. After exsanguination by the caudal vena cava, a ventral incision was made in the trachea and cannulated at the larynx. The thorax was collapsed by rupturing the diaphragm. To fix the lungs, the lungs were inflated at 30 cm of pressure with Z-fix containing 4% paraformaldehyde, by tracheal instillation. After the lungs had fixed for one hour, the heart, lungs, and mediastinal contents were removed *en toto*. Histological sections (5 μm thick in paraffin) prepared from transverse slices of the fixed left lung lobe embedded in paraffin. Slides were stained with Combined Eosinophil and Mast Cell Stain (CEM), which contains Astra Blue Stain, Vital New Red Stain, and Modified Mayers Hematoxylin. CEM stains eosinophils a bright pink color and mast cells a vivid turquoise color.

The centriacinar (terminal bronchiole/acinar duct [TB/AD]) and blood vessel (BV) regions were examined for cellularity and cellular inflammation. The region includes the terminal bronchioles and the extending parenchyma, or acinar duct area around it. Maps were made using the Olympus BH-2 microscope, a black and white camera (model MTI CCD72S), and National Institute of Health (NIH) imaging software. These were marked during observation to note the centriacinar regions and blood vessels being examined during the cellular inflammation and cellularity data collection processes. A semi-quantitative grading scale was used to standardize the results and to give an unbiased picture of the comparative amount of inflammation in the lungs of each rat. The scale for the centriacinar areas, (i.e. bronchiole alveolar duct junctions [BADJ]) and perivascular space (area around blood vessels) used 0 as no inflammation, + as mild inflammation, ++ as moderate inflammation, and +++ as severe inflammation. The degree of inflammation for each BADJ and perivascular space was determined by viewing the slides under an Olympus BH-2 microscope.

Perivascular spaces were further evaluated by noting the number of eosinophils and mast cells present within the perivascular space surrounding each blood vessel. This percentage was determined by adding the number of eosinophils and mast cells together, and dividing by the total number of cells around each blood vessel. Each blood vessel's perivascular space was counted using the Olympus BH-2 microscope. Eosinophils and mast cells were also counted in the epithelium and submucosal layers of the central airways of each lung. These were made into comparable results by dividing by the basal lamina length. The basal lamina is the membrane between the epithelium and the submucosal layer. To find this, the NIH Imaging (V. 1.62f) program was also used. After taking color pictures of the parts of the central airway using the color camera (model OLY-750; Scion Imaging V. 1.62c), the scale was set and a free-form line drawn along the basal lamina in each picture. This line could then be measured to give the length of the basal lamina for that portion of central airway. The different sections were calculated and their lengths were added to attain the length of the central airway basal lamina.

These eosinophils and mast cells are excellent indicators of an allergic response. Both cell types are transported through the blood vessels to the perivascular space during periods of lung injury and allergic response. These cell counts are done to examine the extent of the inflammation in conjunction with the initial inflammation analysis. Since elevated levels of eosinophils and mast cells are characteristic of asthma, counting these cells and finding percentages using total cell count would give an indication of the extent of the asthma in the lungs of the rats.

Study Design: BN rat Experiment 3

Table 3 summarizes the experimental design of this study to examine the effects of exposure to particles following a single sensitization and a single challenge to OVA. A number of biological endpoints were selected for analysis to include pulmonary function testing (PFT), bronchoalveolar lavage (BAL), cell permeability (EtD-1), DNA synthesis (BrdU), and histopathology.

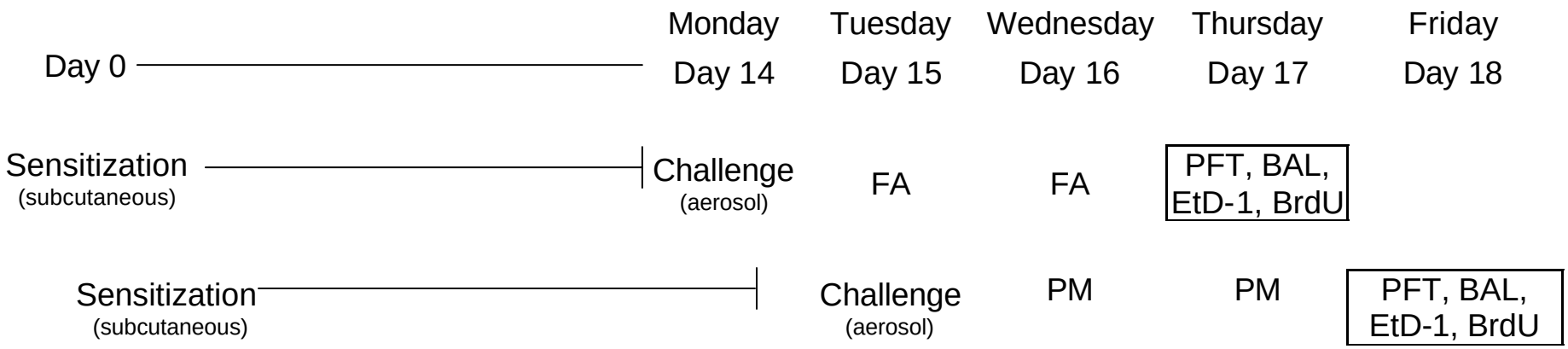
Animals and treatment groups

Brown Norway rats were obtained from Harlan Sprague Dawley Inc. (Pratville, AL). All animals were allowed to acclimate one week prior to the onset of the experiment. All rats were randomly divided into three groups. Group 1 consisted of rats receiving only a single OVA challenge. Group 2, was sensitized with OVA by intraperitoneal injection, but was not subsequently challenged with OVA aerosol. Group 3 received OVA sensitization by a single IP injection, followed 14 days later by a single challenge aerosolized OVA. Each of the three groups were exposed for 2 days to filtered air (FA) or PM prior to pulmonary function testing and necropsy (Figure 1).

Sensitization and challenge with ovalbumin in BN rats

Sensitization to OVA in rats was done by subcutaneous (subQ) injection with a 1.0 ml suspension containing 1 mg chick ovalbumin and 200 mg aluminum hydroxide in normal saline. At the time of sensitization, rats were also given an IP injection of 0.5 ml saline containing 6×10^9 killed B. pertussis organisms (obtained from the Michigan Dept Public Heath, E. Lansing , Michigan 48909) to act as an adjuvant.

Table 3: Rat Ovalbumin Sensitization, Challenge, and PM Exposure Regimen



PM = particulate matter (150 μg/m^3 ammonium nitrate + 100 μg/m^3 carbon black)

	Treatment		Number of Animals		
Experiment	OVA	Particles	PFT/BAL	BrdU	EtD-1
1	N/C	FA	8	6	6
	N/C	PM	8	6	6
2	S	FA	8	6	6
	S	PM	8	6	6
3	S/C	FA	8	6	6
	S/C	PM	8	6	6

N/C = non-sensitized + challenged
S = sensitized
S/C = sensitized + challenged

PFT = pulmonary function testing
BAL = bronchoalveolar lavage
BrdU = bromodeoxyuridine
EtD-1 = ethidium homodimer-1

Two weeks following OVA sensitization, rats were challenged to OVA by a single aerosol delivery. The OVA exposure protocol was adapted from that previously described by Schelegle and colleagues (2001). The total aerosol mass concentration was measured gravimetrically using pre-weighed Teflon-coated glass fiber filters (Pallflex EMFAB, Pall Gelman Sciences, Ann Arbor, MI). Aerosol samples were submitted to the UC Davis Molecular Structure Facility to Measure protein concentration by extraction and amino acid analysis (System 6300, System Gold Software, Beckman Coulter, Inc., Fullerton, CA). OVA sensitized and challenged rats were randomly divided into two groups designated as FA or PM. Rats only receiving OVA sensitization or OVA challenge were also randomly divided into FA and PM groups.

Exposure protocol

All rats were housed as described under the subsection on inhalation chambers (page 5).

Pulmonary function testing

Pulmonary function testing (PFT) was performed two days following exposure to particles or filtered air and/or four days following ovalbumin aerosol challenge as described under exposure design, experiment 1, Measurement of bronchial responsiveness (pages 9-10).

Bronchoalveolar lavage

Following PFT, the lungs were lavaged three times with a single dose of PBS at 35ml/kg BW. The recovered lavage volume was recorded and centrifuged at 4ºC at 2500 rpm for 10 min. The pellet was re-suspended in PBS with 10µl trypan blue (Gibco, Grand Island, NY) to a final volume of 1ml. WBC/ml and cell viability were determined by counting cells with a hemocytometer. Differentials were determined by counting 500 cells from each cytospin preparation (Shandon Southern Instruments, Pittsburgh, PA) stained with Hema 3 (Biochemical Sciences Inc).

BAL supernatants were analyzed for mg protein/ml by utilizing the BioRAD protein assay as per manufacturer's directions. Samples were measured against a known BSA protein standard spectrophotometrically at 595 nm.

Necropsy and tissue fixation

Subsets of animals not undergoing PFT were used to examine (1) cell proliferation, (2) cell permeability, (3) immunohistochemistry, and (4) histopathology within lung tissues. The day following the final exposure period to particles or filtered air, each animal was deeply anesthetized by IP injection with sodium pentobarbital. A cannula was placed in the trachea. Prior to fixation, the abdomen was opened by surgical incision and the lungs collapsed by rupture of each hemidiaphragm followed immediately by intratracheal instillation of 4% paraformaldehyde (Z-fix, Anatech LTD) at a hydrostatic pressure of 30 cm for one hour. The lungs and mediastinal contents were subsequently removed *en bloc* from the thoracic cavity and stored in fixative for later embedment and sectioning.

Cell proliferation

For cell proliferation studies, each animal had surgically implanted subcutaneously a miniosmotic pump (Alzet) filled with bromodeoxyuridine (BrdU) solution (30 mg/ml). These pumps were surgically placed one week prior to necropsy to provide a continuous infusion of BrdU systemically for the purpose of identifying cells undergoing DNA synthesis and/or repair during this one week period of time. A portion of intestine was also excised and placed in fixative for embedment with lung tissue from each animal to serve as a positive control for BrdU immunohistochemistry.

Cell permeability assay: Ethidium homodimer-1

For the assessment of cell permeability following exposure to filtered air or to particles, a subset of animals were anesthetized and lavaged with ethidium homodimer-1 (Molecular Probes Cat#1169) for 10 min before infusion of Karnovsky's fix at 30 cm pressure for one hour. The lungs were removed and stored in fixative for later airway microdissection and analysis using confocal laser scanning microscopy.

OVA-specific IgE immunohistochemistry

Immunohistochemisry was done on 5 µm thick paraffin sections for IgE using ova-specific monoclonal mouse anti-rat IgE antibody purchased from Pharmagen, BD Biosciences (ref.). Briefly, paraffin sections were baked at 56ºC for one hour then rehydrated through increasing concentrations of ethanol after removal of paraffin with three 5-minute xylene washes. Antigen capture was done for two minutes in boiling 0.5M EDTA pH 7.95-8.00 followed by three water washes. Endogenous peroxidase was blocked with 3% peroxide in PBS for 30 min. Nonspecific binding was blocked for 30 min at 37ºC with a 50/50 10% solution of combined horse and rat serum. The slides were incubated with primary antibody diluted 1:10 in blocking serum at 37ºC for 60 min. followed by PBS wash. A Vector Vectastain Kit (Vector Inc., Burlingame, CA) was used to biotinylate the secondary antibody (horse anti-mouse) followed by binding of avidin-biotin –horseradish peroxidase. Diaminobenzidine (DAB) substrate was used to localize antibody binding. Slides were counterstained with nuclear fast red.

Following immunohistochemical staining using mouse monoclonal anti-rat IgE antibody, two hundred random 45x fields were analyzed for IgE positive cells and recorded based on subcompartment of the lung.

OVA-specific serum IgE

A blood sample was drawn from each animal for serum IgE analysis. The serum was separated by centrifugation and frozen at –80ºC prior to IgE analysis. Serum OVA–specific IgE was determined using a solid phase ELISA antigen-specific IgE antibody. Optical densities were used to compare serum from treated rats to serum from a randomly selected positive control and expressed as a percentage of control. Briefly, 96 well plates were coated with 100 µl/well anti-rat IgE (2.5 µg/ml) and incubated overnight at 4ºC, washed with buffer (PBS), blocked with 1% BSA for one hour at room temperature and washed again with buffer. Serum samples diluted 1:5 in blocking serum were added and incubated overnight at 4ºC. Subsequent to washing in PBS, biotinylated OVA in blocking buffer (Sulfo-NHS-LC-Biotinylation Kit; Pierce) (2 µg/ml) was added to each well and incubated at room temperature for one hour. All wells were

washed again with buffer, then incubated with 100µl/well HRP-streptavidin (Zymed) diluted in blocking buffer at room temperature for one hour and washed. DakoTMB (Dako) at 100 µl/well was added and the color reaction was allowed to develop 10 min. Plates were read at a wavelength of 650 nm.

BrdU immunohistochemistry

BrdU immunohistochemistry using anti BrdU mouse monoclonal clone BMC9318 (Boehringer Mannheim), 1:100 dilution, was performed similarly to IgE immunohistochemistry, with the exception that antigen capture was performed by incubating rehydrated tissue sections after endogenous tissue block for three minutes with 0.1% pronase followed by a water rinse. Tissue sections were incubated in 2N HCl for 60 min. Following a 5-min PBS wash, the nonspecific block and subsequent steps were followed as described for IgE immunostaining.

Histopathological scoring

All groups were examined for histopathological changes by light microscopy and scored for cellular changes observed. Levels of inflammation in subcompartments of the lung were objectively scored by a blinded individual on a scale of 0 (no inflammation) to 3 (severe inflammation).

Paraffin sections were stained for eosinophils and mast cells with CEM stain. Eosinophil profiles were counted and normalized to the total area examined in the sub-mucosal regions of terminal bronchioles.

Alcian blue/PAS staining for mucin was analyzed by density measurement (NIH image software) of 50 random captured high-powered fields of the most proximal generations of bronchial mucosa in transverse sections through the mainstem bronchus of the left lobe.

Statistical analysis

All data was expressed as mean $\pm$ SE. Differences between groups and exposures were assessed using analysis of variance (ANOVA; Statview 4.5 AbacusConcepts Inc., Berkley, CA.) A p value less than 0.05 was considered significant. Serum IgE was analyzed using Kruskal-Wallis test.

Study Design: BN rat Experiment 4

The study design for Experiment 4 is shown in Table 4. In this experiment, we examined the effects of particle exposure for up to six days. We utilized BrdU labeling of epithelial cells, histology, pulmonary function testing (PFT) and mRNA expression for eotaxin, IL4 and IL5 in whole lung homogenates enhanced by RT-PCR, as endpoints to measure exacerbation of inflammation following PM exposure. We hypothesized that prolonged exposure to PM following OVA-induced allergic inflammation would increase (1) BrdU labeling of airway epithelial cells, (2) produce an influx of inflammatory cells and exacerbate pulmonary granuloma formation, (3) increase airway hypersensitivity measured by methacholine challenge, and (4) alter mRNA expression of three key Th2 cytokines critical to the development and progression of Type I hypersensitivity response in allergic airway disease.

Table 4. Brown Norway PM Study: Multi-Day PM Exposure

Day	Mon	Tues	Mon	Tues	Wed, Th	Fri	Sat	Sun	Mon, Tues	Wed	Th
Day #	0	1	14	15	16, 17	18	19	20	21, 22	23	24
	Sensitized & unsensitized FA (48) (36S, 12N)		OVA Challenge (30)	FA	FA	FA Necropsy (18)	FA	FA	FA	PFT (12) Necropsy (18)	
		Sensitized & unsensitized PM (48) (36S, 12N)		OVA Challenge (30)	PM	PM	FA Necropsy (18)	FA	PM	PM	PFT (12) Necropsy (18)

PM = particulate matter (150 μg/m^3 ammonium nitrate + 100 μg/m^3 carbon black)

		PFT/EtD-1	Histology (BrdU), Biochemistry/Gene Expression (1)	Histology (BrdU), Biochemistry/Gene Expression (2)
N/C	FA	—	6 (3/14/03)	6 (3/19/03)
N/C	PM	—	6 (3/15/03)	6 (3/20/03)
S	FA	6 (3/19/03)	6 (3/14/03)	6 (3/19/03)
S	PM	6 (3/20/03)	6 (3/15/03)	6 (3/20/03)
S/C	FA	6 (3/19/03)	6 (3/14/03)	6 (3/19/03)
S/C	PM	6 (3/20/03)	6 (3/15/03)	6 (3/20/03)

N/C = non-sensitized + challenged
S = sensitized
S/C = sensitized + challenged

PFT = pulmonary function testing
BAL = bronchoalveolar lavage
EtD-1= ethidium homodimer-1

Necropsy and Tissue Fixation

BN rats designated only for lung fixation and not used for pulmonary function testing were deeply anesthetized by IP injection of sodium pentobarbital. A tracheal cannula was placed through a ventral incision and the chest cavity opened through an abdominal incision and rupture of the diaphragm to collapse the lungs. A 3cc blood sample for serum IgE analysis was drawn from the caudal vena cava. The serum was separated by centrifugation and frozen at –80^{o}C for later analysis. The right lung lobes were isolated from perfusion by ligating the right mainstem bronchus with silk suture. The right lobes were removed, immediately flash frozen in liquid nitrogen and stored at –80^{o} C for later PCR analysis. The left lung was fixed by intratracheal infusion of Z-fix (Anatech LTD.) at 30-cm pressure for one hour. The left lung and mediastinal contents were removed *en bloc* from the thoracic cavity and stored in Z-fix for later embedment and sectioning. A section of gut was also excised and placed in Z-fix to be embedded with the lung tissue from the same animal to serve as a positive control for BrdU immunohistochemistry.

Morphometric Analysis

CEM positive eosinophil counts in sub-epithelial regions of terminal bronchioles were normalized to basal lamina length analyzed by NIH Image 1.68. Granuloma scores in lung sections from FA and PM exposed sensitized and challenged rats determined subjectively by a blinded individual on a scale of 0 (no inflammation) to 3 (severe inflammation).

BrdU Immunohistochemistry

BrdU immunohistochemistry using anti-BrdU mouse monoclonal clone BMC9318 (Boehringer Mannheim), 1/100 dilution, was performed on 5 µm paraffin lung tissue sections. Briefly, 5 µm paraffin sections were baked at 56 ^{o}C for one hour then re-hydrated through increasing concentrations of ethanol after removal of paraffin with three 5-min xylene washes. Endogenous peroxidase was blocked with 3% peroxide in PBS for 30 min. Antigen capture was performed by incubating re-hydrated tissue sections after endogenous tissue block for three minutes with 0.1% pronase followed by a water rinse. Tissue sections were then incubated with 2N HCl for 60 min. Following a 5-minute PBS wash, nonspecific binding was blocked for 30 minutes at 37^{o}C with a 50/50 10% solution of combined horse and rat serum. The slides were subsequently incubated with primary antibody diluted 1/100 in blocking serum at 37^{o}C for 60 min followed by PBS wash. A Vector Vectastain Kit (Vector Inc., Burlingame, CA) was used to biotinylate secondary antibody (Horse anti-mouse) followed by binding of avidin-biotin horseradish peroxidase. Diaminobenzidine (DAB) substrate was used to localize antibody binding. Slides were counterstained with nuclear fast red. BrdU labeling of airway epithelial cells was quantified by counting the positive cells from five randomly selected airways in each rat at specific airway levels and expressing the results as an average percent positive of total cells present in counted airways.

Ova-Specific Serum IgE

The identical assay as described under the study design for Experiment 3 (page 17) for ova-specific serum IgE was also used for this experiment.

Reverse Transcriptase - Polymerase Chain Reactions Eotaxin, IL4 and IL5

Total RNA was isolated from the right middle lobe of each rat by immersing frozen (- 80^0C) in Tripure (Roche) and proceeding according to the manufacturer's instructions. The RNA pellet was resuspended in nuclease-free H_2O and was treated with TurboDnase I (Ambion) to remove genomic DNA. RNA was quantified by spectrophotometer at A260 and A280. RNA quality was assessed by electrophoresis in a 1.0% denaturing agarose gel containing 2.1 M formaldehyde.

Synthesis of cDNA was performed using 1 μM oligo dT primer allowed to anneal to 0.75 μg of total RNA at 65°C for 5 min. Reverse transcription (RT) was performed utilizing Omniscript Reverse Transcriptase (Qiagen) according to the manufacturer's protocol. The cDNAs were used in the polymerase chain reaction (PCR). PCR was performed using synthesized (MWG) primers for eotaxin and IL4 created using rat sequences downloaded form the NCBI database in conjunction with Primer3 primer design software (Citation). The IL5 primers were synthesized from the sequence reported by Kobayashi and colleagues (Kobayahi et al, 2000). Intron spanning primers were created for β-actin and IL-4 (Table 2).

PCR reactions contained 2 μl of RT product, 18 μl of master mix containing 10 μl of 2x QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.5 μM of each sense and anti-sense primer, and 6 μl of water. The initial denaturation step was performed at 95°C for 15 minutes. Temperature cycling consisted of a denaturation step at 94°C for 15 sec followed by annealing step at 58°C for 20 sec and terminated with an elongation step at 72°C for 20 sec. Eotaxin reactions required 35 cycles; IL4 and IL5 each required 45 cycles. No RT controls were performed on trial runs to ensure that genomic DNA was not amplified. No template controls were run for the PCR and the RT-PCR reactions. Correct product was confirmed by melting curve analysis performed at the end of each run. All amplified products were normalized to β-actin. Normalized gene expression was then compared between treatment groups.

Table 5. RT-PCR Primer Sequences

Gene	Sequence		Product Size
β-actin	Sense	5' TGA-GCA-CCA-GGG-TGT-GAT-G 3'	108 bp
	Anti-sense	5' CCG-TGT-TCA-ATG-GGG-TAC-TT 3'	
IL-4	Sense	5' CAA-CAA-GGA-ACA-CCA-CGG-A 3'	117 bp
	Anti-sense	5' CAC-CGA-GAA-CCC-CAG-ACT-T 3'	
IL-5	Sense	5' GGT-GAA-AGA-GAC-CTT-GAT-ACA-GCT-G 3'	78 bp
	Anti-sense	5' AGG-AAC-AGG-AAG-CCT-CAT-CGT 3'	
eotaxin	Sense	5' AGG-TTC-CAT-CCC-AAC-TTC-CT 3'	104 bp
	Anti-sense	5' TTC-AGC-GTG-ACT-CTG-TTG-TT 3'	

Pulmonary Function Testing (PFT)

On the day following three consecutive days of FA or PM, six rats from each treatment group were anesthetized with an IP injection of 0.4ml/100gm body weight alpha-chloralose/urethane in saline. Measurements of airway function were followed as described under the earlier subsection, Measurement of bronchial responsiveness (page 9).

Statistical Analysis

Data were expressed as mean ± SE. Differences between groups and exposures were assessed using analysis of variance (ANOVA; Statview 4.5 Abacus Concepts Inc., Berkley, CA.) A p-value of less than 0.05 was considered significant. Serum IgE was analyzed using Kruskal-Wallis test.

Human Experiments

Human Subject Exposures

This project consisted of two separate controlled human exposure experiments. All subjects were individuals with mild to moderate asthma. The exposure conditions for the first experiment were separate single exposures to each of filtered air, carbon and ammonium-nitrate particles at a total concentration of 300 $\mu g/m^3$, and carbon and ammonium-nitrate particles with O_3 at a concentration of 0.2 ppm. The exposure conditions for the second experiment were single exposures to filtered air, single exposures to carbon and ammonium-nitrate particles, and three serial-day exposures to carbon and ammonium-nitrate particles. The duration of all the exposures was four hours, during which subjects completed four 30-minute exercise periods, separated by four 30-minute rest periods.

For both experiments, each subject attended the laboratory for one characterization session, and subsequently for three or four exposure and bronchoscopy sessions. The characterization session was used to collect physical and pulmonary characteristics, and to familiarize each subject with the procedures of the experiment. Each of the experiments utilized a repeated measures design, with each subject completing each condition within the experiment. The order of the experimental conditions was counterbalanced/randomized within each experiment. For both

experiments, a control exposure condition of filtered air was used. To allow recovery from preceding sessions, a minimum of three weeks separated each of the exposure conditions within each experiment.

All subjects were informed of the risks of the experiment and provided informed consent prior to participation. The procedures for this experiment were approved by the University of California, San Francisco, Institutional Review Board Committee on Human Research. All subjects completed a medical history questionnaire, were current non-smokers, had no history of excessive smoking, and had no serious health problems. Female subjects were not pregnant throughout the project. Subjects had no respiratory-tract illness in the three weeks prior to, or during, each session. Subjects were characterized by physical characteristics, spirometric pulmonary function, non-specific airway reactivity, and allergy skin test. All subjects for both Experiment One and Experiment Two had mild to moderate asthma, but were otherwise healthy. Asthma status was determined using the guidelines of the National Asthma Education Program (National Asthma Education Program Expert Panel, 1997). All subjects had non-specific airway reactivity of < 10 mg/ml methacholine.

Acquisition of Tissues from Human Subjects: Bronchoscopy and Biopsy

The bronchoscopies were conducted in a dedicated room at San Francisco General Hospital. Vital signs were measured pre- and post-bronchoscopy. Throughout the procedure, intravenous access was maintained, and arterial hemoglobin:oxygen percent saturation, the electrocardiograph, and blood pressure were monitored. Atropine, to decrease airway secretions, and if required, midazolam, to maintain subject comfort, were administered intravenously. The posterior pharynx was anesthetized using a 1% lidocaine spray, and 4% lidocaine-soaked cotton-tipped plegets applied to the mucosa over the ninth cranial nerve. Supplemental oxygen was delivered via a nasal cannula at 2 l/min. The bronchoscope (Pentax, Model No. FB 18x), tipped with lidocaine jelly, was introduced through the mouth, and the larynx and airways were anesthetized using 1% lidocaine solution as required. The bronchoscope was directed and wedged into the right middle lobe orifice and subsequently into the lingula.

Six to eight endobronchial biopsies were obtained from multiple sites of the airway bifurcations within the right middle lobe and carina using spiked forceps (Pentax Precision Instrument Corporation). The bronchoscopy was conducted 18 h post-exposure to filtered air, particles or particles plus ozone.

In Vitro Allergen Challenges: Human Biopsy Tissues

Biopsy specimens were immediately placed into cold MEM (Joklik modified Minimal Essential Medium with 1 mM HEPES) and shipped overnight on wet ice. Immediately upon receiving biopsy specimens at UC Davis, lung tissues were evenly distributed into four experimental groups. As a control, one experimental group was immediately placed in a tissue culture vial and snap-frozen in liquid nitrogen; this sample was stored for RNA isolation. The remaining experimental groups were cultured in Bronchial Epithelial Growth Medium (purchased from Cambrex), using an air-liquid interface method. In brief, airway biopsy specimens were placed on top of 3 µm pore size Transwell inserts within a 24 well culture plate, into which 600 microliters of culture medium was added to the lower chamber. The *in vitro* experimental groups were

cultured for 24 hours with medium that included patient-specific allergen, phorbol myristate acetate (PMA) plus A23187 (a calcium ionophore), or no additions. The patient-specific allergen was defined by prior skin-prick testing at UC San Francisco using nine local aeroallergens. The nine allergens tested for this study included house dust mite plus *aspergillus fumigatus*, birch mix, Chinese elm, cat, dog, mountain cedar, mugwort sage, olive tree, and perennial rye. The allergen that produced the strongest skin-prick response for each individual subject was utilized in cultures at a 1:100 dilution. Allergens used for each subject analyzed within the UC Davis study are listed in Table 6. PMA (50 ng/ml) plus A23187 (250 ng/ml) was utilized as a non-specific activator of cytokine signaling in tissue samples to assess non-antigenic stimulation by particulate matter.

Following 24-hour culture of biopsy specimens, tissues were separately placed into tissue culture vials and snap-frozen in liquid nitrogen. Samples were stored at -80°C until RNA extractions could take place. Snap-frozen samples were homogenized in TRIzol® reagent (a guanidine isothiocynate-based buffer purchased from Invitrogen) and extracted according to manufacturer's instructions. The total amount of RNA isolated for each sample was determined by Ribogreen® RNA quantitation reagent (Molecular Probes), which consists of a fluorescent nucleic acid stain. Isolated RNA from each sample was stored at –80°C until analyzed. For each RNA sample, cDNA was synthesized using Taqman® Gold RT-PCR kit (purchased from Applied Biosystems), which includes reverse transcriptase and nucleotides. For each experimental condition, 100 nanograms of total RNA was utilized for cDNA synthesis. Following cDNA synthesis, each sample was assessed for RNA integrity by real-time PCR analysis of 18S ribosomal RNA, using specific Taqman® primers and probe (Applied Biosystems) and the ABI PRISM 5700 Sequence Detection System. Real-time PCR methods, as opposed to traditional PCR methods, allow for the semi-quantitative detection of fluorescent amplified products during the early phases of the reaction as opposed to only end-point reactions. By monitoring the kinetics of a PCR reaction, it is possible to determine a cycle threshold (Ct) value that is set at the exponential phase of the amplification reaction. For this study, RNA integrity was established when a Ct value was within the range of 12 to 14.

In order to rapidly assess the expression profile of inflammatory and immune-related genes within experimental groups for this study, we utilized real-time PCR analysis and the Human Taqman® Cytokine Expression Plate (Applied Biosystems). In brief, the Human Taqman® Cytokine Expression Plate is a pre-developed assay that contains primers and probes for 12 human cytokine targets. For a 96 well plate, each individual cytokine assay is loaded into eight wells. In addition, for each individual cytokine assay well, primers and probes for 18S ribosomal RNA are also provided as an endogenous control; amplification is quantified in a multiplex reaction that utilizes two different fluorochromes detected at different wavelengths. Because of the sensitivity required for this assay, the Human Taqman® Cytokine Expression Plate was analyzed with the laser-based ABI PRISM 7900 Sequence Detection System. The cytokines that were evaluated in this study include interleukin-1 alpha, interleukin-1 beta, interleukin-2, interleukin-4, interleukin-5, interleukin-8, interleukin-10, interleukin-12 p35, interleukin-12 p40, interleukin-15, interferon gamma, and tumor necrosis factor alpha. The mRNA expression for all 12 cytokine targets was determined for all experimental groups

generated from human subject airway biopsy specimens (control or cultured), each sample was analyzed in duplicate.

Subject #	Allergen
101	Birch mix
102	House dust mite
104	Dog
106	Olive
108	Mountain Cedar
110	Birch mix
111	Cat
113	House dust mite
118	Perennial rye
119	Olive

Table 6. Listing of human subjects with primary allergens

RESULTS

Ovalbumin and PM exposures (all BN rat experiments)

The ovalbumin and PM aerosol exposure data for each exposure have been summarized on Tables 7, 8, 9 and 10. Except for the initial temporal study of the allergic response that used only ovalbumin aerosols (Table 7), each exposure series was assigned a code number, Experiment 3 (Tables 8 and 9) and Experiment 4 (Tables 10 and 11). For all exposures described, the ovalbumin aerosols were stable and repeatable. The largest variability was seen in the protein concentrations. We attribute this to difficulties analyzing or extracting protein. The gravimetrically determined total mass concentrations were less variable, and we consider these the more accurate measurements. The nebulized ovalbumin solutions contained 10.56 g/liter total salts from the PBS used. Therefore, for 25.0 g/liter ovalbumin solution in PBS, 70.3% of the mass concentration should be protein. The extraction and amino acid analysis always yielded lower protein concentrations than expected, even when applied to the original solutions.

Table 7.
Ovalbumin in Phosphate Buffered Saline Aerosol
for Initial Temporal Study of Allergic Response: Experiment 2

	Target	One Week	Two Weeks	Three Weeks	Four Weeks
Total Mass Concentration, mg/m^3	11	11.07 ± 0.08	11.16 ± 0.25	11.38 ± 0.36	11.47 ± 0.34
Number of Samples		2	4	6	8
Protein Content[a], mg/m^3		6.22 ± 0.13	5.74 ± 0.63	5.61 ± 0.53	5.84 ± 0.63
Number of Samples		2	4	6	8
Aerosol Size					
MMAD[b], µm		1.50	1.53 ± 0.04	1.57 ± 0.08	1.59 ± 0.08
σ_g[c]		2.73	2.59 ± 0.20	2.65 ± 0.17	2.65 ± 0.14
Number of Samples		1	2	3	4

[a] Determined by amino acid analysis
[b] Mass median aerodynamic diameter (MMAD)
[c] Geometric standard deviation

mg/m^3: milligrams per cubic meter
µm: micrometer
sg: sigma g

Table 8.
Ovalbumin in Phosphate Buffered Saline Aerosol: Experiment 3

		Group N/C		Group S/C	
	Target	Filtered Air	PM	Filtered Air	PM
Total Mass Concentration, mg/m^3	11	N/A	11.09 ± 0.20	11.56 ± 0.21	10.80 ± 0.15
Number of Samples			2	2	2
Protein Content[a], mg/m^3		N/A	4.46 ± 1.10	5.05 ± 0.15	4.45 ± 0.59
Number of Samples			2	2	2
Aerosol Size					
MMAD[b], µm		1.35	1.10	1.23	1.37
σ_g[c]		3.59	2.14	2.18	2.01
Number of Samples		1	1	1	1

[a] Determined by amino acid analysis
[b] Mass median aerodynamic diameter (MMAD)
[c] Geometric standard deviation

N/C: Not sensitized/challenged
S/C: Sensitized/challenged
µm: micrometer
sg: sigma g

Table 9. PM Exposure; Experiment 3
Simulated Particulate Matter Aerosol (PM)
2-day exposures for 6 hrs/day

	Target	Group N/C	Group S	Group S/C
NH_4NO_3, µg/m^3 ± SD	150	128 ± 16	127 ± 24	141 ± 32
Number of Samples		8	8	8
Carbon[a], µg/m^3 ± SD	100	112 ± 14	102 ± 20	108 ± 21
Number of Samples		8	8	8
Mass Monitor[b], µg/m^3 ± SD		220 ± 40	190 ± 40	240 ± 70
Number of Samples		16	14	14
Total Mass Concentration, µg/m^3		245 ± 11	225 ± 36	251 ± 49
Number of Samples		4	4	4
Mean NH_4NO_3 Concentration Added to Mean Carbon Concentration, µg/m^3		240	229	249
Slurry				
NH_4NO_3, g/liter		5.10	5.10	5.10
Carbon, g/liter		4.08	4.08	4.08
Ratio, NH_4NO_3:Carbon		1:0.80	1:0.80	1:0.80
Aerosol Mass Concentrations				
Ratio, NH_4NO_3:Carbon		1:0.88	1:0.80	1:0.77
Aerosol Size				
MMAD[c], µm ± SD		1.34 ± 0.02	1.02 ± 0.06	1.42 ± 0.10
σ_g[d] ± SD		2.38 ± 0.07	2.81 ± 0.07	2.18 ± 0.28
Number of Samples		2	2	2

[a] Analyzed by Dr. Kochy Fung of Atmospheric Assessment Associates, Inc.
[b] Exp. Fac. monitor serial no. 557899
[c] Mass median aerodynamic diameter (MMAD)
[d] Geometric standard deviation

N/C: Not sensitized/challenged
S: Sensitized
S/C: Sensitized/challenged
NH_4NO_3: Ammonium nitrate
µm: Micrometer
µg/m^3: Micrograms/cubic meter
SD: Standard deviation

Table 10.
Ovalbumin in Phosphate Buffered Saline Aerosol: Experiment 4

		Groups N/C and S/C	
	Target	Filtered Air	PM
Total Mass Concentration, mg/m^3	11	11.55 ± 0.33	10.57 ± 0.20
Number of Samples		2	2
Protein Content[a], mg/m^3		6.38 ± 0.34	5.69 ± 0.21
Number of Samples		2	2
Aerosol Size			
MMAD[b], µm		1.75	2.10
σ_g[c]		2.57	2.43
Number of Samples		1	1

[a] Determined by amino acid analysis
[b] Mass median aerodynamic diameter (MMAD)
[c] Geometric standard deviation

N/C: Not sensitized/challenged
S/C: Sensitized/challenged
µm: micrometer
sg: sigma g

Table 11. PM Exposure: Experiment 4
Simulated Particulate Matter Aerosol (PM)
3 or 3+3 day-exposures for 6 hrs/day

	Target	Groups N/C, S and S/C	
		3 Days	3+3 Days
NH_4NO_3, µg/m^3 ± SD	150	151 ± 31	149 ± 27
Number of Samples		12	24
Carbon[a], µg/m^3 ± SD	100	114 ± 25	114 ± 23
Number of Samples		12	24
Mass Monitor[b], µg/m^3 ± SD		227 ± 39	204 ± 42
Number of Samples		22	48
Total Mass Concentration, µg/m^3		294 ± 54	295 ± 39
Number of Samples		6	12
Mean NH_4NO_3 Concentration Added to Mean Carbon Concentration, µg/m^3		265	263
Slurry			
NH_4NO_3, g/liter		5.10	5.10
Carbon, g/liter		4.08	4.08
Ratio, NH_4NO_3:Carbon		1:0.80	1:0.80
Aerosol Mass Concentrations			
Ratio, NH_4NO_3:Carbon		1:0.76	1:0.77
Aerosol Size			
MMAD[c], µm ± SD		1.52 ± 0.14	1.48 ± 0.11
σ_g[d] ± SD		2.64 ± 0.22	2.68 ± 0.26
Number of Samples		3	6

[a] Analyzed by Dr. Kochy Fung of Atmospheric Assessment Associates, Inc.
[b] Exp. Fac. monitor serial no. 557899
[c] Mass median aerodynamic diameter (MMAD)
[d] Geometric standard deviation

N/C: Not sensitized/challenged
S/C: Sensitized/challenged
S: Sensitized
NH_4NO_3: Ammonium nitrate
µm: Micrometer
µg/m^3: Micrograms/cubic meter
sg: Sigma g
SD: Standard deviation

PM inhalation exposure conditions (BN rats)

Target concentrations for PM levels were 150 µg/m^3 NH_4NO_3 + 100 µg/m^3 C. Tables 9 and 11 are exposure data summaries for the PM aerosols. Multiple measurements of a given parameter are expressed as mean ± SD. NH_4NO_3 and elemental carbon concentrations are reported. The mass monitor data represents the most frequent (hourly) determination of PM concentration during exposure. The total mass concentration determination was a more direct measurement, but only one pair of samples drawn simultaneously could be made for each exposure interval. Mean NH_4NO_3 concentrations were added to mean carbon concentrations. These sums serve as a check on the analytical methods because they should equal the measured total mass concentrations. The concentration of NH_4NO_3 and of carbon mixed in each slurry, and the ratio of NH_4NO_3 to carbon, is listed. A ratio is also given for NH_4NO_3 to carbon measured in the aerosol. Carbon loss in the system is reflected in a ratio in the aerosol smaller than what was in the slurry. Carbon stratification and concentration in the reservoir or nebulizer is reflected by a ratio greater in the aerosol than in the slurry. Aerodynamic size of the particles is the final information listed on Tables 9 and 11.

The desired target concentrations of NH_4NO_3 and carbon were achieved throughout these studies. Mean PM concentrations measured for each group with three of the analytical methods used, gravimetry (total mass concentration), ion chromatography (for NH_4NO_3) and selective thermal oxidation (for C) agreed with an average difference of 4.85% higher for the gravimetric determinations. These results represent excellent agreement among three different analytical methods performed in two different laboratories. The most likely causes for the difference could be slight moisture sorption as the total mass concentration samples were collected during the five-hour sampling period, different sampling times or a small weighing inaccuracy. Since the NH_4NO_3 samples and C samples were collected for 60 minutes during the first and second halves of a given six-hour interval and the total mass concentration samples were collected for most of the period, the agreement also demonstrates temporal stability in maintaining the exposure atmospheres.

As previously reported (Pinkerton et al 2000), for nitrate analysis by ion chromatography, initial testing revealed that the modified polysulfone Supor™ filters were a better choice in our system than the Teflo™ filters that are more commonly used for ambient air monitoring. After extraction by the CARB procedure (CARB Monitoring and Laboratory Division) for Teflon® filters, they yielded about 9% less nitrate than the Supor™ filters. Ion chromatograms selected as typical examples from exposure to PM are shown on Figure 2. Chromatograms of the nitrate calibration standards are equivalent to the chromatograms from the extracted PM samples, indicating that the anionic species present in the PM aerosol was nitrate of high purity. All deposited PM on filters or impactor stages appeared to be in a dry, solid phase as predicted since the chamber relative humidity (44.0 ± 11.9% RH at 24.7 ± 0.5° C, mean ± SD for all exposures) was maintained below the deliquescence point of NH_4NO_3 (61.2% at 25° C) (Clegg et al 1998, Clegg et al 2001). Nitrate loss is a frequent problem in ambient air sampling with acidified aerosols in the liquid phase. Nitric acid (HNO_3) can be formed, and this volatile acid

is lost in the gas phase (Clegg et al 1998, Clegg et al 2001). However, nitrate loss was neither expected nor evident under our more controlled atmosphere generation conditions. NH_4NO_3 particles in the solid phase are not volatile, exhibiting a low vapor pressure (Clegg et al 1998, Clegg et al 2001). In addition, as reported previously (Pinkerton et al 2000), Dr. Kleinman, generating aerosols at UC Irvine with the same chemical composition, tested nitrate loss during sampling by placing nylon filters behind the Teflon® filters he normally uses for collecting samples for nitrate analysis. He found no nitrate present on the Nylon filters that very effectively trap HNO_3 (Kleinman, 2001).

Carbon was analyzed by Dr. Fung by his method of selective thermal oxidation that speciates elemental and organic carbon (Fung 1990). As expected, elemental carbon was by far the predominant species present in the PM from the exposure atmospheres. The carbon analysis results are expressed as elemental carbon. Figure 3 is a photograph of a typical pair of quartz fiber filters on which PM was collected and analyzed for carbon content. Organic carbon, however, was a ubiquitous contaminant. Relatively small amounts of organic carbon were detected in samples from the PM aerosol chamber, filtered air chamber and to a minimal extent on filter blanks analyzed with the samples. Existing organic carbon contaminants on the filters before use were essentially eliminated by baking the filters at 750º C for 24-hours in an inert atmosphere. For all PM carbon samples analyzed, elemental carbon averaged 89.9% of the total carbon measured. The origin of any organic carbon found was possibly the rodent diet or the rats themselves. During these exposures, a total of 10 samples for carbon analysis were collected from the filtered air chamber. Elemental carbon in the filtered air chamber was not detectable.

Results of the ovalbumin and PM particle size measurements by seven-stage cascade impactor (Mercer 1970) are included as the final item summarized on each table. Overall, including all the studies, the MMAD of the ovalbumin particles averaged 1.51 µm with a sigma g of 2.53 and the PM averaged 1.36 µm with a sigma g of 2.54. Rats readily inhale particles of this size, and these inhaled particles have a relatively high probability of pulmonary deposition (Anjilvel & Asgharian 1995). As indicated in other sections of this report, the ovalbumin aerosols were very effective at eliciting the expected allergic airway responses in the brown Norway rats primed and sensitized to ovalbumin. The simulated PM generated and characterized for these studies would be classified as being in the fine (0.1 to 2.5 µm) PM size fraction in ambient air.

A scanning electron photomicrograph of the ovalbumin particles is included on Figure 4. On Figure 5 is a light photomicrograph of the PM collected on a Nuclepore® membrane filter (0.2 µm pore size, 25 mm diameter, Whatman, Inc., Clifton, NJ) during exposure PM 40 from about 10 liters of chamber air. The photomicrographs show the basic appearance of the particles collected and add validity to the aerodynamic size measurements.

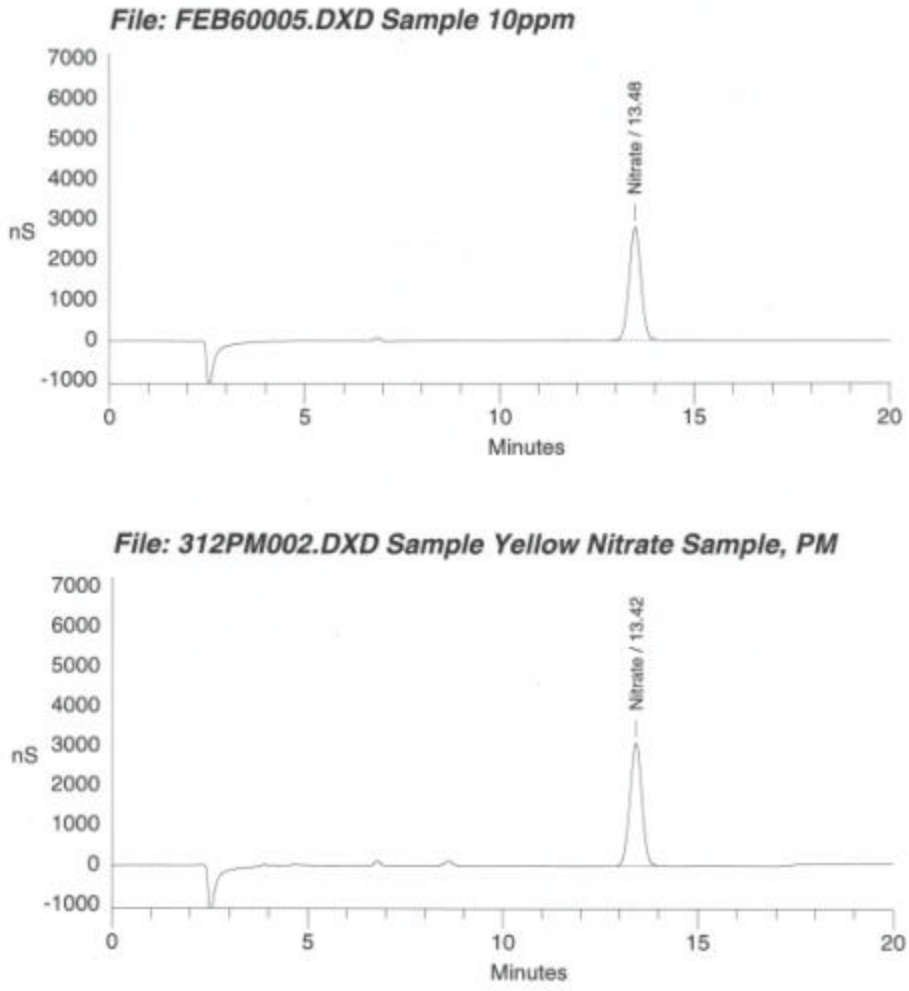


Figure 2. On top is an anion chromatogram (Model DX-120 ion chromatograph with PeakNet software, Dionex Corp., Sunnyvale, CA) of a high purity standard nitrate solution (EM Industries, Inc., Gibbstown, NJ). The bottom anion chromatogram is from a sample eluted from particles collected on a Supor®-800 filter (47 mm diameter, Pall Gelman Sciences, Ann Arbor, MI) from the chamber containing PM aerosol during exposure PM 41. Conductivity in microsiemens is on the ordinate, and retention time is on the abscissa of the chromatograms. The chromatograms are nearly identical, indicating that the anionic species present in the PM aerosol was nitrate of high purity.

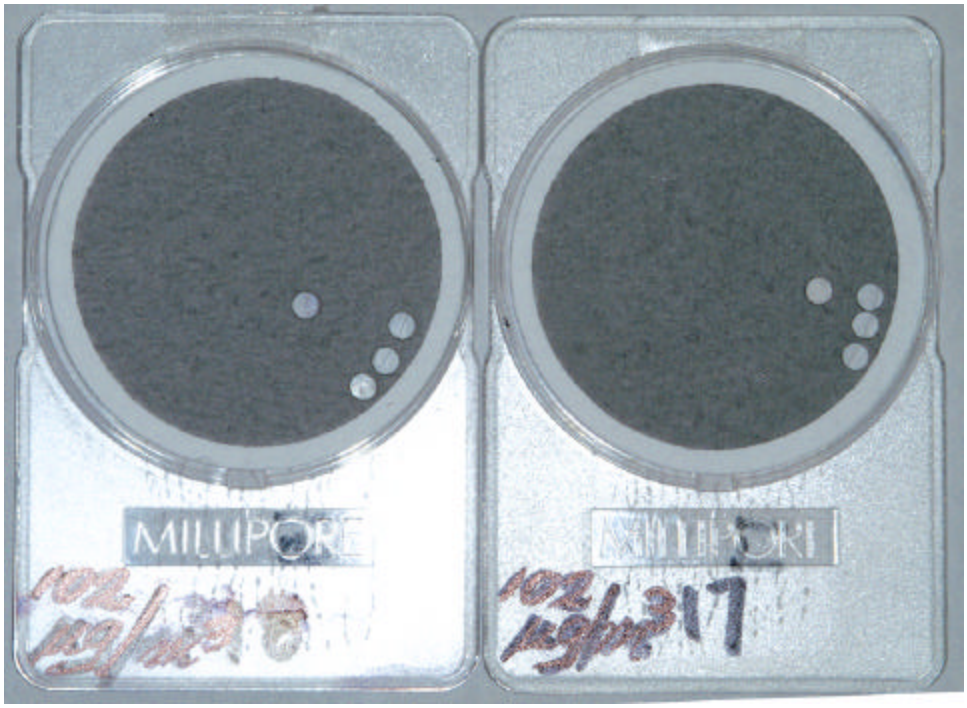


Figure 3. Pair of PM samples for carbon analysis collected simultaneously from each side of the chamber during exposure PM 40 on quartz fiber filters (QM-A, 47 mm diameter, Whatman, Inc., Clifton, NJ). Analysis of each filter resulted in identical concentrations of 102 µg/m^3 elemental carbon. Disks 3 mm in diameter were punched out of each filter for carbon analysis (Fung, 1990). Holes closer to the edge of a deposit were made to clean the punch mechanism before sample disks were removed.

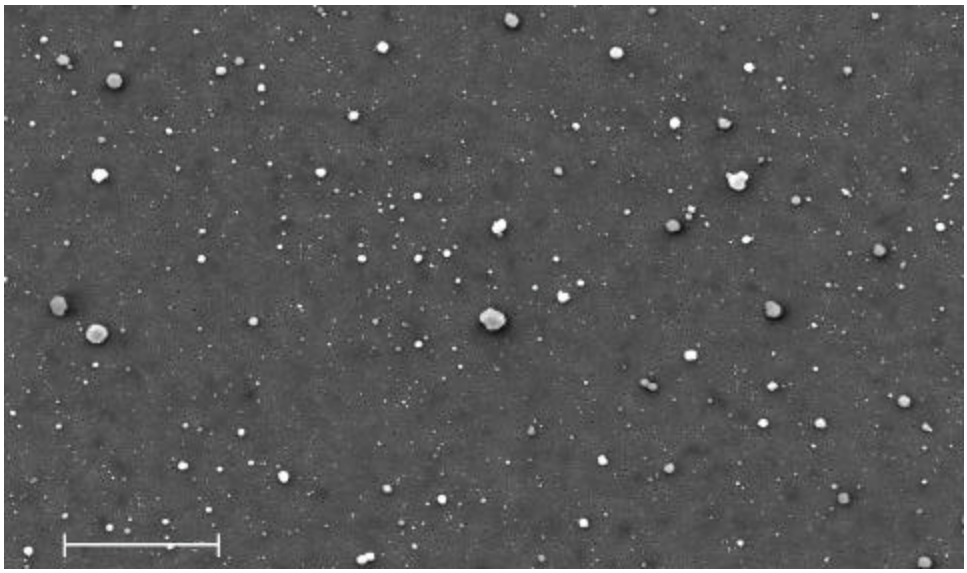


Figure 4. Scanning electron micrograph of ovalbumin with salt residue particles collected on a 0.2 µm pore size Nuclepore® filter (Whatman, Inc., Clifton, NJ). The marker shows a length of 20 µm. The sample was sputter coated with gold for stability in the electron beam. Inhalation Exposure Facility, California National Primate Research Center, University of California at Davis

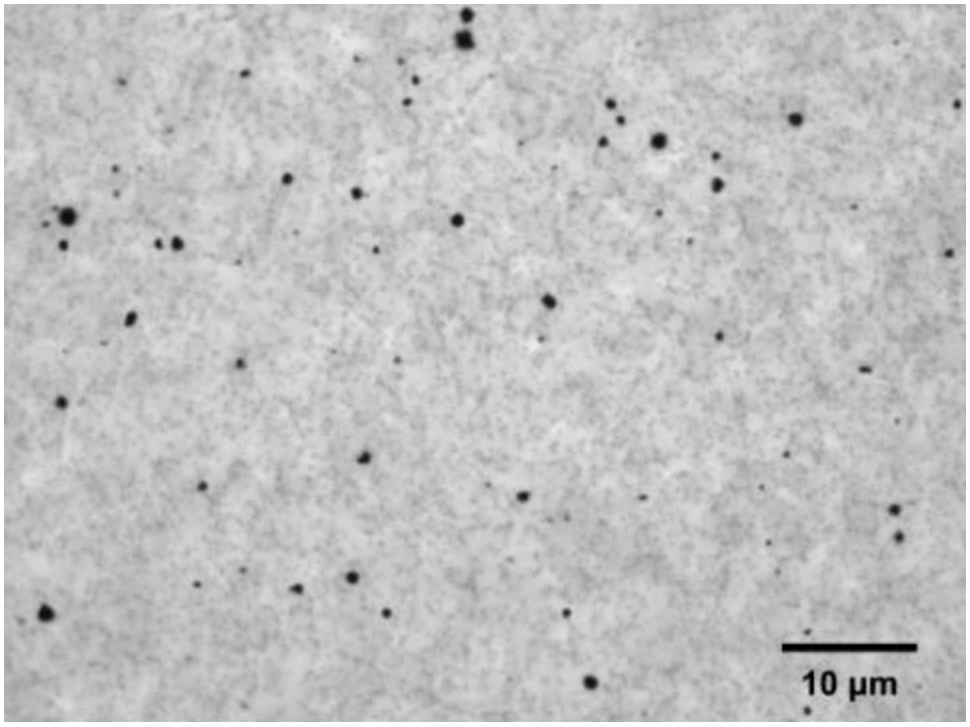


Figure 5. Light micrograph of the PM aerosol collected on a 0.2 µm pore size Nuclepore® filter (Whatman, Inc., Clifton, NJ).

Results: BN rat Experiment 1

These findings reflect the effects of only repeated aerosol exposure challenge with ovalbumin. No PM exposure was done in this initial experiment.

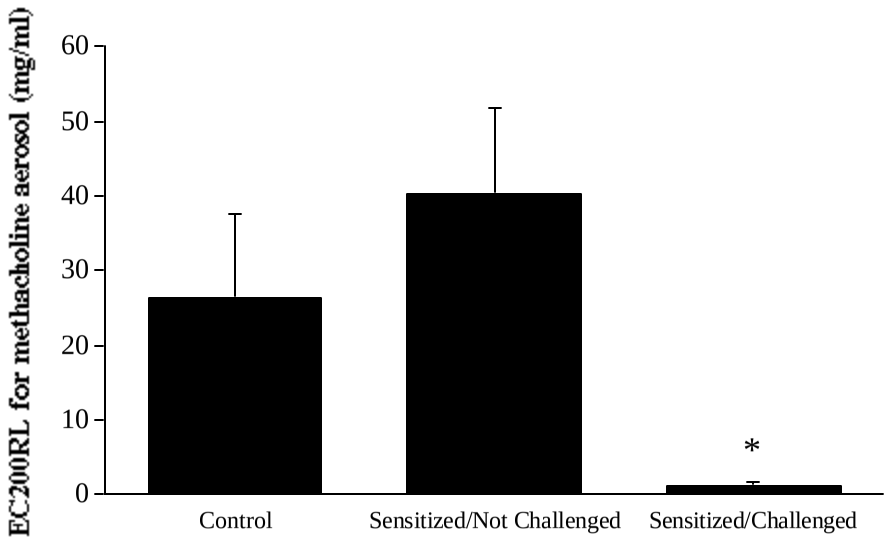
Bronchial responsiveness:

Sensitization and aerosol inhalation challenge with ovalbumin of Brown Norway rats resulted in a significant ($p < 0.05$) reduction in the effective concentration of metacholine required to double airway resistance (EC200RL) compared to Brown Norway rats that were sham treated (control) and/or sensitized with ovalbumin and inhaled a saline aerosol (Table 11 and Figure 6). There was no significant difference between Brown Norway rats that were sham treated (control) and Brown Norway rats that were sensitized with ovalbumin and inhaled a saline aerosol.

Table 12. Effect of ovalbumin sensitization and aerosol challenge on airway hyperresponsiveness (EC200RL) in Brown Norway rats.

group	number of animals	methacholine doubling dose mean ± sem (mg/ml)
control	8	26.47 ± 11.08
sensitized/saline aerosol	8	40.19 ± 11.62
sensitized/challenged	8	1.14 ± 0.71*

* Represents a significant difference ($p < 0.05$) from both control and sensitized/saline aerosol groups. EC200RL represents the “effective concentration” of metacholine required to double airway lung resistance. From Table 12 it is highly apparent BN rats sensitized and challenged with ovalbumin develop significant airway hyper-reactivity.



* significantly different from control and sensitized/not challenged groups

Figure 6. Effect of ovalbumin sensitization and aerosol challenge on airway hyperresponsiveness (EC200RL) in Brown Norway rats.

Quantitative histopathology:

The central axial airway of the left lung as indictated by the arrows (Figure 7) was examined by histochemical staining of tissue sections in all animals (Figures 8 and 9).

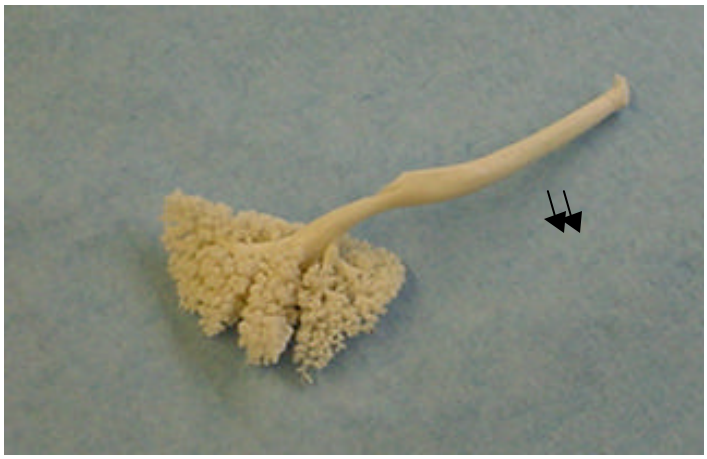


Figure 7 - Left lung corrosion cast (arrows indicate the major axial airway and approximate location for histological evaluation of airway anatomy and cell composition).

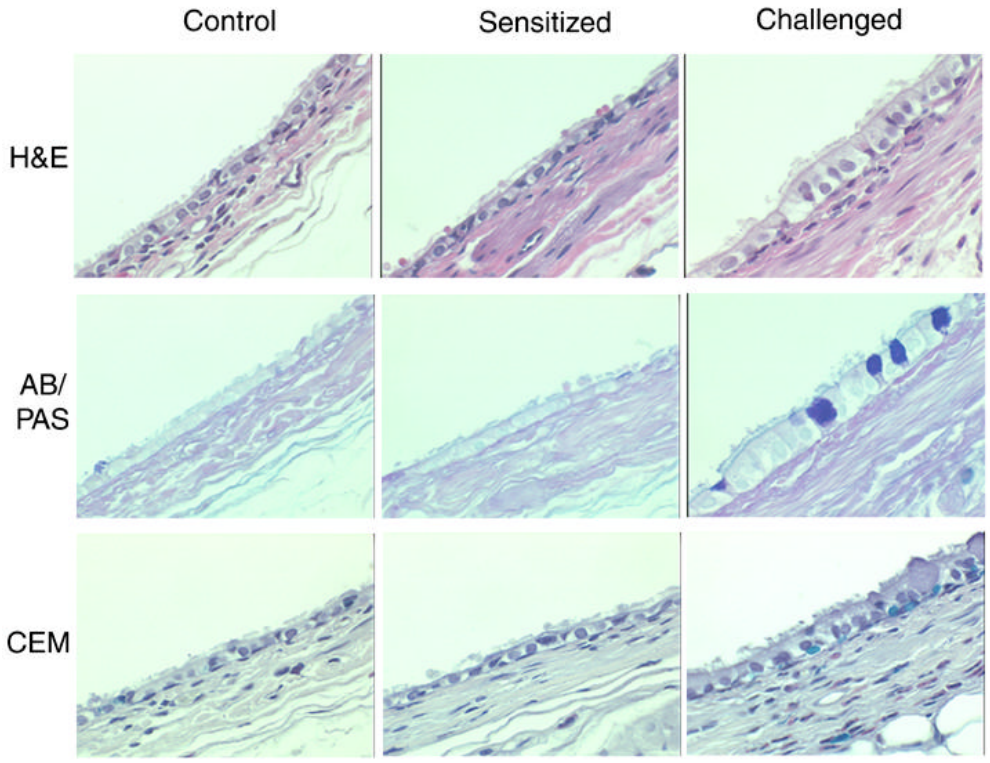
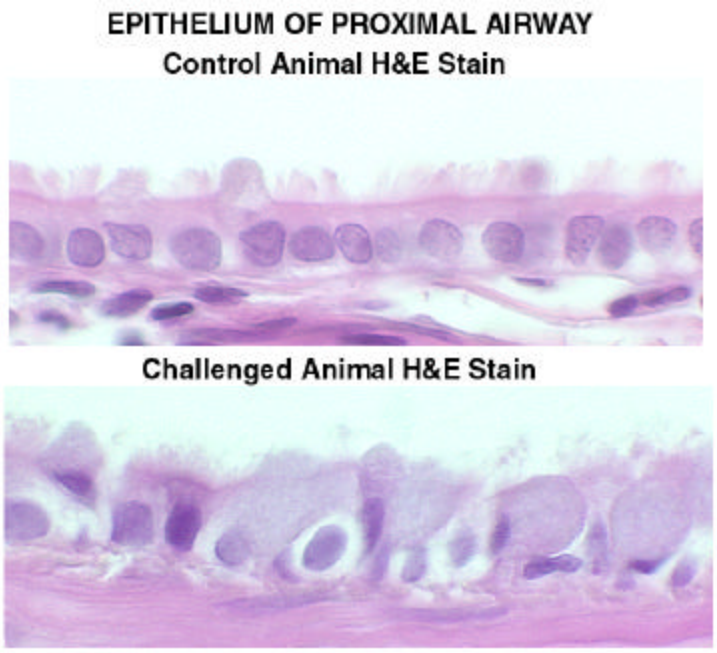


Figure 8. Histochemical staining of central airway. Hematoxylin and eosin (H&E), alcian blue/periodic acid Schiff (AB/PAS), combined eosinophil and mast cell (CEM).

The volume of epithelium was expressed per surface area of airway basal lamina (BL) (Figure 10). For the same airway the presence of mucosubstances within epithelial cells (Figure 11) was expressed as a volume per surface area of airway basal lamina (BL). Sensitization with ovalbumin alone did not affect either morphologic parameter compared with control. In contrast, a significant increase in both epithelial cell volume and intracellular mucosubstance volume were observed following ovalbumin sensitization and subsequent aerosol challenge with ovalbumin ($p < 0.05$).

Figure 9. Alcian blue/Periodic Acid Schiff staining of central airway epithelium



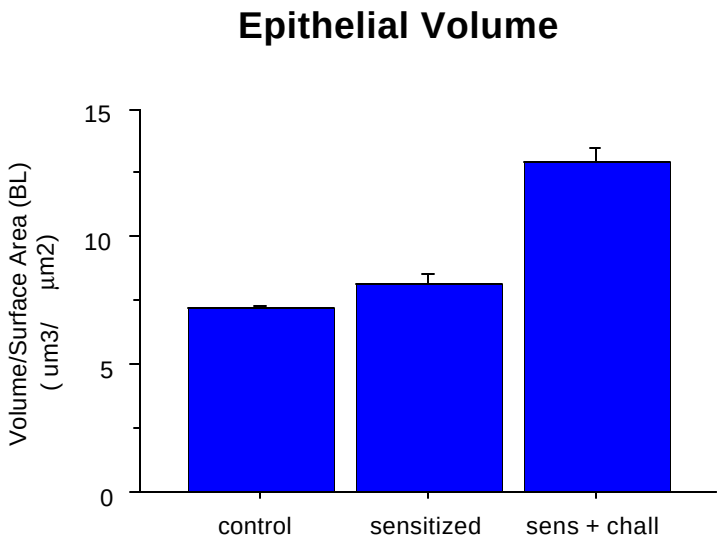
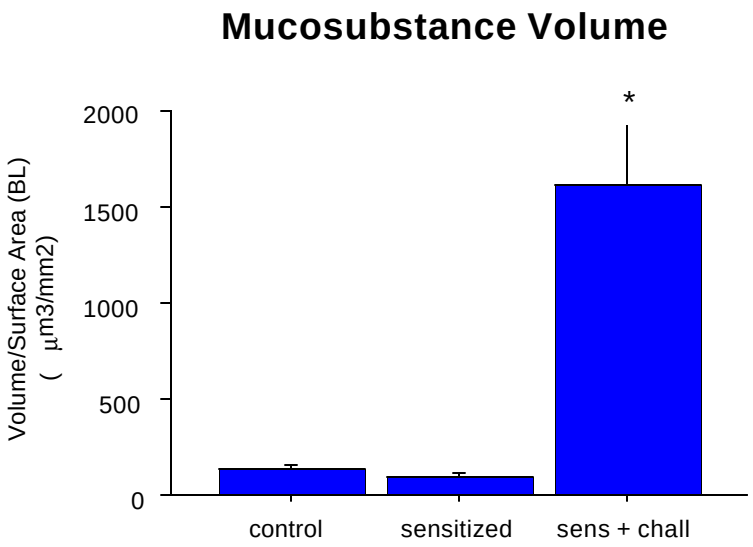


Figure 10. Epithelial cell volume of the central airway.

Figure 11. Volume of intracellular mucosubstances of the central airway.



The number of eosinophils (**Figure 12**) and mast cells (**Figure 13**) were also determined within the epithelium of the central airway and expressed per airway basal lamina length. Sensitization alone to ovalbumin did not change cell number compared to filtered control animals. In contrast, sensitization followed by ovalbumin aerosol challenge significantly increased eosinophil number in this airway.

Figure 12. Number of eosinophils

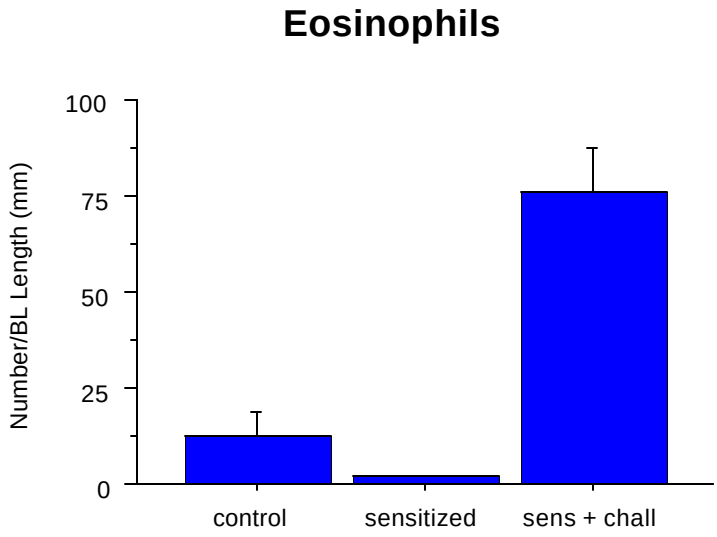
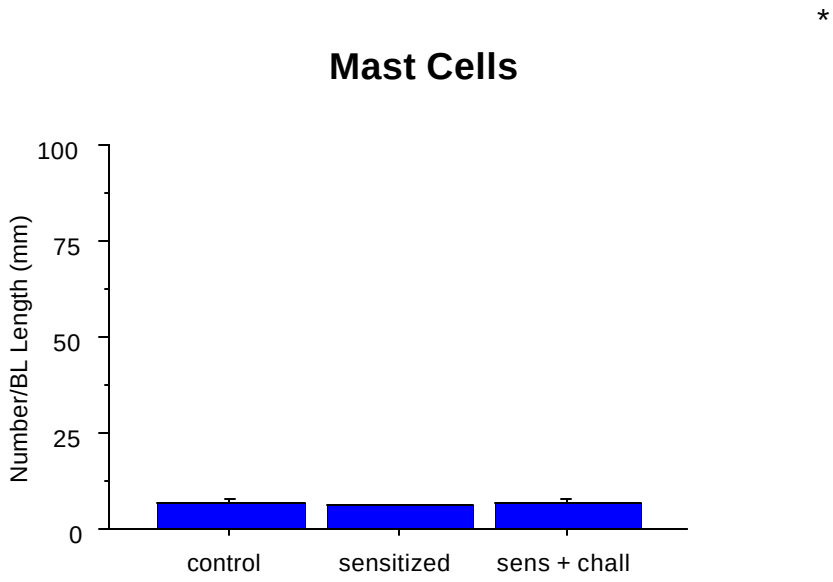


Figure 13. Number of mast cells.



SUMMARY

The bronchial responsiveness data collected to date for this model demonstrate the usefulness of ovalbumin-induced airway hyperresponsiveness in Brown-Norway rats as a model of induced asthma. The morphologic changes observed in the airways also confirm a significant alteration in the normal composition of the epithelium with a thicker epithelial lining and hypertrophy of cells containing mucosubstances, both hallmarks of an asthmatic condition of the airways. These studies suggest that this model may serve as a sensitive approach to examine the effects of particulate inhalation on airway function and structure.

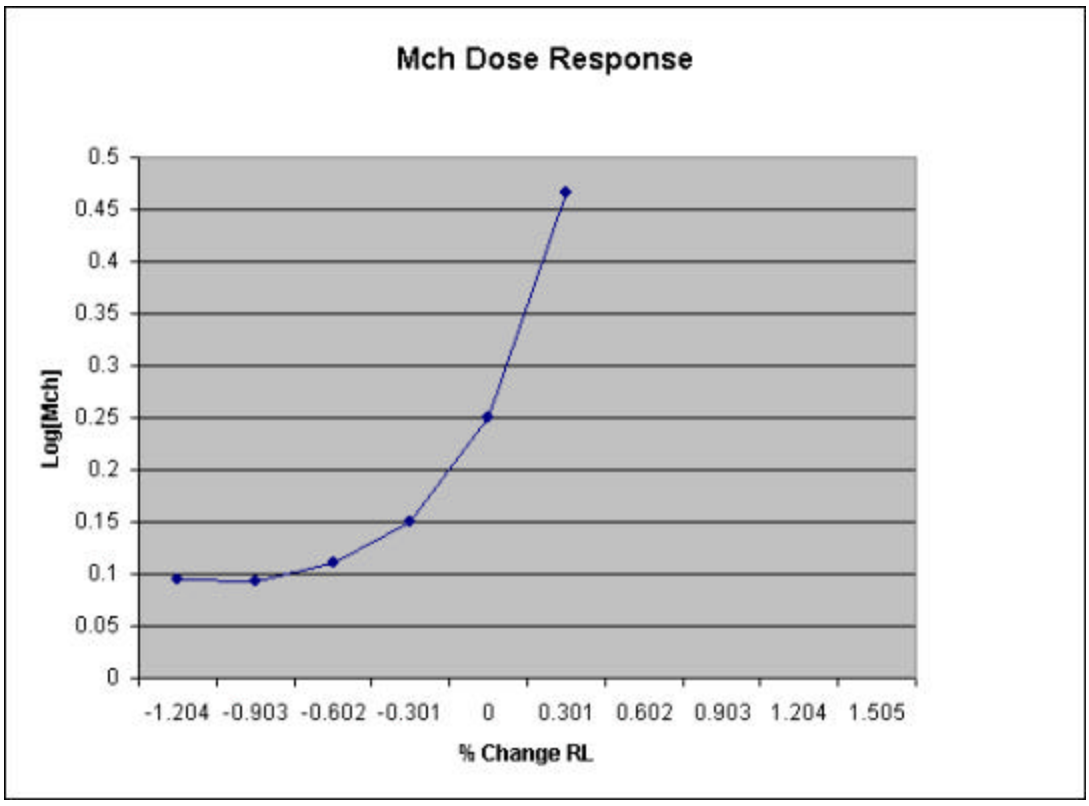
Results: BN Rat Experiment 2

The results of this experiment reflect the temporal effects of increasing the number of aerosol inhalation challenges with ovalbumin over a period of 4 weeks. No exposure of BN rats to PM was done as part of experiment 2.

Pulmonary function testing

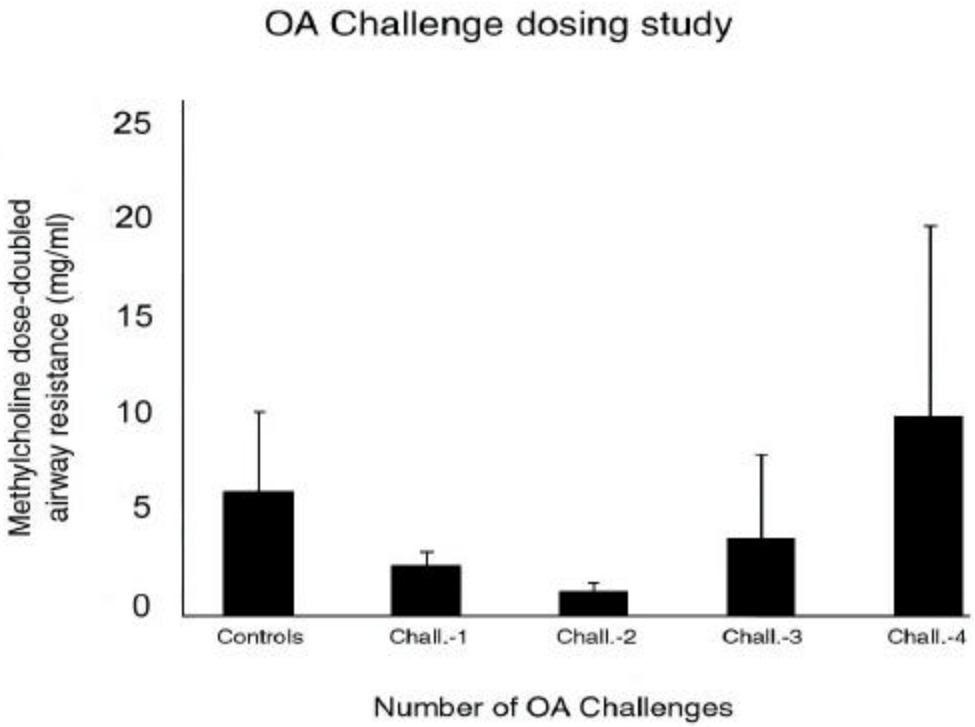
The methylcholine dose response curve for a single animal is shown in Figure 14, along with the log dose and corresponding resistance values and percentages. EC200RL is a measure of the methylcholine concentration which is associated with a doubling of airway resistance. The results of four sequential weekly ovalbumin challenges on EC200RL values are shown in Figure 15 along with the individual animal values in tabular form below the figure.

Figure 14. EC 200RL assay or the effective concentration of methacholine to double lung resistance.



The above graph demonstrates as the concentration (log dose) of metacholine (Mch) is increased, the percent change in lung resistance (RL) also increases. From dose response studies such as these, the effective concentration of metacholine required to double lung resistance (EC200RL) is determined. The effective dose to double airway resistance is shown in Figure 15 with serial OVA challenge.

Figure 15. EC200RL. The effective dose required to double lung resistance.



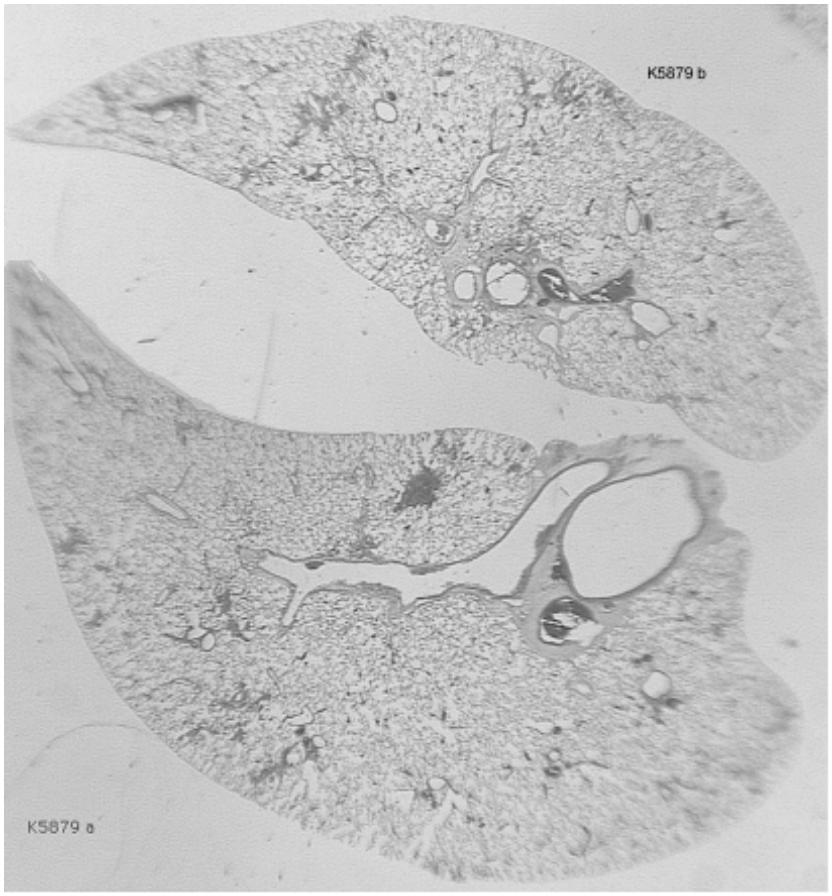
Controls		1 Challenge	2 Challenge	3 Challenge	4 Challenge
1.[illegible]	Week 1	1.606	1.167	8.404	8.385
6.703		2.531	0.433	0.606	24.005
3.75			0.6	1.323	3.072
11.916	Week 2				3.072
1.183					3.354
8.905	Week 3				
3.373					
1	Week 4				
3.269					

All controls were sensitized with ovalbumin, except one (dose-doubled airway resistance) (n=9). A significant difference in EC200 was noted between sensitized animals challenged two times to avalbumin aerosol versus sensitized animals challenged four times to ovalbumin aerosol, suggesting development of physiological tolerance to ovalbumin in BN rats with repeated exposure to ovalbumin.

Temporal histopathology: Severity of allergic lesions
Centriacinar (BADJ)

Centriacinar regions were identified on transverse lung sections (Figure 16). These regions were scored (0, +, ++, +++) for inflammatory changes as shown in Figure 17. As as the number of aerosol challenges with ovalbumin increased, an increase in the frequency and severity of inflammation in centriacinar regions were noted (Table 12). From animals exposed twice to animals exposed three times, the percent cellular involvement increased. However, in animals challenged three times, the involvement dropped off to levels of rats challenged once. In rats exposed four times, the percentage cellular involvement increased again to a percentage significantly higher than in the previous three animal groups. The controls had little cellular involvement. The filtered air controls showed no involvement, while filtered air sensitized controls, animals not subsequently challenged by aerosolized ovalbumin, showed percentages slightly higher than the non-sensitized rats did. Although there appeared to be no pattern, the animal group challenged twice was the only group to show statistically significant difference from the filtered air controls (Table 13 and Figure 18).

Figure 16 – Transverse lung tissue sections.
From these sections, centriacinar regions were identified at higher magnification.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K5879 a

Figure 17 - Centriacinar Region (BADJ) scoring

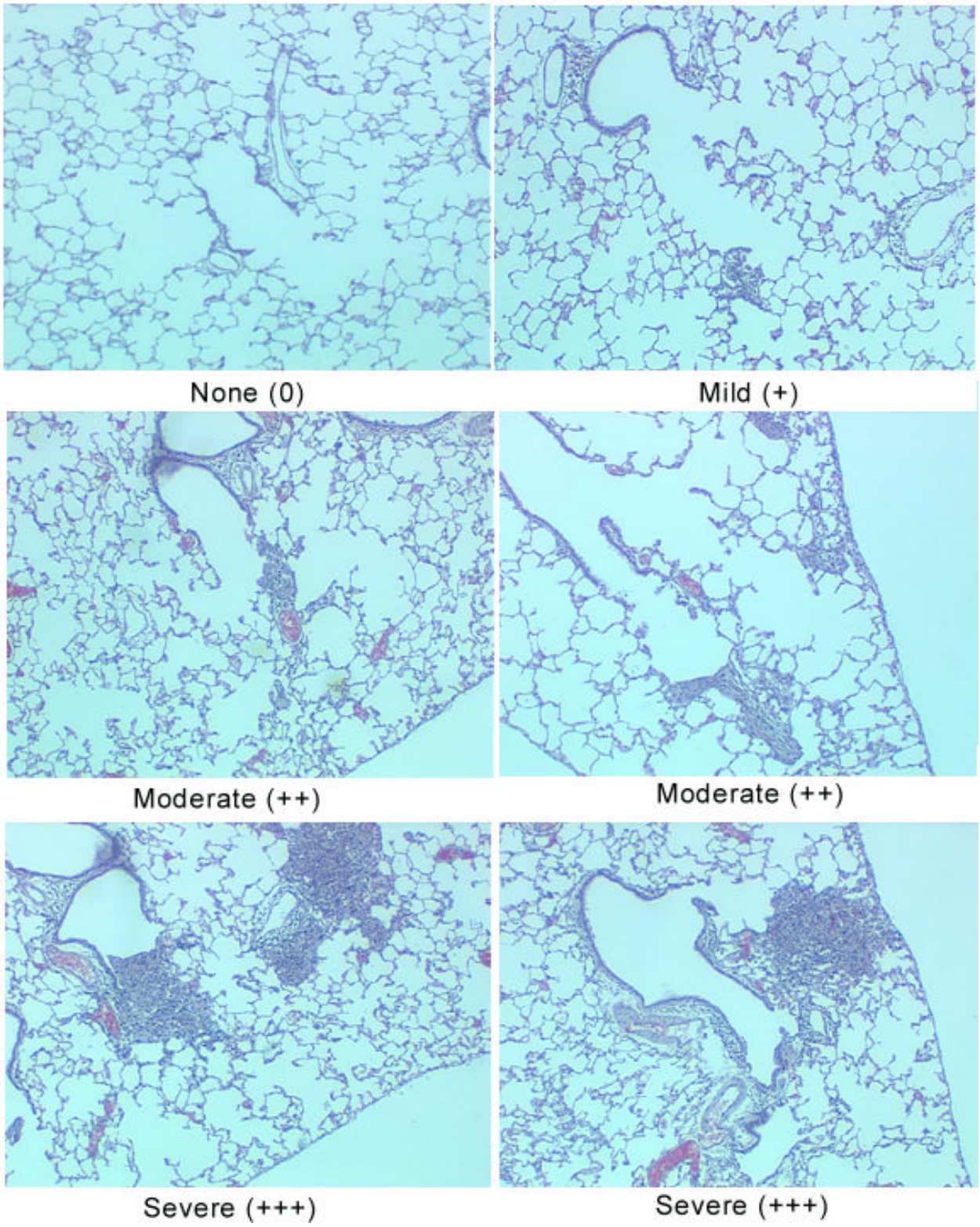
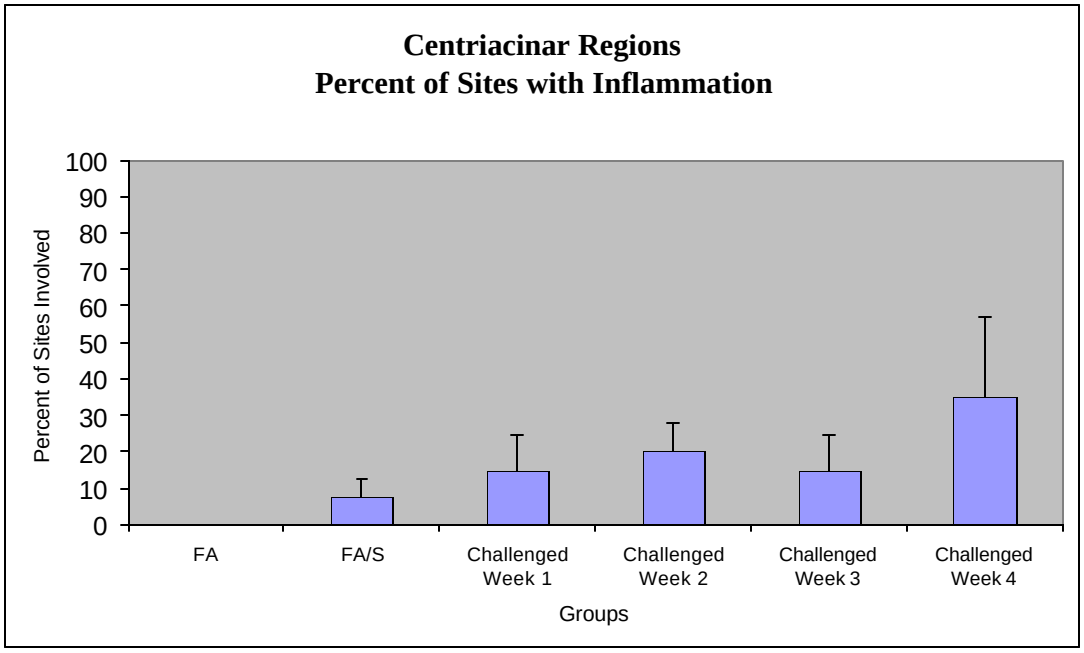


Table 13 - Centriacinar Regions: Percentage of Sites with Inflammation

	Total Sites Examined (# of animals)	Sites Showing Involvement	Degree of Severity of Sites Examined			
			0	+	+ +	+ + +
Filtered Air	20	0	20	0	0	0
	(4)	0%	100%	0%	0%	0%
Filtered Air/S	40	3	37	2	1	0
	(8)	7%	93%	5%	2%	0%
Challenged	20	3	17	0	2	1
Week 1	(4)	15%	85%	0%	10%	5%
Challenged	20	4	16	1	1	2
Week 2	(4)	20%	80%	5%	5%	10%
Challenged	20	3	17	3	0	0
Week 3	(4)	15%	85%	15%	0%	0%
Challenged	20	7	13	1	1	5
Week 4	(4)	35%	65%	5%	5%	25%

Figure 18 Centriacinar regions
*- p<0.05 compared with FA control
†- p<0.05 compared with FA/S control



Perivascular space around blood vessels

The perivascular spaces around blood vessels were scored as shown in Figure 19. The cellular involvement of the perivascular space did not fluctuate as dramatically as the cellular involvement noted within the centriacinar regions. The involvement in the rats remained at or just below 100 percent in the 4 different exposure groups. While the controls remain high at 87.5 percent for the filtered air and 91.1 percent for the filtered air (FA) sensitized rats, all challenged groups were significantly different from the filtered air controls. Although the differences between the filtered air controls and the challenge groups did not appear to be drastic, they were significant. Therefore, a determination was made that the ovalbumin did have an effect on the cellular inflammation in the perivascular space of the blood vessels. It could also be shown that the quantity of exposure did not have an effect on the amount of involvement, just that any exposure augmented cellular involvement. (Table 14; Figure 20) It should also be noted in the BN rat, a high level of cellular influx around blood vessels occurs even in FA controls with or without OVA sensitization.

Figure 19 - Blood Vessel scoring of perivascular cell influx

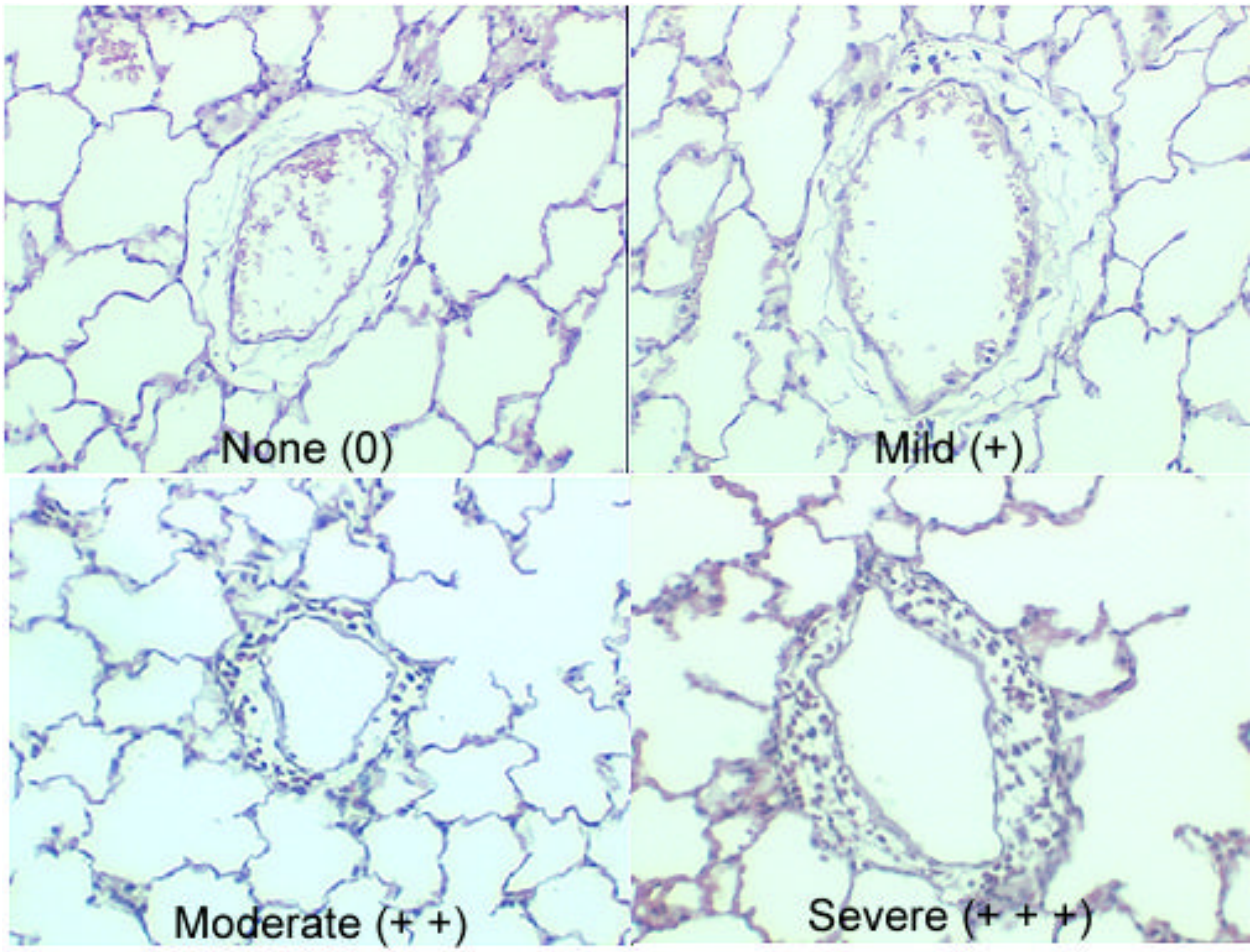
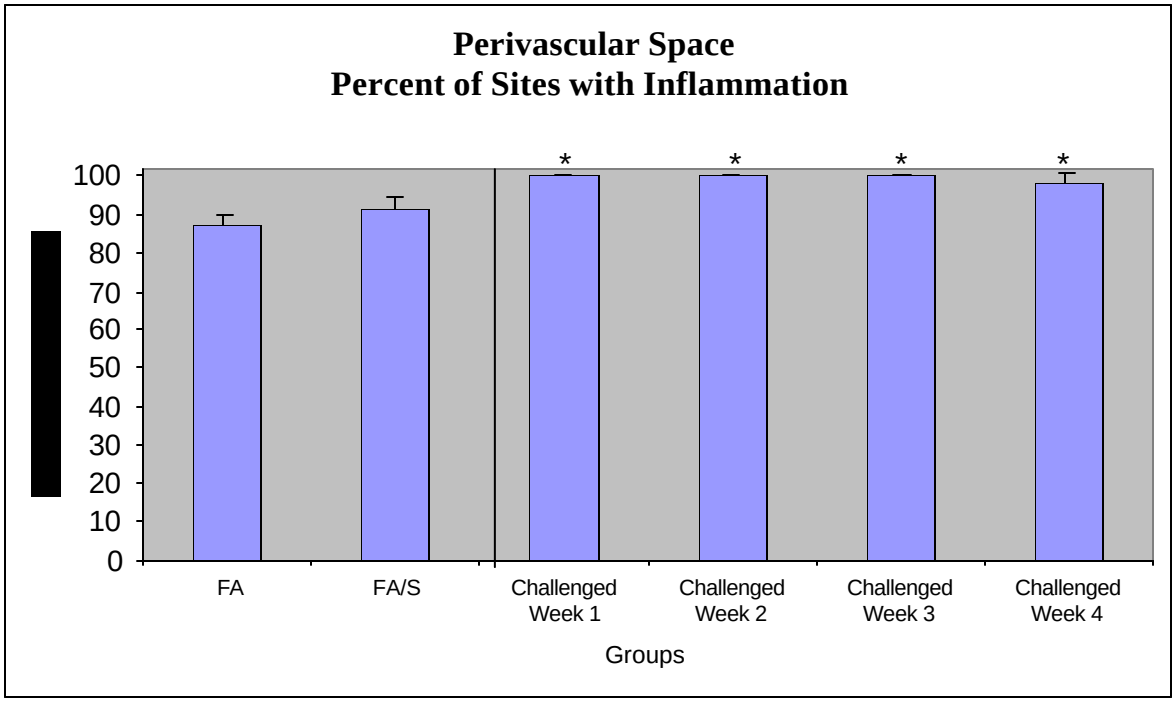


Table 14 - Perivascular Space: Percentage of Sites with Inflammation

	Total Sites Examined (# of animals)	Sites Showing Involvement	Degree of Severity of Sites Examined			
			0	+	+ +	+ + +
Filtered Air	40	35	5	23	8	4
	(4)	88%	12%	58%	20%	10%
Filtered Air/S	79	72	7	45	20	7
	(8)	91%	9%	57%	25%	9%
Challenged	40	40	0	8	19	13
Week 1	(4)	100%	0%	20%	48%	32%
Challenged	40	40	0	6	9	25
Week 2	(4)	100%	0%	15%	22%	63%
Challenged	40	40	0	6	15	19
Week 3	(4)	100%	0%	15%	37%	48%
Challenged	40	39	1	3	16	20
Week 4	(4)	98%	2%	8%	40%	50%

Figure 20: This graph indicates the high number of cells in the blood vessel areas.



Histopathology: Cellularity of lesions by site

Perivascular space around blood vessels

Eosinophils

Eosinophils were counted in the perivascular space of blood vessels as shown in Figure 21. The data showed a pattern shaped like a bell curve, with the values initially increasing before dropping off. The percentage of eosinophils rose with the first two challenges before decreasing slightly in rats with three aerosol challenges. The fourth challenge to rats reduced the eosinophil to total cell ratio to 29 percent. (Figure 13)

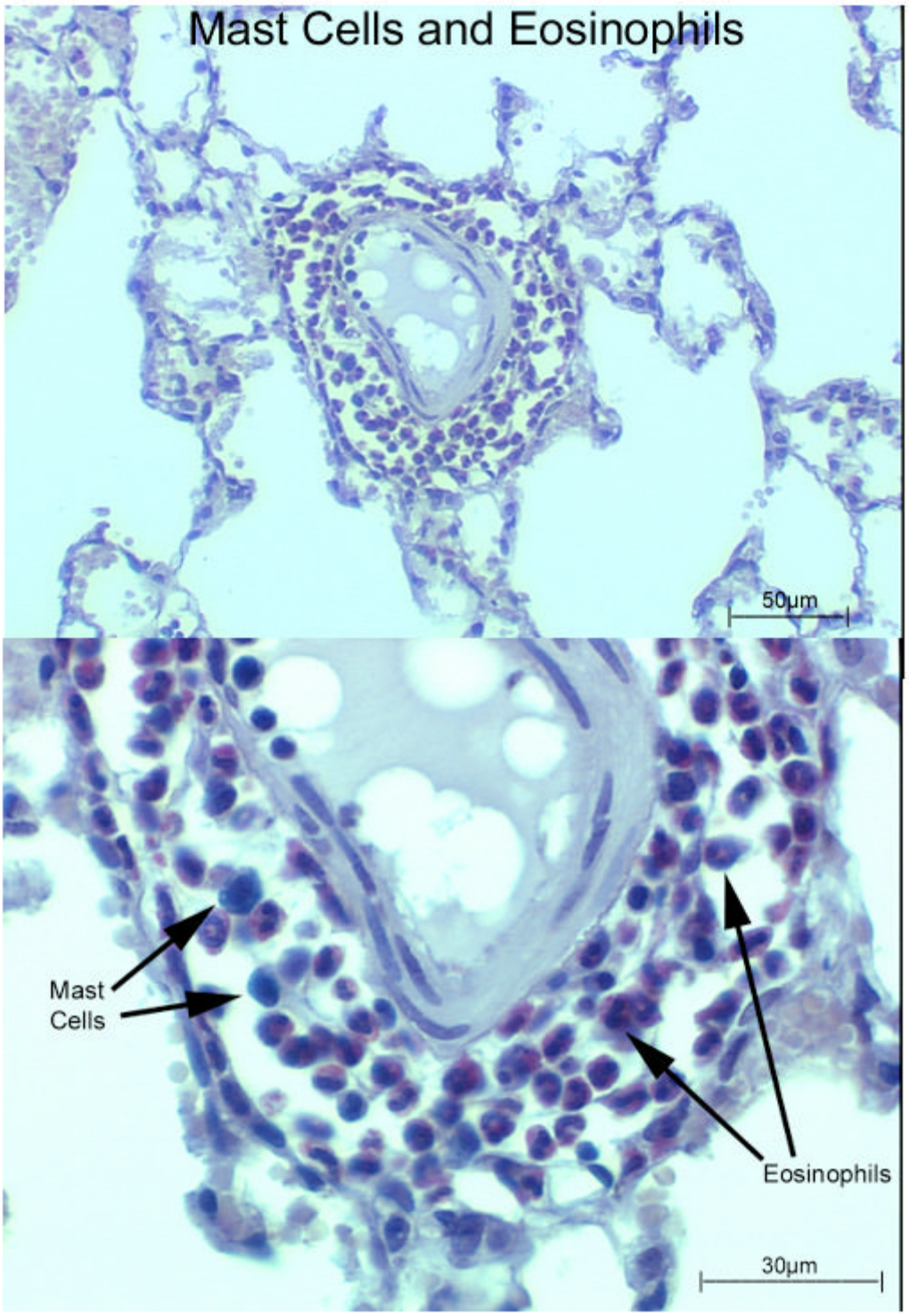
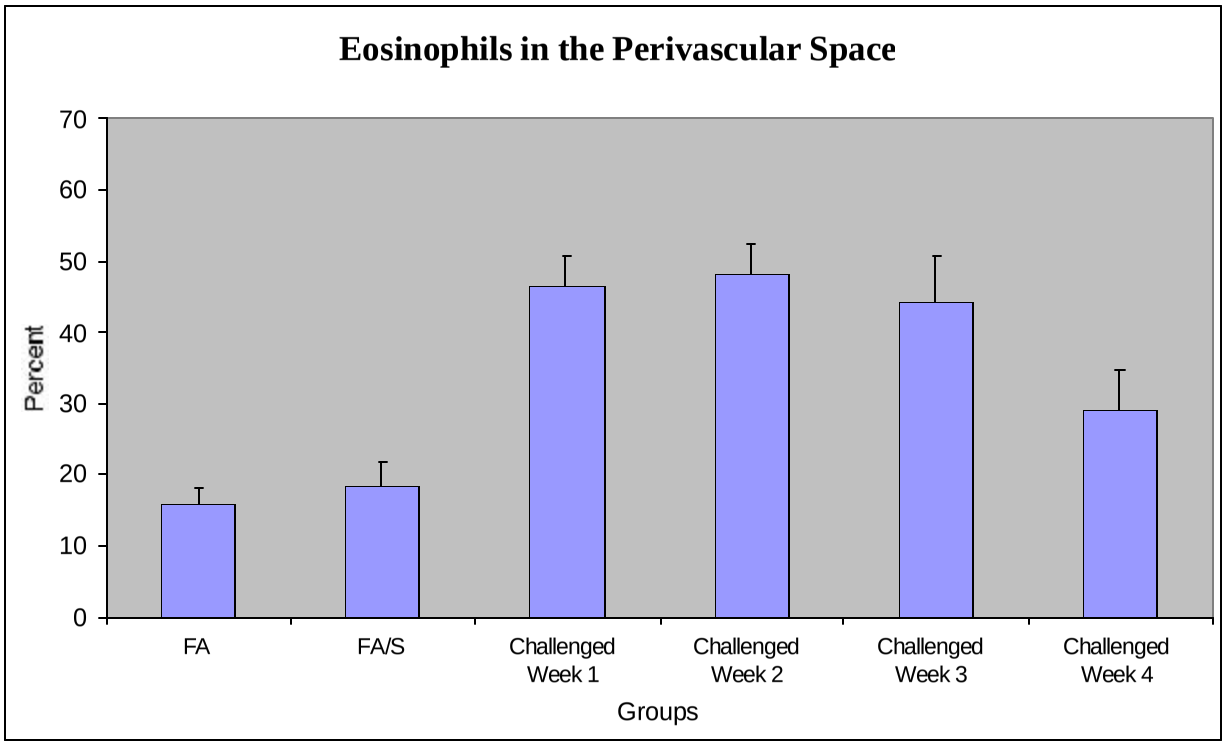


Figure 21. Perivascular mast cells and eosinophils

Figure 22 - The graph shows that OVA has a significant effect on the presence of eosinophils in the perivascular space of the blood vessels.



Mast cells

The mast cells showed a trend similar to that of the eosinophils. However, these percentages were markedly lower than those for eosinophils. BN rats exposed to repeated challenges of aerosolized ovalbumin demonstrated only slight increases in these cells in the perivascular space (Figure 23). However, these changes were not statistically significant.

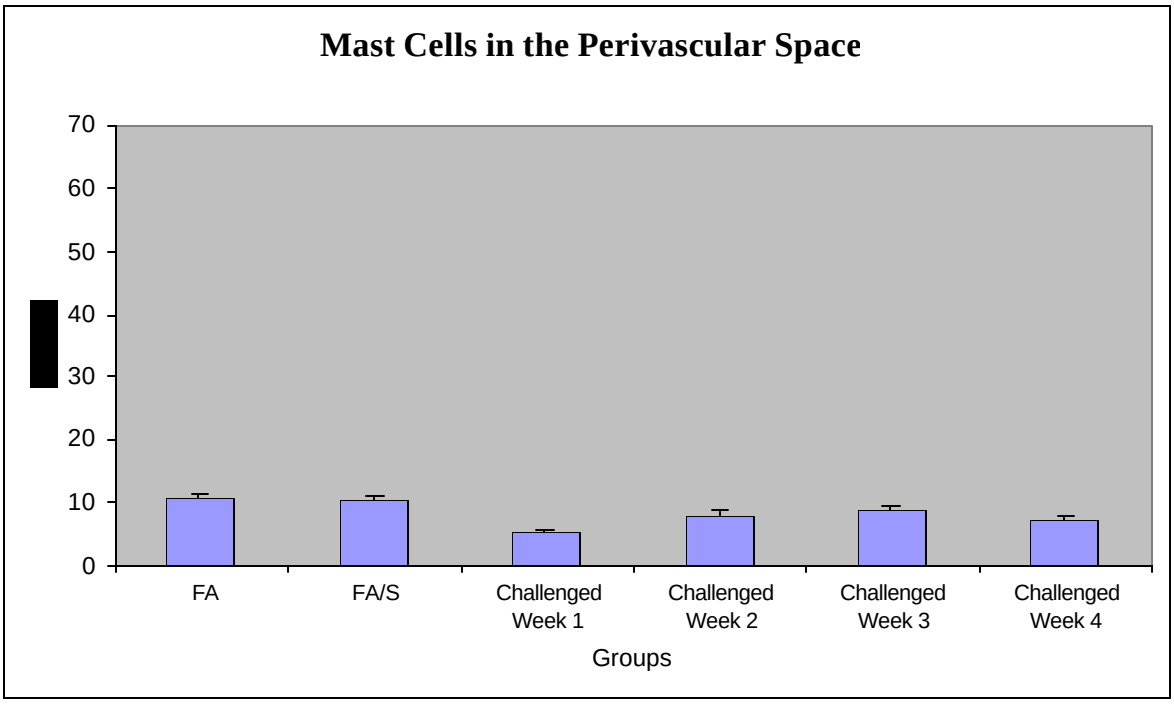
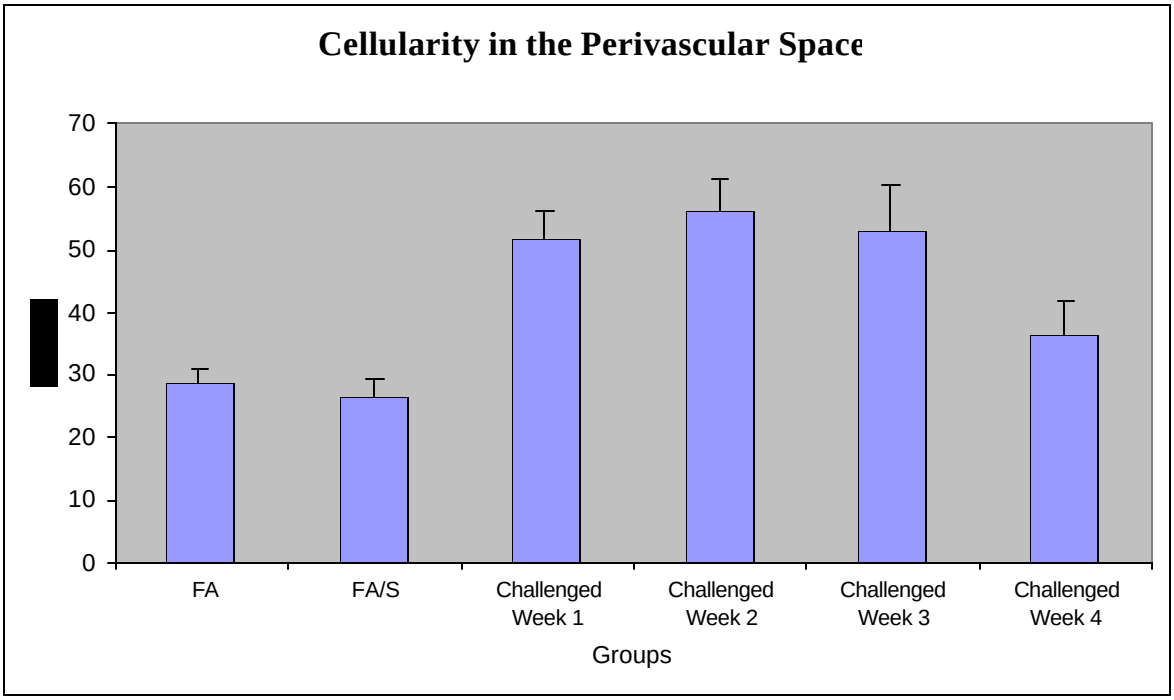


Figure 23: The mast cells demonstrate a similar pattern to the eosinophils, however they represent only a small percent of the total number of cells present in the perivascular space. Therefore, these changes are considerably less and do not attain a level of statistical significant compared with control animals or with repeated OVA aerosol challenge.

Combined (eosinophils and mast cells)

The combined cellularity showed a bell curve trend almost identical to the one produced by eosinophils. The single challenge rats showed cellularity greater than that in the controls. After continuing to rise in Week 2 of exposure, the cellularity diminished in the final two weeks of aerosolized ovalbumin challenges. (Figure 15)

Figure 24: The total cellularity reflects the patterns of the eosinophils and mast cells and indicates that after initially having an allergic reaction to the OVA, the lungs of the rats were able to adapt and recover by the fourth week.



Central Airways

Epithelium (Mucin)

The amount of mucin stored within the airway epithelial cells of the central axial pathway is illustrated in Figure 25. A significant increase in the volume of mucin per basal lamina (BL) length was noted following two, three and four sequential weeks of ovalbumin aerosol challenge (Figure 25).

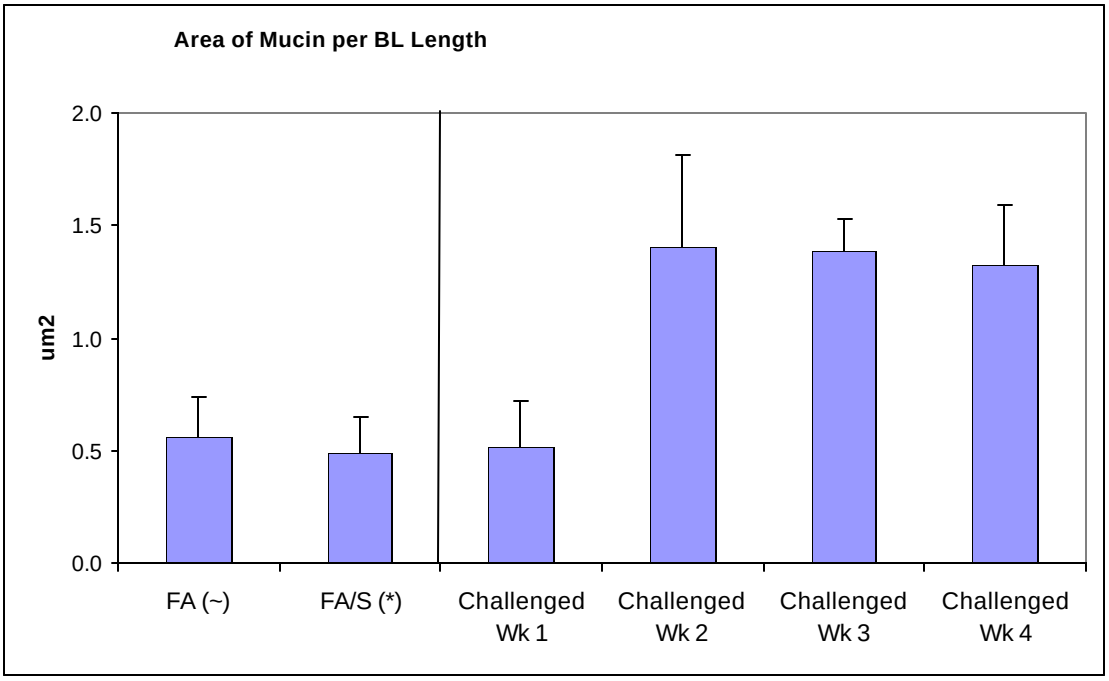
Epithelium (Inflammation)

With each subsequent exposure, the mast cell to basal lamina length ratio in the epithelium of the central airways increased. The eosinophil count increased over the first three exposures before decreasing significantly in animals challenged four times. Since mast cells were acknowledged to indicate an allergic reaction, the rising quantity of mast cells throughout the four challenge-groups indicated that there was allergic activity occurring around the central airways.

After rising for the first three weeks of challenges, the quantity of eosinophils present dropped off in the fourth week of challenges. In the perivascular region, the eosinophil count increased during only the first two challenges, rather than all three challenges as in the epithelium of the central airways. The similarity of the patterns is identifiable and important.

The difference between the eosinophil and mast cell counts occurred in the actual number present. The mast cells had a significantly higher ratio of cells to the basal lamina than did the eosinophils. The ratios were calculated by dividing the respective cell count by the length of the basal lamina to get comparable numbers. The filtered air and filtered air sensitized controls had similar levels of mast cells and eosinophils. The only significant difference concerning eosinophils occurred between the filtered air sensitized and the rats challenged three times. Statistical significance indicates that there is an appreciable and important difference between the two being compared. It shows how the exposures had a significant effect by the third challenge (Figure 26).

Figure 25



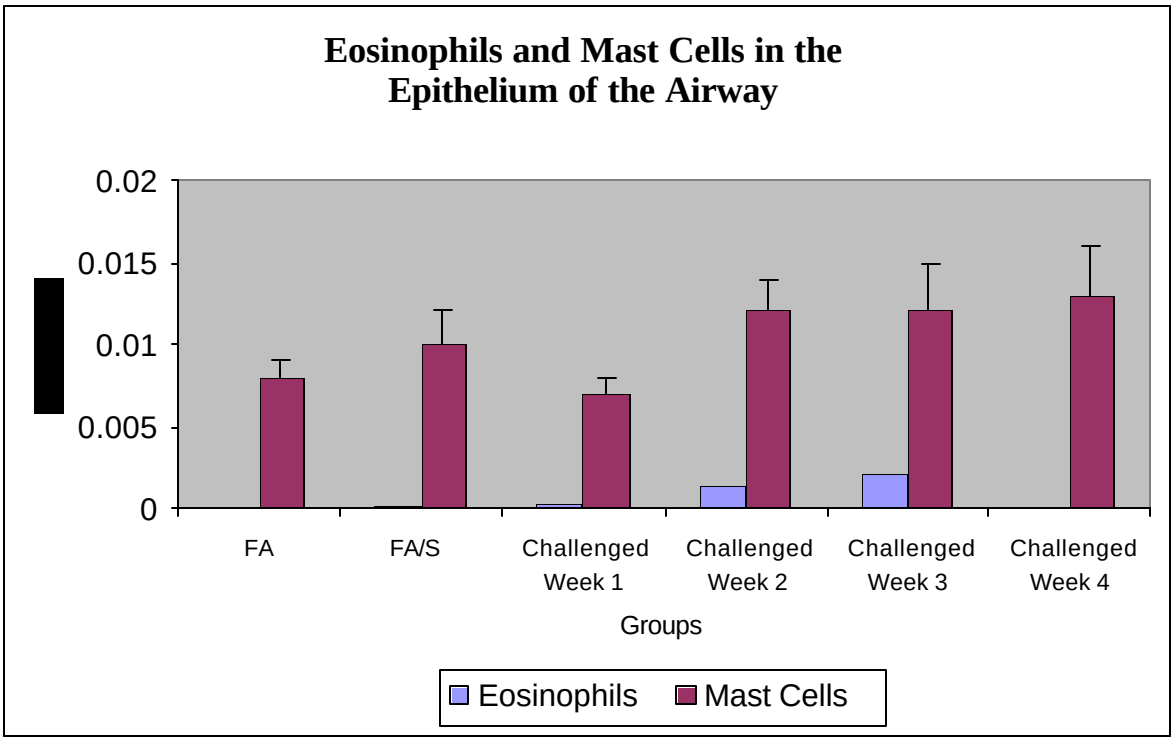
Volume of mucin present within the central airways of Brown Norway rats

Group

FA (~)

	FA/S (*) Challenged Wk 1 Challenged Wk 2 Challenged Wk 3 Challenged Wk 4
um2 mucin	
	0.563
	0.485
	0.514
	1.405
	1.388
	1.323
std err	
	0.176
	0.162
	0.203
	0.41
	0.145
	0.268
n	
	4
	8
	4
	4
	4
	4

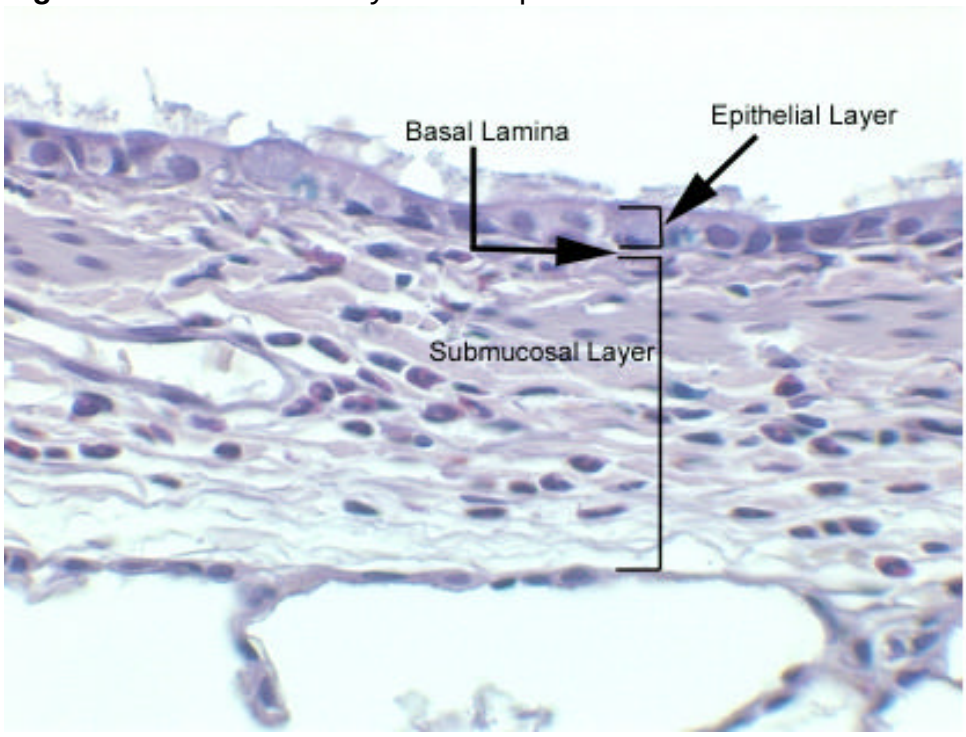
Figure 26 - Similar to the graphs above, this eosinophil and mast cell count showed that the OVA does increase the number of cells. Note that the eosinophils declined in Week 4 after increasing for the first three weeks. It should be noted eosinophils are present in the airway epithelium of BN rats even in the absence of OVA sensitization.



Submucosal layer: Interstitial vascular and airway wall

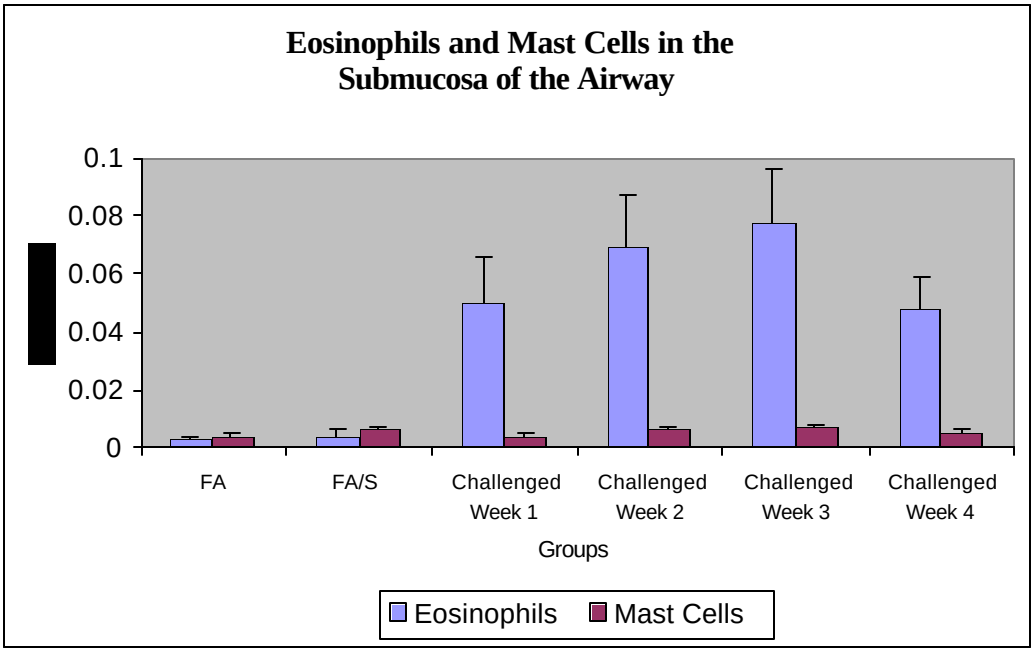
Measurements of cellular change within the submucosal layer (Figure 27) were opposite that of the epithelium in that the eosinophils occurred in a much higher cell to basal lamina length ratio than did the mast cells. The eosinophils followed the same trend in the submucosa as in the perivascular space of the blood vessels. The ratio dropped considerably in the group exposed four times after increasing over the first three exposure groups. In each of the four weeks, the eosinophils were significantly different from both the filtered air controls and the filtered air sensitized controls.

Figure 27 – Central airway wall composition.



The filtered air and filtered air sensitized control groups had a similar percentage of mast cells to the challenged groups (Figure 28). However, no statistical significance was noted when compared with control animals. The eosinophils showed greater difference from the controls than the mast cells did; all four animal groups had much higher percentages than the controls and as a result, the correlation was more statistically significant (Figure 28). After increasing for the first three exposures, the quantity of both the eosinophils and mast cells fell after a fourth week of exposure to aerosolized ovalbumin. As before, the mast cell pattern mimicked that observed in the perivascular space. The eosinophils also increased and decreased like a bell curve, but their percentage increased in the third week, while eosinophil percentages decreased in the third week in the perivascular space. The same general trend occurred in all of the data and graphs from the previous aspects of this experiment.

Figure 28 - As before, the graph shows a large increase in eosinophils following the OVA challenges. The mast cells show a similar pattern, but to a lesser degree. Both cell types, especially eosinophils, are reduced in the fourth week, suggesting tolerance with repeated OVA challenge.



Summary: We found repeated ovalbumin aerosol challenge increased centriacinar inflammation (Figures 17-18, Table 13) as well as eosinophil numbers (Figure 28) in the lungs of OVA-sensitized BN rats. However, these changes did not attain a level of statistical significance with repeated OVA challenge.

Results: BN rat Experiment 3

Note: In our study, we found BN rats which had not been sensitized with OVA by IP injection but had received a single aerosol challenge to OVA demonstrated a significant response to this challenge. Such a finding suggests that in some manner, these rats had been previously exposed to OVA. Therefore, the findings for animals receiving only OVA aerosol challenge are not reported (with the exception of the OVA-specific serum IgE levels). This experiment represents the first study in which BN rats were exposed to PM in addition to OVA.

Pulmonary function testing

Rats sensitized (S) to OVA followed by exposure to PM showed decreased sensitivity to methacholine challenge compared to FA controls. However, this difference did not reach a level of statistical significance. In a similar manner, rats sensitized and challenged (S/C) with OVA, followed by exposure to PM also showed decreased sensitivity to Mch challenge compared to FA controls (Figure 29), but once again this decrease did not attain a level of statistical significance.

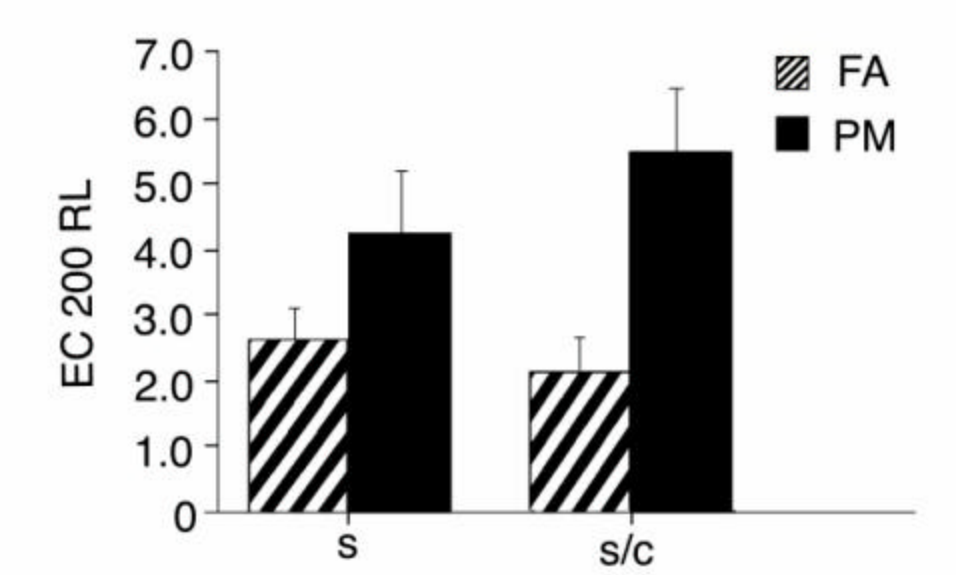


Figure 29. Methacholine dose to double lung resistance

BAL leukocytes, cell viability and cell differential

There was no difference in total cell counts or cell viability between treatment groups, however the percentage of macrophages in sensitized and challenged rats decreased significantly from both controls while the percentage of lymphocytes increased significantly in the sensitized and challenged rats when compared to the challenged rats that were not sensitized. The lymphocyte percentage also increased in the group that was sensitized, but not challenged. However, this change did not reach a level of statistical significance. Challenge with OVA significantly increased the percentage of eosinophils over unchallenged controls, whereas sensitization with OVA rather than challenge increased percentage of neutrophils compared to unsensitized controls. The effects on total white blood cells (WBC), and differential counts between exposure to filtered air and particulate matter was also analyzed as total cells per ml of recovered lavage. There was no significant difference between FA and PM exposures in any treatment group with regards to total WBC or percentage of cell viability. In the sensitized but not challenged group, there was a significant decrease in lymphocytes per ml BAL. This same trend was observed in the other two treatment groups, but did not reach a level of significance. There were no significant changes in cell number or consistent trends for macrophages, eosinophils or neutrophils in the BAL.

BAL total protein

There were no significant changes in protein/ml in recovered BAL between groups or for FA versus PM exposures.

Cell permeability assay

Airways treated with ethidium-1-homodimer (EtD-1) as a measure of increased permeability were examined for each group. In the two sensitized groups, a trend of increased numbers of cells positive for EtD-1 was observed in the lungs of rats exposed

to PM compared with the matched animals exposed only to FA (Figures 30-31). However, these changes did not attain a level of statistical significance.

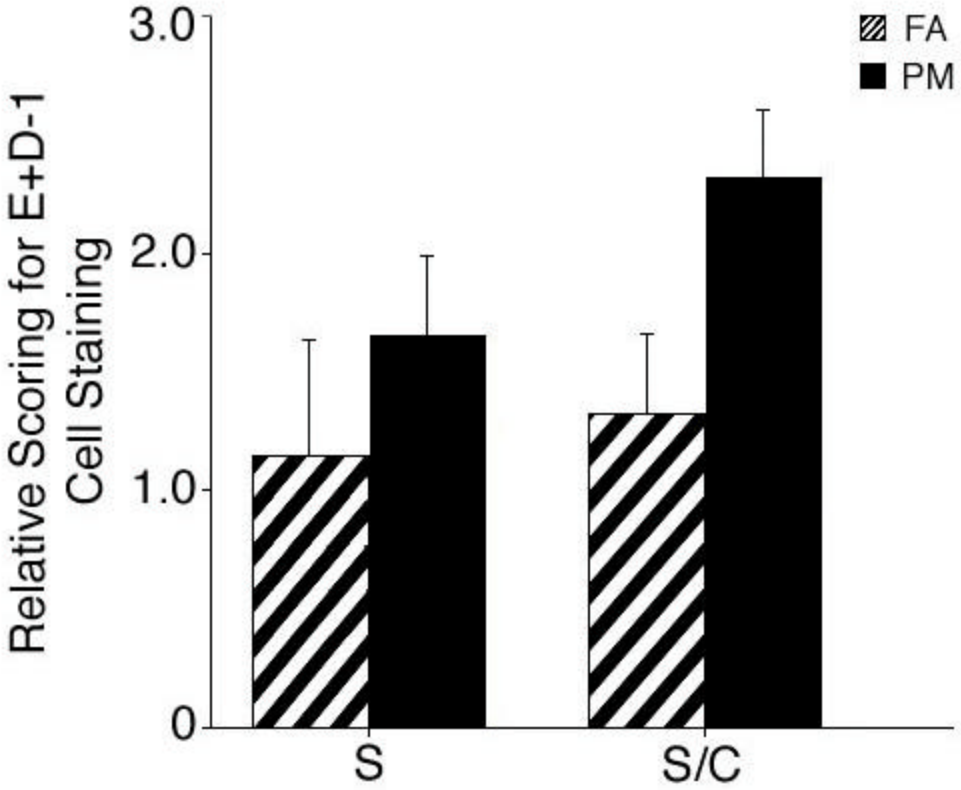
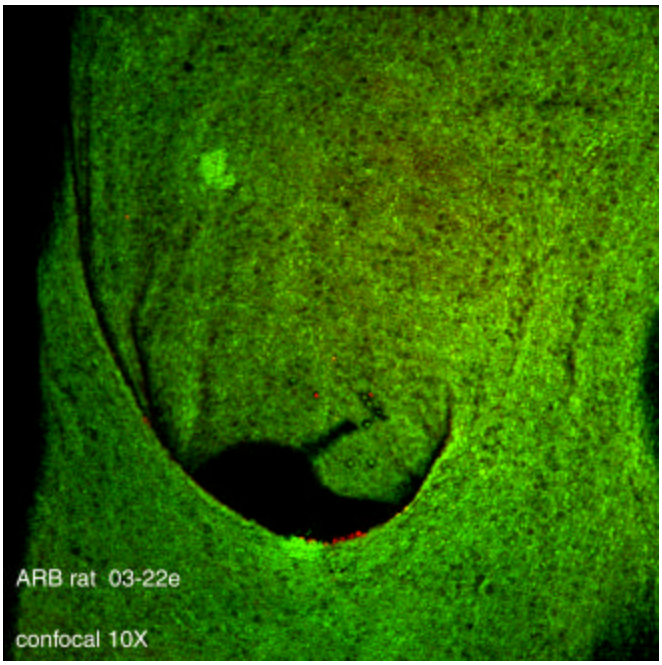
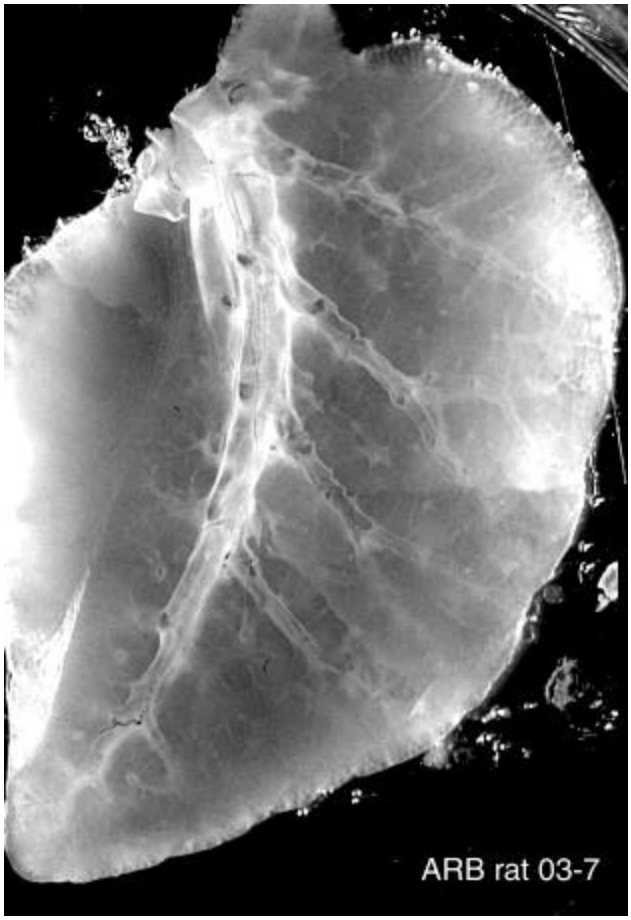


Figure 30. Epithelial cell permeability

Figure 31. Epithelial cell permeability (red dots) at an airway bifurcation along the central axial airway of a microdissected airway.





OVA-specific serum IgE

Rats in all groups demonstrated a consistent trend for increased serum IgE in rats exposed to PM, but only reached a level of significance in the group receiving OVA aerosol challenge, but no prior sensitization to OVA by subcutaneous injection (N/C) (Figure 32).

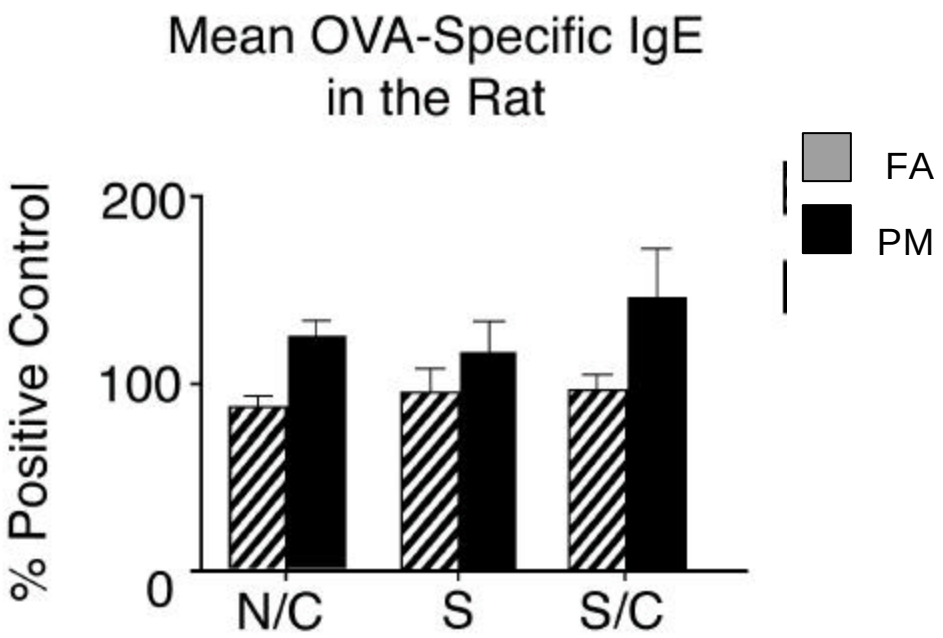


Figure 32. OVA-specific serum IgE

N/C: Not Sensitized/Challenged with OVA aerosol.
S: Sensitized with OVA
S/C: Sensitized with OVA/Challenged with OVA aerosol
(A significant increase in OVA-specific IgE was noted following PM exposure compared with FA controls only in N/C group, $p<0.05$. All other groups demonstrated a similar trend following PM exposure, but did not attain a level of statistical significance.)

IgE immunohistochemistry

IgE positive cells present in each subcompartment of the lung were low. Cytoplasmic staining and morphology suggested that these cells were plasma cells. Cell counts were lowest in the airways and highest in the perivascular space, mucosa, and submucosal regions. Significant numbers of positive cells were also located in the lung parenchyma. There were no detectable differences between FA and PM exposures with respect to distribution of IgE positive cells or numbers of cells staining positive for IgE.

BrdU immunohistochemistry

The primary cells examined for BrdU uptake were epithelial cells lining airways from mid-level bronchioles to the termination of the airways (i.e., terminal bronchioles). Analyzed as a percentage of total cells, or normalized to airway basement membrane length, the epithelium of bronchioles in sensitized and challenged rats showed statistically significant increases in BrdU labeling following exposure to PM compared with matched FA controls. This same trend was observed for the other treatment groups, but did not reach a level of significance in the sensitized/not challenged or the OVA only challenged groups.

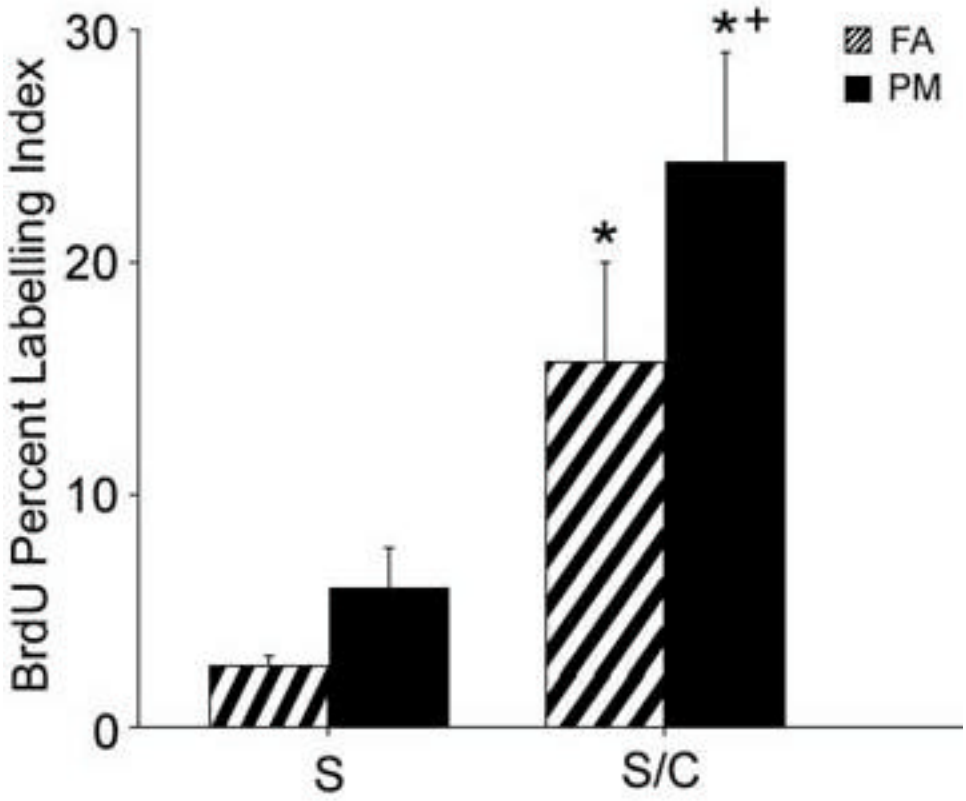


Figure 33. BrdU uptake in airway epithelial cells

BrdU incorporation into epithelial cells lining the airways of the lungs. The labeling index for BrdU is indicated as a percentage of the total epithelial cells present. The asterisk designates a significant difference ($p<0.05$) from the corresponding sensitized (S) animals. The cross designates a significant difference from sensitized/challenged (S/C) animals exposed to filtered air.

Histopathological scoring

The levels of inflammation in subcompartments of the lung were objectively scored by a blinded individual on a scale of 0 (no Inflammation) to 3 (severe inflammation). Sensitized and challenged rats demonstrated increased mean scores in PM exposed rats in the bronchi, terminal bronchioles, and perivascular regions but a slightly lower average level of inflammation in the parenchyma compared with animals exposed to filtered air. However, due to a high degree of individual variability, none of the differences between groups attained statistical significance. There was no significant difference between extent of granulomas in lung sections from FA and PM exposed sensitized and challenged rats determined by utilizing a 42 point graticule and counting points hitting a granuloma in 50 random 10x fields. However, the percent of rats showing severe granuloma formation was 80% in PM rats, while severe granuloma formation was only seen in 33% of the FA rats.

Eosinophil counts normalized for the area counted in the sub mucosal region of terminal bronchioles were determined and compared between exposure groups. Eosinophil counts per 100μm^2 demonstrated higher mean values in sensitized groups when exposed to PM but the difference did not reach statistical significance. Mast cell numbers on average were lower in PM exposed animals, but again not to a level of significance.

Alcian blue/PAS staining for mucin was analyzed within the most proximal generations of the bronchial airway formed by the major axial pathway of the left lobe. There were no changes in mucin volume per basal lamina surface area between groups or between animals exposed to FA and PM. (Figure 34).

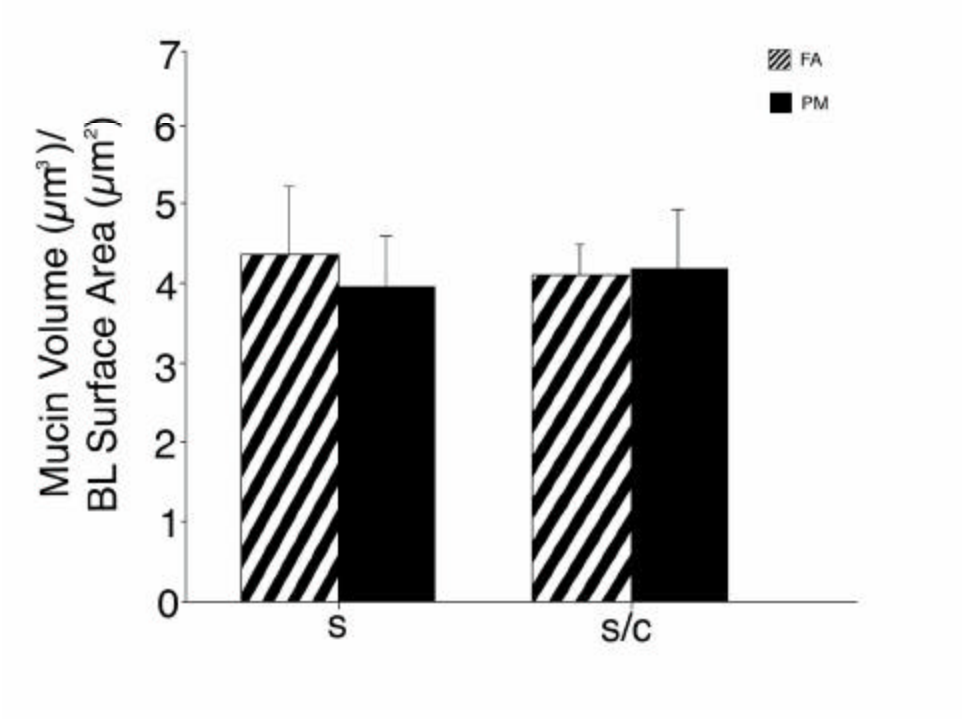


Figure 34. Mucin volume of airway epithelium

Results: BN rat Experiment 4

Tissue Inflammation

CEM positive eosinophil counts in the sub-epithelial compartment of terminal bronchioles, normalized to basal lamina length, were significantly higher following OVA challenge in the PM exposed BN rats when challenge was followed by three days of continuous PM exposure. A similar non-significant increase was observed for the BN rats receiving FA for three days following challenge. Eosinophil counts were not affected by PM exposure relative to FA controls. Eight days after OVA challenge, both FA and PM (six days) exposed groups showed non-significant increases over corresponding unchallenged controls. At the eight-day time point, PM exposed rats in both challenged and unchallenged groups tended to have lower eosinophil counts, when compared with similar groups receiving FA, that were not statistically significant.

Centriacinar inflammation scores in rats receiving three or six days of PM following OVA challenge were not significantly different from those groups receiving FA. In BN rats receiving three days of continuous PM, and following six days of PM in the sensitized but not challenged group, there was a tendency for PM exposed groups to have lower mean scores than corresponding FA controls but this difference did not reach a level of significance.

BrdU Labeling of Epithelial Cells

In mid-level bronchioles examined in paraffin sections OVA challenge followed by three days of PM exposure resulted in mean BrdU labeling of epithelial cells with a non-significant decrease in BrdU labeling compared to corresponding FA controls. Challenge was found to cause a non-significant increase in labeling over that observed in unchallenged groups. Following six days of PM exposure (eight days after OVA challenge) BrdU labeling of epithelial cells in mid-level bronchioles was significantly elevated compared to corresponding unchallenged controls. There was no effect on BrdU labeling of mid-level bronchiolar epithelium due to PM.

In transverse paraffin sections of terminal bronchioles, a significant increase in BrdU labeling was observed due to OVA challenge between the PM exposed groups following three days of PM exposure and a non-significant increase was present between the FA groups. Following six days of PM exposure, the reverse occurred, with challenge causing a significant increase in the FA group and a non-significant increase in the PM group. There were no effects on BrdU labeling of epithelial cells due to PM at the level of the terminal bronchioles for three or six days of PM exposure following OVA challenge.

Serum IgE

Serum IgE levels were consistently elevated compared to unchallenged controls by three days of continuous PM following OVA challenge. Five days later, following two days of FA and an additional three days of PM, this trend was still apparent in the PM groups but was no longer observed for FA. PM had no significant effect on IgE levels in BN rats at either time point studied.

Cytokine mRNA Expression

OVA challenge followed by three or six days of PM had no effect on sensitized or sensitized/challenged BN rat mRNA expression levels for eotaxin and IL5 purified from the right medial lobe. Eotaxin and IL5 mRNA expression was also not affected by challenge following sensitization at the time points examined in this study.

Three continuous days of carbon and ammonium nitrate PM caused a significant increase in IL4 mRNA expression in sensitized and challenged BN rats compared to levels in rats receiving FA. When exposed to PM, OVA challenge significantly increased IL4 mRNA expression. Levels of IL4 mRNA were significantly deceased in sensitized and challenged rats exposed to PM following two days of FA and an additional three days of PM when compared to rats three days after challenge. In contrast, there were no effects due to PM or OVA challenge following six days of PM (Figure 35).

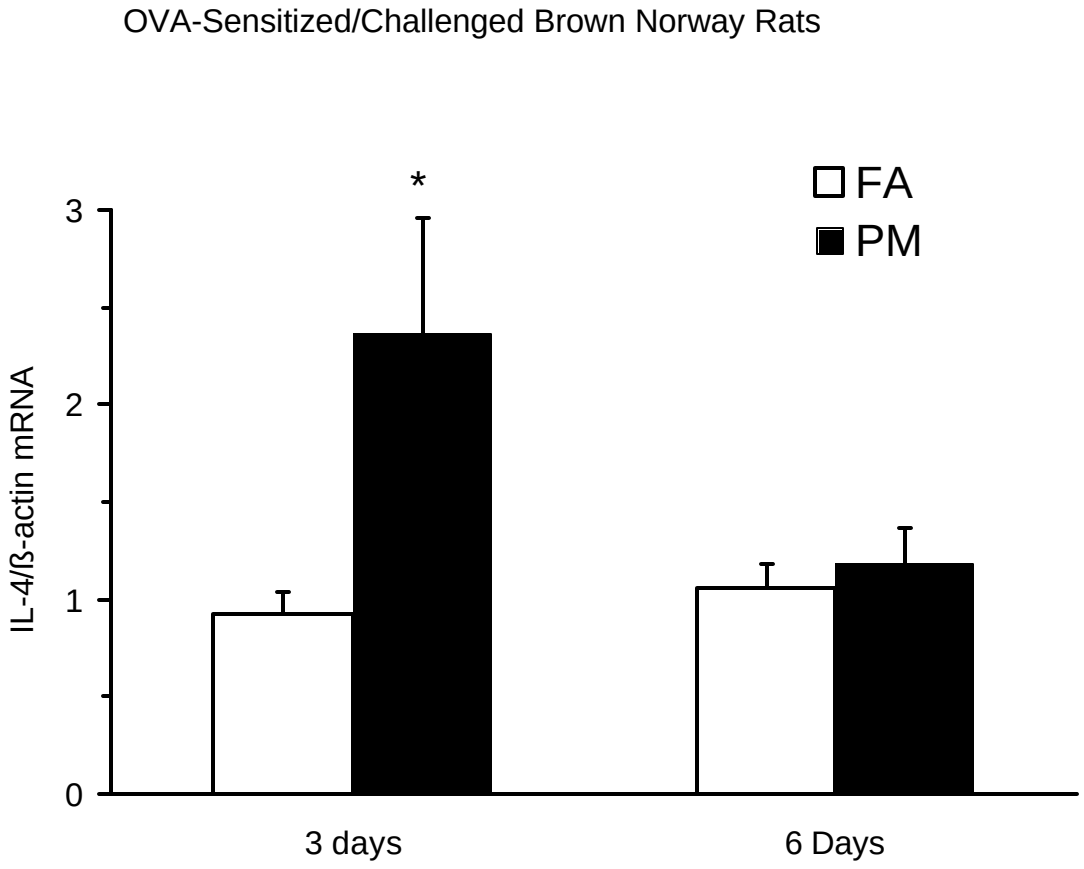


Figure 35. mRNA levels in lung tissues of BN rats exposed to FA or PM.

Pulmonary Function Testing

Pulmonary function testing was only performed in groups that received three continuous days of PM exposure following OVA challenge. Challenge caused an increase in airway sensitivity to methacholine challenge that reached a level of statistical significance between groups exposed to PM but not in groups exposed to FA.

In the sensitized-only groups, PM appeared to decrease airway sensitivity but the difference failed to be statistically significant.

Overall results of BN rat Experiment 4

We found particle exposure for up to six days to show no significant change in BrdU labeling of airway epithelial cells compared with filtered air controls. We observed eosinophilic inflammation to be significantly increased by OVA challenge following three days of particle exposure, but not following six days of particle exposure. Eotaxin and IL5 mRNA levels measured in lung tissue homogenates by RT-PCR were similar for each treatment group. In contrast, IL4 mRNA expression was significantly increased in sensitized and challenged rats following exposure to airborne particles for 3 days. However, with progressive particle exposure up to six days, IL-4 mRNA levels returned to control levels (Figure 35).

These findings suggest PM may initially increase eosinophilic inflammation and epithelial damage following OVA challenge, but may become attenuated with progressive PM exposure. Decreases in inflammation and BrdU labeling with progressive PM exposure suggest an association between eosinophils and the PM-induced epithelial damage. Increases in IL4 mRNA expression in BNRs with allergic airways following exposure to PM may reflect either increased pulmonary recruitment of Th2 T cells or alternatively increased expression by individual cells. Increases in IL4, a pro-Th2 inflammatory cytokine may explain the apparent exacerbation of allergic airway diseases such as asthma in allergen exposed atopic individuals during periods of high ambient PM.

Results: Human Subjects Experiments

In order to quantitatively evaluate the effect of carbon and ammonium nitrate particle exposure on cytokine gene expression profiles in cultured human airway biopsy specimens, a comparative Ct method was utilized. In brief, the effect of any exposure regimen on the expression of any individual cytokine gene (either an increase or decrease) can be calculated if a baseline sample is used as a calibrator, and assigned a value of “1”. Following determination of individual Ct values for each experimental sample assay well, values were normalized using 18S ribosomal RNA Ct values (also determined on a per well basis). Duplicate assay wells were assessed for each experimental sample. For each sample evaluated by this method, the calibrator Ct value was obtained from a comparatively treated sample collected from filtered air exposure conditions. For example, the control snap-frozen sample from a carbon and ammonium nitrate particle exposure was directly compared with the control snap-frozen sample obtained following filtered air exposure. It is important to note that relative expression levels reflect a change in expression (either increase or decrease) relative to the baseline calibrator, and does not allow for direct comparisons between individual cytokines and cannot be used to determine absolute quantity of mRNA. For example, a lack of change in relative interleukin-8 expression levels following carbon and ammonium nitrate particle exposure does not mean this cytokine mRNA is not present in lung samples. Rather, interleukin-8 mRNA is present in the tissue, but transcription is not affected by the exposure. Of the 12 cytokines evaluated on the Human Taqman® Cytokine Expression Plate, eight cytokines were consistently detected in airway biopsy

samples by this method. These cytokines include interleukin-1 alpha, interleukin-1 beta, interleukin-2, interleukin-8, tumor necrosis factor alpha, interleukin-10, interleukin-12p35, and interleukin-15.

Effect of Single Carbon and Ammonium Nitrate Particle Exposure

Analysis of airway biopsy specimens immediately following receipt of tissues allows for determination changes in mRNA expression following the overnight shipment process. This may also reflect some of the early changes in cytokine transcription following exposure protocols. An early pilot test in which lung samples that were snap-frozen immediately upon collection at UC San Francisco were compared with lung samples snap-frozen at UC Davis following receipt showed minimal differences in cytokine expression (data not shown). Therefore, for the remainder of the study, we evaluated only samples processed at UC Davis. For single carbon and ammonium nitrate particle exposures, we have evaluated eight different subjects relative to eight filtered air subjects (Figure 36). Although most of the subjects (7/8) were identical between groups, we were not always able to obtain biopsy samples from every exposure regimen. For the purposes of evaluating global changes in cytokine expression profiles, we have pooled the values obtained for each exposure/culture group, regardless of whether or not they are matched for human subjects. After a single carbon and ammonium nitrate particle exposure, relative mRNA expression for several cytokines were found to be markedly elevated in the samples that were immediately snap-frozen following receipt of shipments; these cytokines include interleukin-1 alpha, interleukin-1 beta, and interleukin-12p35. Following overnight culture, only interleukin-1 beta remained elevated. Stimulation of cultures with patient-specific allergen resulted in a cytokine response that paralleled findings in the control snap-frozen samples, although interleukin-1 beta expression was not as responsive but interleukin-15 was more responsive. Stimulation of cultures with the non-specific cellular activators PMA with A23187 resulted in a similar increase in expression for the same group of cytokines.

Effect of Single Carbon and Ammonium Nitrate Particle Exposure with Ozone

For carbon and ammonium nitrate particle and ozone combined exposures, we have evaluated seven different subjects relative to eight filtered air control subjects (Figure 37). As with single carbon and ammonium nitrate particle exposures, the majority of the subjects were identical between groups (7/8). In contrast with carbon and ammonium nitrate particle exposures, control snap-frozen samples showed no change in expression for the cytokines evaluated in this study. For several cytokines, including interleukin-1 alpha, interleukin-1 beta, and interleukin 15, mRNA expression appeared to decrease as compared with lung samples collected under filtered air exposure conditions. Following overnight culture, expression of interleukin-2 was markedly elevated, regardless of patient-specific allergen stimulation. Stimulation of cultures with PMA with A23187 resulted in an increase in modestly enhanced expression of several cytokines, although the response was not as pronounced as the single carbon and ammonium nitrate particle exposure regimen (Figure 36).

Effect of Serial-Day Carbon and Ammonium Nitrate Particle Exposure

For serial-day carbon and ammonium nitrate particle exposures, we have evaluated five different subjects relative to eight filtered air control subjects (Figure 38). Three out of five subjects were identical between groups. In comparison with single carbon and ammonium nitrate particle exposures, serial-day exposures resulted in a similar cytokine expression profile. These cytokines include interleukin-1 alpha, interleukin-1 beta, and interleukin-12p35. In the allergen-stimulated cultures, cytokine expression was more exaggerated in the serial-day exposure regimen, whereas PMA with A23187 stimulation resulted in marked down regulation of many cytokine genes. One notable difference in the serial-day exposure regimen was the consistently increased expression of interleukin-10 in control snap-frozen, control cultures, and allergen stimulated cultures.

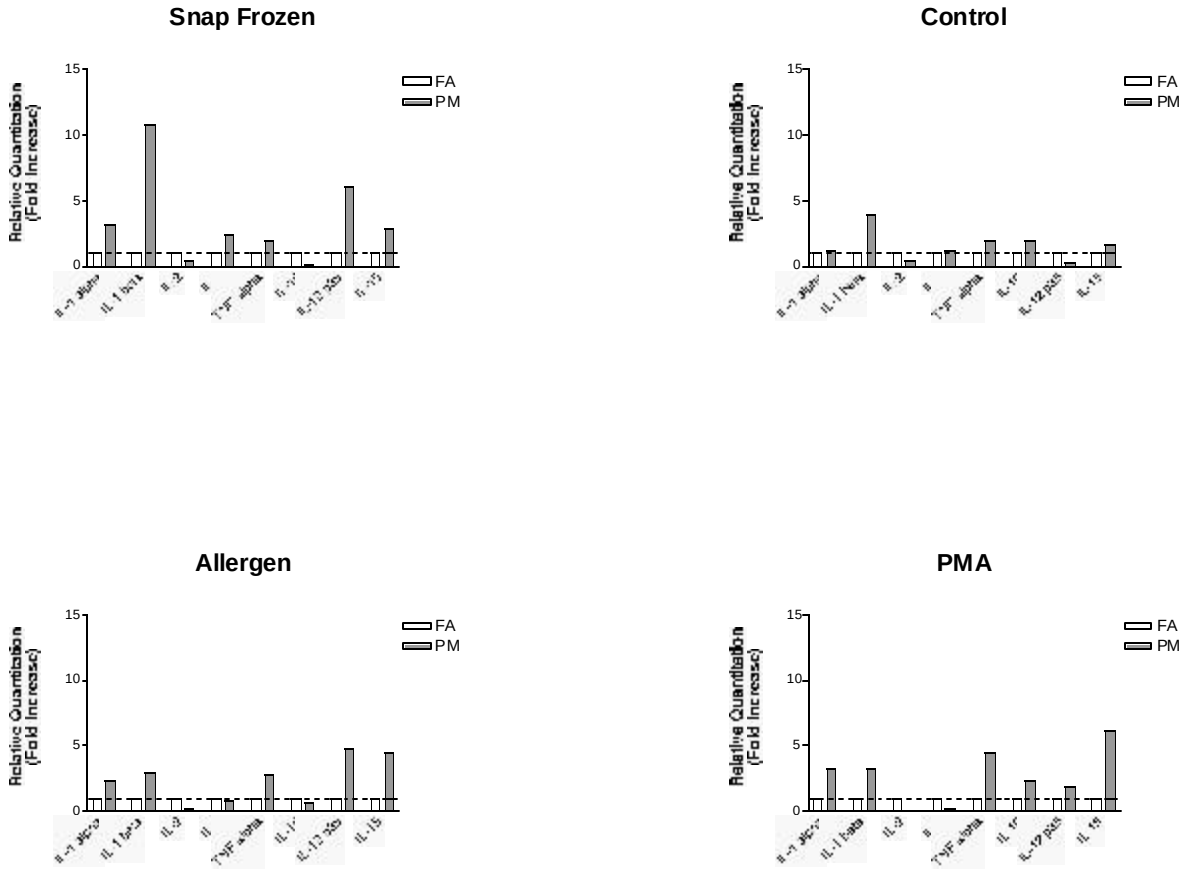


Figure 36. Effect of Single Carbon and Ammonium Nitrate Exposure on Cytokine Expression in Cultured Airway Biopsy Tissue. Airway biopsy samples were collected from human subjects as described in Methods. Upon receipt of samples at UC Davis, one tissue specimen was immediately frozen for RNA isolation (snap-frozen). The remaining tissue specimens were cultured overnight with the addition of patient-specific allergen (Allergen), PMA with A23187 (PMA), or no additions. Each graph

represents the relative increase or decrease of mRNA expression for each individual cytokine in a pool of eight single carbon and ammonium nitrate exposure subjects in comparison to a pool of eight filtered air control subjects. The dotted line at "1" represents the baseline expression value for each cytokine assay.

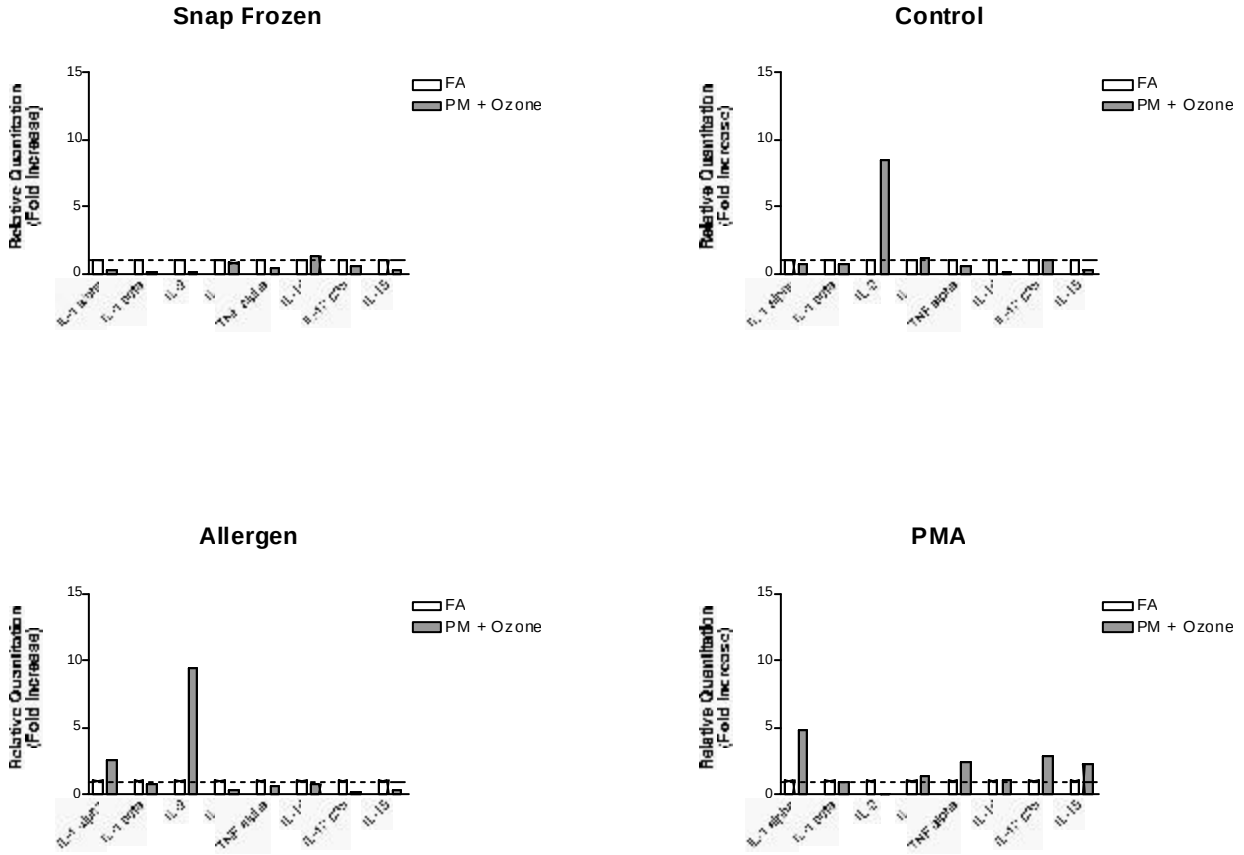


Figure 37. Effect of Combined Single Carbon and Ammonium Nitrate (PM) Exposure and Ozone Exposure on Cytokine Expression in Cultured Airway Biopsy Tissue. Airway biopsy samples were collected from human subjects as described in Methods. Upon receipt of samples at UC Davis, one tissue specimen was immediately frozen for RNA isolation (Snap-Frozen). The remaining tissue specimens were cultured overnight with the addition of patient-specific allergen (Allergen), PMA with A23187 (PMA), or no additions. Each graph represents the relative increase or decrease of mRNA expression for each individual cytokine in a pool of seven combined carbon and ammonium nitrate exposure and ozone exposure subjects in comparison to a pool of eight filtered air control subjects. The dotted line at "1" represents the baseline expression value for each cytokine assay.

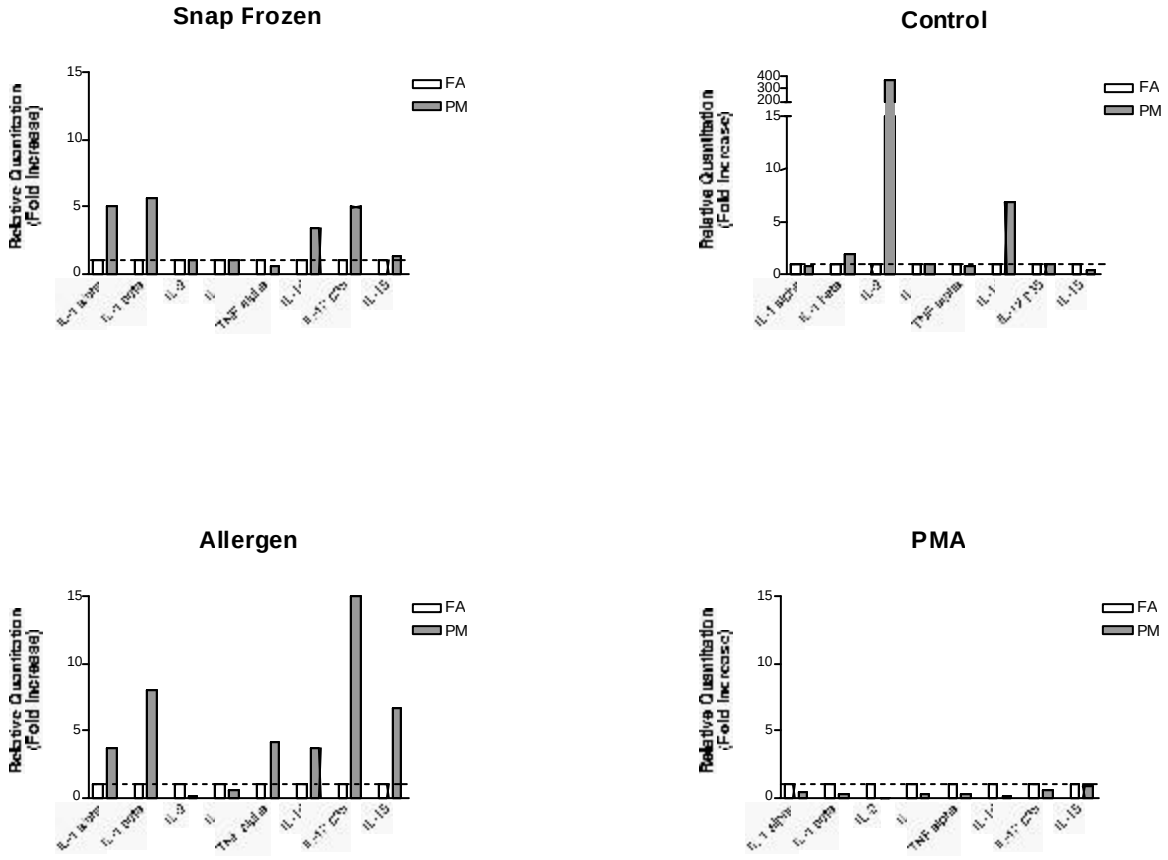


Figure 38. Effect of Serial-Day Carbon and Ammonium Nitrate (PM) Exposure on Cytokine Expression in Cultured Airway Biopsy Tissue. Airway biopsy samples were collected from human subjects as described in Methods. Upon receipt of samples at UC Davis, one tissue specimen was immediately frozen for RNA isolation (Snap Frozen). The remaining tissue specimens were cultured overnight with the addition of patient-specific allergen (Allergen), PMA with A23187 (PMA), or no additions. Each graph represents the relative increase or decrease of mRNA expression for each individual cytokine in a pool of five serial-day carbon and ammonium nitrate exposure subjects in comparison to a pool of eight filtered air control subjects. The dotted line at "1" represents the baseline expression value for each cytokine assay.

DISCUSSION: OVERVIEW

For this study the Brown Norway rat was chosen as a prospective model for a variety of reasons. This animal model is highly convenient due to the availability of the

strain and the ease in handling and caring for BN rats under experimental conditions. Due to the need for sufficient numbers of animals to perform the necessary experiments and exposure conditions to airborne particles in this study, BN rats were deemed as ideal to fulfill these requirements.

The literature is replete with information on the BN rat as a model of allergic airway disease. The use of ovalbumin to sensitize these rats, followed by simple aerosol challenge with ovalbumin is highly reproducible. Although it is well established that cats also develop a form of allergic airway disease, this is a natural process that cannot be experimentally induced and therefore not easy to use for our studies in PM. Recent investigations from our laboratory at UC Davis have also demonstrated the utility of the Rhesus monkey to produce an allergic airway condition with repeated administration of house dust mite allergen and ozone. However, the costs involved in using non-human primates are prohibitively high. Therefore, we felt development of the BN rat as a model would best fit the needs of our study to examine the potential mechanisms of particle toxicity in the respiratory system of a sensitive animal.

The use of BN rats as a model of allergic airways does have some deficits. We found although BN rats could be treated to produce a number of anatomical features characteristic of an allergic airway such as mucous cell hypertrophy, inflammation and eosinophil influx (Experiment 1), it was also easy to overwhelm the respiratory system with these changes. BN rats can also rapidly adapt tolerance to ovalbumin (Experiment 2), thus making them less responsive to further OVA challenge. We also found in some instances the presence of a subset of BN rats already exquisitely sensitive to the effects of ovalbumin and/or with pre-existing high levels of OVA-specific IgE. Such conditions presented difficulty in the unambiguous interpretation of our findings for each of the endpoints selected for study.

Bronchial responsiveness, eosinophil cell infux and centriacinar inflammation in some instances were markedly greater in a few animals compared with others. Once again, we assumed these differences to be due to pre-existing hypersensitive animals. However, these inflammatory and cellular changes could not be detected in the lungs without histological assessment. Serum analysis could be useful to identify such animals, but was not found to be practical for these studies due to timing, costs and the potential discomfort to which the animals would be subjected. Due to the random nature of this condition, such changes could be found in any treatment group. Our approach to sensitize all animals with OVA, regardless of group assignment, lead to exquisitely sensitive animals that could be present in any group.

Despite these potential confounders, our experiments were able to identify a number of differences to implicate PM-induced effects in this animal model. In the following discussion we have reported all our observations, many of which do not attain a level of statistical significance. The purpose of this discussion is to place our conclusions into perspective with the corollary work performed in human asthmatics. Although it is difficult to make direct comparisons between our animal model and human asthmatics, a number of conclusions can be drawn. Perhaps the most important is the

potential role of the airway epithelium as a target of PM-induced respiratory effects. Airway biopsies obtained from humans included both epithelium and interstitium. The measures derived from these biopsies focused in large measure on cytokine expression. In our animal studies we found the epithelium to be significantly impacted by exposure to particles expressed as an increase in DNA synthesis (and/or repair) through BrdU cell labeling (Experiment 3). We further found a transient increase in cytokine gene expression for IL-4 (Experiment 4). These findings in humans and animals suggest a potential common link of the airway epithelium as a critical site for PM-induced respiratory effects.

Discussion: BN rat Experiment 1

As summarized in Table 15, we have shown the BN rat can be sensitized and challenged with ovalbumin to produce physiological and cellular changes to mimic an allergic airways condition. We found this condition can be done in a highly controlled manner. Our potential goal with this model is to allow us to determine the impact of lung particle deposition on cellular responses for distinct anatomical regions of the tracheobronchial tree and ventilatory units of the lungs in rats. With such a model, we hoped to facilitate comparisons of an animal model with *in vitro* responses of airway biopsy tissues from asthmatic individuals exposed to identical particles.

Table 15. Summary of Results for Experiment 1.

Parameter measured	control	S/NC	S/C
bronchial responsiveness	-	-	+
epithelial volume	-	-	+
mucosubstance volume	-	-	+
eosinophils	-	-	+
mast cells	-	-	-

This table summarized the effects of ovalbumin sensitization without subsequent ovalbumin aerosol challenge (S/NC) or with challenge (S/C) on a variety of parameters. A negative sign (-) designates no change from control. A positive sign (+) designates a significant change from control.

Discussion: BN rat Experiment 2

The purpose of this experiment was to compare the effects of a single oOVA challenge to multiple OVA challenges to create an allergic airways condition in the BN rat. A summary of these findings is presented in Table 16.

Table 16. Summary of results for Experiment 2.

Parameter measured	single OVA challenge	multiple OVA challenge
Bronchial responsiveness	-	-
Centriacinar inflammation	-	-
Perivascular inflammation	-	-
Eosinophils	-	-
Mast cells	-	-
Epithelial cell mucosubstance volume	-	+

This table summarizes very few differences exist between a single ovalbumin (OVA) challenge vs. multiple OVA challenges in rats previously sensitized to OVA by subcutaneous injection. One exception is the relative volume of intraepithelial mucosubstances.

Repeated exposure to aerosolized ovalbumin following sensitization generally increased the recruitment of cells in the centriacinar regions of the lungs, however, this was highly variable from animal to animal.

The perivascular cell recruitment was consistent in all ovalbumin-challenged animals. The filtered air controls also demonstrated perivascular cell recruitment, but to a lesser degree than OVA-challenged rats. Exposure of the rats to aerosolized ovalbumin provided sufficient challenge to make the inflammation greater than that of the filtered air rats, though the number of challenges did not make a difference in the level of inflammation.

Eosinophil counts in the blood vessels of Brown Norway rats showed that while the exposure to aerosolized ovalbumin did affect the lungs, these rats also appeared to eventually acquire tolerance. Increased eosinophil number by OVA challenge from one week to two weeks was followed by a drop in eosinophil count following challenge for a third and four time. The initial increase suggests repeated OVA challenges caused an increase in the severity of the inflammation. However, by the third OVA challenge the rats had developed a tolerance to the aerosolized OVA and were similar to those animals challenged a single time with OVA. By the fourth week of OVA challenge, the rats had drastically reduced the eosinophil percentage in the perivascular space. After the third week, the allergic effect sought by these experimental conditions had been reduced. Four weeks of challenge to aerosolized OVA actually reduced the degree of inflammation to levels similar to that noted following a single challenge in these rats.

Relevance of Outcomes from Experiments 1 and 2

Based on the observations from Experiments 1 and 2, we found the anatomical and cellular changes reflective of an allergic airways condition could be achieved in the BN rat with a single ovalbumin aerosol challenge following two weeks of subcutaneous

sensitization with ovalbumin. The concluded the advantages of using a single OVA challenge included: (1) not overwhelming the respiratory system with an intense inflammatory and cellular response that could mask the effects of exposure to particulate matter and (2) convenience in better testing particle-induced effects in the lungs by optimizing the sensitization/challenge protocol in BN rats with ovalbumin.

Discussion: BN rat Experiment 3

Experiment 3 represents our first study to examine the effects of exposure to nitrate and carbon black on a rat model of allergic airways disease in the BN rat. Table 17 summarizes our findings. We further discuss the implications of these findings in this section.

Table 17. Summary of results for Experiment 3.

Parameter measured	FA	particle/exposed
Bronchial responsiveness	-	-
Cell permeability	-	-
OVA-specific IgE	-	-
BrdU labeling of epithelial cells	-	+
Epithelial mucosubstance volume	-	-

For the data reported in this table, both filtered air (FA) and particle-exposed animals in this table have been sensitized and challenged with ovalbumin. A positive sign (+) represents a significant particle-induced effect compared with the FA control group.

Using a single OVA challenge protocol, we failed to demonstrate any significant changes in BAL cell viability, numbers of recovered leukocytes/ml lavage, or protein levels between groups. Exposure to PM for two days following OVA challenge also failed to produce changes in these measures compared with filtered air controls. However, a significant increase in eosinophils following challenge was also noted. Interestingly, a significant decrease in lymphocytes was noted between FA controls and rats exposed to PM in the sensitized but not challenged group. A similar trend in decreased number of lymphocytes was also seen in the other two groups. This phenomenon could be due to changes in chemokine synthesis and release following T-cell activation in the presence of PM resulting in changes in the inflammatory cell milieu present in the lung airspaces.

Repeated allergen challenge following sensitization has been found to result in a decrease in airway hypersensitivity suggesting the development of tolerance following continual exposure to antigen (Palmans et al 2000). However, this decrease did not attain a level of statistical significance and therefore should be interpreted with caution. Gamma delta T cells have been shown to be an important effector cell in the development of tolerance leading to decreases in airway hypersensitivity independent of $\alpha\beta$ T cells (Lahn et al, 1999). Gamma delta T cell-modulated decreases in airway hypersensitivity are regulated at least in part by TNF-α (Kanehiro et al, 2001). Gamma

delta T cell-deficient mice have exhibited significantly decreased migration of B cells to airways (Svensson et al, 2003). Lymphocyte numbers in the BAL are at least in part due to migration of cells from the blood in the Brown Norway rat allergic model (Schuster et al 2001). These findings are compatible with seemingly contradictory studies which have demonstrated progressive airway remodeling with increases in collagen deposition, airway smooth muscle mass, goblet cell hyperplasia, epithelial disruption leading to increased BrdU labeling, and inflammatory cell recruitment following repeated allergen exposure in sensitized Brown Norway rats. A decrease in airway sensitivity has been shown to be independent of immunoglobulin synthesis and various cytokines (IL-4, IL-5, IFN-γ) (Lahn et al, 1999). (Palmans et al, 2000; Salmon et al, 1999). These studies, along with our findings of decreased BAL lymphocytes and decreased airway hypersensitivity in the presence of PM when compared to FA controls, suggest a possible mechanism for PM effects in which PM accelerates the development of airway tolerance. This result resembles the effect seen when repeated OVA challenges are given to sensitized Brown Norway rats, by directly increasing $\lambda\delta$ T- cell number or activation, or indirectly, through increases in TNFα in the presence of PM.

Following a single OVA challenge in sensitized Brown Norway rats, we did not find changes in mucin production seen with repeated allergen challenge in this model of allergic airway disease. In Experiment 2, we found two or more challenges were necessary to increase mucin production significantly in this model (Figure 25, page 56). This result suggests acute (two-day) exposure to PM is insufficient to produce goblet cell hyperplasia. We were also unable to detect changes in smooth muscle mass or collagen deposition with the airways following a single aerosol challenge to OVA following sensitization. However, eosinophils were significantly increased by sensitization and challenge, suggesting the development of allergic airway disease in the late phase of a Type I hypersensitivity response. In a similar model, eosinophil accumulation in the lung parenchyma was found to peak at 48 hours and to persist for six days (Schneider et al, 1997). Therefore, we may have failed to see a significant alteration in eosinophils following exposure to PM compared to FA due to the fact that sensitization and challenge may have induced a large eosinophil peak at the 48-hour time point following OVA challenge, thus masking a possible PM effect on this cell type. We could not determine changes in the inflammatory response in the presence of PM from the single time point used in this study. Therefore, the question remains whether PM exacerbates asthma symptoms by prolonging the influx of eosinophils into the lungs.

Carbon black and indoor suspended particulate matter have previously been shown to have significant adjuvant activity in the development of an allergic response to OVA when measured by popliteal lymph node assay through increasing serum IgE (Lovik et al, 1997; Ormstad et al, 1998). We have demonstrated significantly increased levels of serum IgE in the presence of PM, suggesting a similar adjuvant effect from inhalation challenge with OVA. Increased levels of IgE may result in increased degranulation of mast cells following further exposure to allergen, thus worsening the acute immediate phase of a Type I hypersensitivity response. This finding might explain the worsening of asthma symptoms during periods of high ambient levels of PM in compromised individuals with allergic airway diseases repeatedly exposed to PM.

In contrast to the finding of elevated serum IgE, we found no change in the number or distribution of IgE positive cells present in the lungs and identified by immunohistochemistry. This finding is not surprising since mast cells are not found in high numbers in this model. In addition, eosinophils possess only a low-affinity FC epsilon antibody receptor (FcεR). The cells identified in lung tissues which appeared as IgE positive were likely B cells found primarily in secondary lymphatic tissue. These cells are known to be present following activation by antigen-activated dendritic cells which migrate following major histocompability antigen (MHCII) binding of antigen in airways. Clonal expansion of IgE OVA-specific B cells may be increased in the secondary lymphatic tissues located in other organ systems that may be responsible for the increases in serum IgE we saw following exposure to PM.

To our knowledge, this is the first study to demonstrate an increase in damage to bronchiolar epithelium following exposure to ammonium nitrate and carbon black, the two most common components of PM in the Western United States (including California). Ammonium nitrate and carbon are assumed unlikely to cause significant oxidative stress or direct toxicity to epithelial cells. Cell turnover and/or DNA repair associated with a significant elevation in BrdU incorporation was present in airway epithelial cell following exposure to PM in sensitized and challenged Brown Norway rats. Epithelial cells are an important source of eotaxin and therefore increased damage may increase release of this and other cytokines, thereby exacerbating the inflammatory response (Cook et al, 1998). Eotaxin is chemotactic for eosinophils that are thought to be responsible at least in part for the damaging pathological events of asthma. Th-2 lymphocytes, major players in allergic inflammation, also possess CCR3 (cc-chemokine) receptors and therefore could potentially be modified with respect to releases of other cytokines in response to epithelial eotaxin release (Guo et al, 2001). Subsequent up-regulation of IL4 and IL5 could exacerbate serum IgE levels and asthma severity, respectively (Humbert et al, 1997). Eotaxin and CCR3 expression was found to be increased in Sephadex particle-induced rat lung inflammation (Harrington et al, 1999).

Sensitized and challenged Brown Norway rats develop interstitial granulomas. Granulomas are a frequent finding in control rats as well, possibly due to inhalation of allergens from rat chow or other uncontrolled sources, although the severity is greatly reduced without sensitization or challenge. Granulomas are not characteristic of human asthma and their presence could be a limitation of the model used for this study. In experimental models of granulomatous lung disease in Brown Norway rats induced by antigen coated beads, hexachlorobenzene ingestion, or injection of Sepharose beads, however, the development of granulomas has been shown to be the result of a Th-2 response (Michielsen et al, 2001; Shang et al, 2002). This Th-2 granulomatous response has been shown to be associated with hyperresponsive airways, increased IgE levels and eosinophilic inflammation (Michielsen et al, 2001). The increase in the number of rats severely affected with granulomatous inflammation in sensitized and challenged animals following exposure to PM may indicate, therefore, an augmentation of the Th-2 response or airway hypersensitivity in rats exposed to PM and may therefore still function as an acceptable model of allergic airway disease for the study of airborne particles.

Sensitized and challenged Brown Norway rats appear to be a sensitive model of allergic airway disease for the study of particulate matter toxicity. These studies suggest PM may have an adjuvant effect when sensitized rats are exposed to OVA. PM appears to increase epithelial damage in bronchioles of Brown Norway rats and, therefore, may pose a significant risk to compromised individuals with allergic airway diseases such as asthma by exacerbating the allergic response.

Discussion: BN rat Experiment 4

A summary of our findings for Experiment 4 is presented in Table 18. The purpose of this study was to determine if progressive repeated PM exposure (from 3 days to 6 days) would further augment responses in the lungs of our model of allergic airways disease. The discussion which follows attempts to place our findings in perspective to what has bben reported previously in the literature.

Table 18. Summary of results for Experiment 4.

Parameter measured	**particle exposure**	
	3-day	**6-day**
Bronchial responsiveness	-	ND
Eosinophils	-	-
BrdU labeling of epithelial cells	-	-
OVA-specific IgE	-	-
Cytokine expression	+	-

This table summarizes particle-induced effects in BN rats sensitized and challenged with ovalbumin. A negative sign (-) designates no significant change compared to OVA-sensitized/challenged filtered air controls. A positive sign (+) designates a significant effect due to particle exposure compared with control animals. ND signifies the assay was not done.

The model of allergic airway disease in BN rats used in this study involved a single sensitization and a single OVA challenge before PM exposures. In BN rat Experiment 3, we demonstrated this single challenge protocol produces a significant increase in eosinophil influx consistent with allergic airway disease. Under the same protocol, significant increases in submucosal eosinophil numbers were observed only between groups that received carbon and ammonium nitrate PM. FA (filtered air) following challenge resulted in a non-significant increase in eosinophils at three days. PM as used in this study appears to result in a greater magnitude of change in sensitized rats exposed to allergen challenge. This may be a reflection of the apparent decrease in eosinophil numbers in unchallenged rats compared to rats receiving FA. Decreases in eosinophil numbers in BN rat allergic models has been previously reported by blocking activation of protein tyrosine kinase Syk, inhibition of the common β subunit of IL3, IL5 and GM-CSF and inhibition of IL4 (Allakhverdi et al, 2002) (Molet et

al, 1999) (Stenton et al, 2002). The presence of cells expressing INFγ decreases the Th2 responses including eosinophil numbers (Minshall et al, 1998). In this study PM appeared to attenuate baseline inflammation without preventing exacerbation of inflammation following challenge. Th2 cytokines are necessary for the vascular adhesion, extravasations, chemotaxis and activation of eosinophils (Gauvreau et all, 1999). The necessary cytokines may be suppressed in PM exposed sensitized but not challenged BN rats by γΔ T cells and/or other cytokines such as INFγ. INFγ would subsequently be decreased by challenge in sensitized BN rats (Haczku, 1996). Gamma delta T cells from sensitized BN rats decreased eosinophilia in BN rats but did not inhibit the subsequent late airway responses following challenge (Isogai et al, 2003).

Eight days following OVA challenge, sensitized BN rats still demonstrated increases in mean submucosal eosinophil counts following challenge, but the increases were no longer significant for either FA or PM groups, despite three additional days of exposure to PM. The lower mean value in PM verses FA suggests PM shortens rather than prolongs the residence time for eosinophils in the airway submucosa of sensitized /challenged BN rats. In contrast, human sputum eosinophils, eotaxin and IL5 attained the highest levels seven hours after allergen challenge and eosinophils remained significantly elevated seven days after challenge (Gauvreau et al, 1999). A trend towards lower eosinophil counts in PM exposed groups compared to FA controls was observed before and after OVA challenge in BN rats eight days following challenge.

This same trend was observed when lung tissue sections were scored for the presence of granulomatous changes. Granulomas are normally present at low levels in control BN rats (Ohtsuka et al, 1997). Granuloma formation appears to be regulated at least in part by IL4 and IL10 in a mouse granuloma model (Wynn et al, 1997). Granuloma models in BN rats have also been linked to Th2 cytokines (Michielsen et al, 2001) (Shang et al, 2002). PM exposed groups tended to have lower scores than corresponding FA controls suggesting inflammation caused by OVA challenge in sensitized BN rats is attenuated by subsequent exposure to PM. Granuloma scores were similar for all groups when compared at three and six days, suggesting little change during this period in FA and PM exposed groups.

BrdU labeling of airway epithelial cells of mid-level bronchioles appeared to be decreased by three or six days of PM following challenge in sensitized and challenged BN rats and in sensitized BN rats following three days of PM. Labeling was increased by challenge but differences did not reach a level of significance. There was no effect due to PM on BrdU labeling of epithelial cells at this level. This is in contrast to our previous study utilizing the same PM and sensitization/challenge protocol in which we found a significant increase in BrdU labeling following challenge and two days exposure to PM (BN rat Experiment 3). This finding suggests, at the mid-level of airway generations, PM effects may occur early on during the initial inflammatory response following challenge in BN rats. Nontoxic PM has previously been demonstrated to increase BrdU incorporation signaling unscheduled DNA synthesis and/or cell proliferation (Timblin et al, 1998). BrdU labeling was not observed, however, following three consecutive days of iron/soot exposure in non-allergic Sprague-Dawley rats (Zhou et al, 2003).

Eight days following challenge, levels of BrdU labeling were markedly decreased in sensitized only rats of both FA and PM groups despite similar percentages of BrdU

positive cells in sensitized/challenged groups at three- and six-day PM exposure periods. BrdU labeling in sensitized/challenged rats was not increased from three-day levels by an additional three days of PM exposure, again suggesting that PM effects occur during the early post-allergen challenge period.

BrdU labeling of epithelial cells was different when terminal bronchioles were examined. Values for percent BrdU positive cells were lower for all groups compared to the labeling in mid-level bronchioles. Levels of epithelial labeling following 3 days of PM exposure were nearly identical to the comparable FA group, however, the increase in labeling due to challenge was significant only between the PM exposed groups. In agreement with our findings for eosinophils, this appeared to be due to a non-significant decrease in BrdU labeling in the sensitized group exposed to PM when compared to sensitized rats exposed to FA. BrdU labeling in this model is likely a direct consequence of eosinophil influx and activation. Eosinophils release granules that have been shown to injure airway epithelial cells (Allakhverdi et al, 2002).

Following six days of PM exposure BrdU labeling of epithelial cells was decreased compared to labeling level observed in terminal bronchioles following three days of exposure to PM. In contrast, the group exposed to FA after challenge showed only a small decrease in labeling, while in the sensitized-only group, BrdU labeling was markedly decreased eight days after the OVA challenge. These shifts resulted in the FA groups showing the significant increase in labeling due to OVA challenge at the six-day exposure time point. Decreased BrdU labeling following continued PM exposure for six days may be related to PM-induced tolerance (BNR 2002).

We observed a non-significant increase in IgE following challenge in both the FA and PM exposed groups three days following challenge, and in the group exposed to PM for six days eight days after challenge. Atopic individuals produce higher IgE levels following exposure to allergens (Wan et al, 2000). In sensitized mice, however, birch pollen and carbon particles together did not increase serum IgE (Fernvik et al, 2002). The BNR-sensitized group exposed only to FA showed marked variability in IgE levels, possibly obscuring changes in this group. Sensitized and challenged rats following both three or six days of PM exposure showed slight increases in serum IgE levels compared to the corresponding FA groups, suggesting a slight adjuvant effect due to PM in this study. Previous studies have demonstrated adjuvant effects due to PM exposure (Lambert et al, 1999) (Lovik et al, 1997) (Ormstad et al, 1998). In the present study increases in IgE due to PM failed to reach a level of significance.

Levels of mRNA expression for eotaxin and IL5, normalized to levels of β-actin, demonstrated no significant changes due to PM following exposure for three or six days. Challenge at these time points also failed to alter eotaxin or IL5 mRNA expression in BN rats. In mice sensitized and challenged with OVA, eotaxin was increased 24 hrs after challenge (Scheerrens et al, 2002). Asthmatic patients had three-fold increases in IL5 in BAL three hours after challenge and a 20-fold increase 24 hours after challenge (Teran et al, 1999). We may have failed to detect changes in these cytokines in BN rats sensitized and challenged to OVA due to the longer interval following challenge. We cannot confirm a PM effect on these cytokines at an early time point following challenge from the present study. Interestingly, the large standard errors observed for serum IgE levels in the sensitized-only rats for the six-day exposure interval exposed only to FA was also observed for eotaxin and IL5. The same single outlier in this group caused

these errors. In contrast, this animal had IL4 levels and eosinophil counts within the normal range observed for this group (individual rat data not shown). It is interesting to speculate whether extremely high eotaxin and IL5 levels without high eosinophil numbers suggests failure of normal chemotaxis signaling in the BN rat.

In sensitized BN rats exposed to carbon and ammonium nitrate PM following OVA challenge, we observed significant increases in IL4 mRNA expression due to PM and due to OVA challenge following three consecutive days of PM exposure. IL4 mRNA expression in sensitized/challenged BN rats was significantly decreased five days later following three additional days of PM exposure when compared with three days of exposure. In a murine allergic model, increases in IL4 in BAL have been reported following exposure to residual oil fly ash (ROFA) (Gavett et al, 1999). IL4 in BN rats is essential for the development of the late airway response following allergen challenge (Molet et al, 1999). Increases in IL4 due to PM exposure could increase the Th2 inflammatory response and B cell isotype switching to IgE. Both events, highly influenced by IL4 levels, could exacerbate allergic inflammation following allergen challenge. Increases in IL4 mRNA expression in BN rats with allergic airways following exposure to PM may reflect either increased pulmonary recruitment of Th2 T cells or alternatively increased expression by individual cells. Both challenge and PM exposure appear to be necessary for significant increases in IL4 mRNA expression to be observed.

Mean airway sensitivity to Mch challenge was decreased in unchallenged controls exposed to PM compared to FA. In our study, utilizing sensitized BN rats, challenge increased sensitivity for both FA and PM groups; however, the magnitude of increase reached significance only between groups exposed to PM.

The tendency for PM-exposed unchallenged controls to show a non-significant decrease in sensitivity relative to FA control, and the resulting increase from challenge following three days of PM exposure to lead to a significant change not observed with FA, mimics the trends observed for IL4 mRNA expression, BrdU labeling in terminal bronchioles and submucosal eosinophil counts measured at the same time point.

Ambient $PM_{2.5}$ and soot plus iron PM were shown by other investigators (Shukla et al, 2002; Zhou et al, 2003) to activate NFκB. NFκB activation regulates genes for TNFα and INFγ. Dust, ROFA, ambient particles and inert carbon have all been shown to stimulate macrophages to produce TNFα (Brown et al, 1996; Dick et al, 2003; Jiménez et al, 2002; Roberts et al, 2003) (Ulrich et al, 2002). Inert carbon particles are phagocitized by macrophages and epithelial cells. TNFα from stimulated macrophages can induce a systemic inflammatory response (Fujii et al, 2002). TNFα was shown to increase activated γΔ T cells that can be associated with decreased airway hypersensitivity (Kanehiro et al, 2001) (Lahn et al, 1999). Only γΔT cells of naïve BN rats decreased late airway responses following challenge (Isogai et al, 2003).

INFγ increase stimulates macrophages to release IL12 (Isogai et al, 2003) (Isler et al, 1999). IL12 augments INFγ production, suppresses Th2 cytokines and decreases eosinophilia (Gavett et al, 1995) (Mountford et al, 1999). IL12 can prevent airway hyper-responsiveness and increases in IL4 and IL5 in sensitized and challenged mice (Schwarze et al, 1998).

Determination of levels of NFκB activation, cytokines of innate immunity and Th1 cytokines following exposure to carbon and ammonium nitrate PM in our BN rat model

of allergic airway disease awaits further studies. PM in this model may have stimulated non-significant decreases in airway sensitivity, eosinophils and IL4 in sensitized but not challenged BNRs. PM-induced activation of NFκB or induction of TNFα, INFγ, γΔT cells and/or IL12 could potentially reduce levels of these endpoints following three continuous days of PM compared to sensitized rats receiving only FA. Decreases in eosinophil numbers could result in the decreases observed in BrdU labeling of terminal bronchioles. Subsequent attenuation of these effects following challenge with OVA, which would drive a strong TH2 response, could explain the observed significant increases following challenge in PM-exposed groups not observed for FA. Significant change only when PM is present in BN rats suggests PM could create the impression of more severe disease due to PM in atopic individuals exposed to allergen because the magnitude of change is greater, despite a non-significant change in the maximum level of inflammation and airway sensitivity compared to FA.

The observed changes in eosinophil numbers and BrdU positive cells suggest an association between airway epithelial injury and eosinophil influx. PM exposure beyond three days was associated with a decrease in the inflammation observed in this study. PM was noted to consistently cause non-significant decreases in baseline levels of inflammation in sensitized rats not subjected to OVA challenge.

Overall Summary: BN rat Experiments 1-4

BN rats demonstrate increased inflammation and cellularity with progressive exposures to aerosolized ovalbumin (Experiment 1). This response to ovalbumin mimics to a limited degree asthmatic conditions observed in humans. Cell inflammation within centriacinar regions, perivascular spaces, and central airways, are attractive features in the Brown Norway rat as a model for allergic airways to mimic human asthma. Aerosolized allergen exposure causes eosinophilic airway inflammation and airway hyper-responsiveness in the Brown Norway rats (Kleinman 2001). Other rats, such as the Sprague-Dawley rats, do not have the same reactions to ovalbumin sensitization and aerosolized ovalbumin challenges as do Brown Norway rats (Anjilvel & Asgharian 1995). It also demonstrates that the rat is capable of responding adequately to an allergic insult all four weeks. The Brown Norway rat also is able to reduce the inflammation and cellularity, and thus reduce its asthmatic-like qualities. Therefore, the optimal condition for generating an asthmatic-like condition for study in the laboratory animal (i.e. Brown Norway rat) would be to limit aerosol challenge with ovalbumin to one or two episodes following sensitization (based on the results of Experiment 2).

Subsequent experiments (Experiment 3) to use this allergic airway model for short-term exposure to aerosols of carbon and NH_4NO_3 particles resulted in a significant increase in BrdU labeling of epithelial cells in OVA sensitized and challenged animals compared with filtered air controls. We also noted a trend of increased OVA-specific serum IgE and eosinophilic inflammation in the airway submucosa following short-term particle exposure. These findings suggest the BN rat allergic airway model may serve as a sensitive tool to better understand PM effects on sensitive airways of the respiratory system which mimic the asthmatic condition.

Our final studies (Experiment 4) to better understand the effects of progressively longer exposure to particles were done in BN rats exposed to particles for up to six days. We utilized BrdU labeling of epithelial cells, histology, pulmonary function testing

(PFT) and mRNA expression for eotaxin, IL4 and IL5 in whole lung homogenates enhanced by RT-PCR, as endpoints to measure exacerbation of inflammation following PM exposure.

We hypothesized that prolonged exposure to PM following OVA-induced allergic inflammation would increase (1) BrdU labeling of airway epithelial cells, (2) inflammatory cell influx pulmonary granuloma formation, (3) airway hypersensitivity measured by methacholine challenge, and (4) mRNA expression of three key Th2 cytokines critical to the development and progression of Type I hypersensitivity response in allergic airway disease.

We found particle exposure for up to six days results in no significant change in BrdU labeling of airway epithelial cells compared with filtered air controls. We did observe eosinophilic inflammation to be significantly increased by OVA challenge following three days of particle exposure, but not following six days of particle exposure. Eotaxin and IL5 mRNA levels measured in lung tissue homogenates by RT-PCR were similar for each treatment group. In contrast, IL4 mRNA expression was significantly increased in sensitized and challenged rats by exposure to airborne particles for three days, but with progressive particle exposure up to six days, IL-4 mRNA levels returned to control levels.

These findings suggest PM may initially increase eosinophilic inflammation and epithelial damage following OVA challenge, but may become attenuated with progressive PM exposure. Decreases in inflammation and BrdU labeling with progressive PM exposure suggest an association between eosinophils and the PM-induced epithelial damage. Increases in IL4 mRNA expression in BNRs with allergic airways following exposure to PM may reflect either increased pulmonary recruitment of Th2 T cells or alternatively increased expression by individual cells. Increases in IL4, a pro-Th2 inflammatory cytokine, may explain the apparent exacerbation of allergic airway diseases such as asthma in allergen-exposed atopic individuals during periods of high ambient PM.

Discussion: Human Subjects Experiments

The immunological basis for the development of allergic asthma lies in the selection of T-helper lymphocyte subpopulations early in life. The process of determining T-helper lymphocyte subpopulations is termed immune deviation (for review see (Holt 1998)). In the immunologically naïve state (Th-0), T-helper cells produce multiple cytokines. Antigen recognition triggers clonal expansion of Th0 cells through one of two pathways, depending upon the balance of cytokines present. Th0 cells automatically secrete low levels of interleukin-4 following initial antigenic stimulation. If there are no other signals, interleukin-4 promotes the differentiation of Th0 into Th2 cells. This subset secretes interleukin-4, interleukin-5, interleukin-10, and interleukin-13, and is thought to be important in IgE-mediated host defense (i.e. allergic reactions). Furthermore, the differentiation of naïve Th0 lymphocytes into Th2 cells only occurs in the presence of interleukin-4 (Swain et al, 1990; Abehsira-Amar et al, 1992; Hsieh et al, 1992). If, however, there are other cytokine signals present, Th0 cells can differentiate into Th1 cells. The Th1 subset secretes interleukin-2 and interferon gamma, and is thought to be important in phagocyte-mediated host defense. Th1 and Th2 cells are antagonistic, and each type secretes inhibitory cytokines to reduce the

population of the other. It has been hypothesized that oneTh subtype gains dominance early in life and this dominance is retained as long-term immunologic memory. In support of this concept, Japanese schoolchildren showed a strong inverse association between delayed hypersensitivity to M. tuberculosis and atopy (Shirakawa et al, 1997). Positive tuberculin responses predicted a lower incidence of asthma, lower serum IgE levels, and cytokine profiles biased toward Th-1 type. Thus, positive exposure and response to M. tuberculosis may, by modification of immune profiles (Th1 type), inhibit atopic disorder.

In adult asthmatics, ozone exposure significantly enhances airways inflammation, suggesting that air pollutant exposure may exacerbate allergic airways (Scannell et al, 1996). A recent epidemiologic study of asthmatic children strongly correlates air pollutant exposure with an increase in respiratory symptoms (Gent et al, 2003). The ability of ozone to enhance allergic sensitization in rodent models has also been documented. Chronic exposure to ozone levels greater than 0.13 ppm result in greater anaphylactic sensitivity to intravenous challenge with ovalbumin (Osebold et al, 1988). In the same ovalbumin mouse model, total cells containing IgE increased 9.4-fold in mice that received aerosolized ovalbumin; exposure to ozone resulted in an additive effect on IgE cell numbers. A recent study by U. Neuhaus-Steinmetz and colleagues (Neuhaus et al, 2000) further showed a shift towards a Th2 cytokine profile in both IgE-high responder (BALB/c) and IgE-low responder (C57BL/6) mice following a combination of ozone and allergen exposures.

Along with ozone, carbon and ammonium-nitrate particles are primary components of ambient air pollution. Epidemiologic studies suggest that persistent exposure to particulate matter does increase acute respiratory symptoms in asthmatics (Penttinen et al, 2001; von Klot et al, 2002). Nasal exposure to diesel exhaust, a major source of particulate matter, can result in significant modulation of immune responses that can promote or exacerbate airway challenges to allergen (Diaz-Sanchez et al, 1997; Devouassoux et al, 2002). *In vitro* exposure of human airway epithelial cell cultures to ultrafine (<0.18 micron) particles resulted in upregulated expression of a chemokine that is critical for antigen presenting (dendritic) cell recruitment, further supporting an adjuvant role of particulate matter (Reibman et al, 2003). In this current study, the experiments were designed to directly assess the role of particulate matter exposures, alone or in conjunction with ozone, in the exacerbation of airways inflammation in adult asthmatics. We tested the hypothesis that exposure to carbon and ammonium-nitrate particles, or a combination of these particles and ozone, would result in an increase in airway inflammatory cells and cytokines in individuals with allergic asthma.

Using a sensitive bioassay for experimental testing of human lung immune response, our results from this study indicate exposure to particles can directly modulate mucosal immune responses within the lung in adult asthmatics. Regardless of the duration of exposure to particles alone or in combination with ozone, the cytokine network within the lung microenvironment was altered. Of the 12 different cytokines evaluated in this study, we focused on the gene expression profile of eight cytokines that were consistently detected in the majority of samples evaluated. These cytokines included interleukin-1 alpha, interleukin-1 beta, interleukin-2, interleukin-8, tumor necrosis factor alpha, interleukin-10, interleukin-12p35, and interleukin-15. Of the

aforementioned cytokines, interleukin-1 alpha, interleukin-1 beta, interleukin-8, interleukin-10 and tumor necrosis factor alpha function primarily during acute inflammatory responses to induce neutrophil recruitment and modulate macrophage activation. Interleukin-2, interleukin-12p35, and interleukin-15 function primarily in the adaptive arm of immunity, by inducing T cell proliferation and modulating T/natural killer cell function. The cytokines that were not consistently detected in this assay were interleukin-4, interleukin-5, interferon gamma, and interleukin-12p40; these cytokines are closely associated with effector T cell functions. Although interleukin-5 and interferon gamma were occasionally detected in our gene assay, we did not include these cytokines in our final analysis because expression was not consistent among all samples evaluated. It has been reported that interleukin-4 and interleukin-5 mRNA is elevated in airway biopsies obtained from human asthmatics (Ying et al, 1997). The lack of strong expression for these cytokines in our human airway biopsy samples (regardless of exposure regimen or culture condition) may be explained by the status of the immune response in the mild asthmatics, particularly if they have not had recent episode with aeroallergen. Alternatively, the lack of expression independent of the presence of specific allergen may be due to limited numbers of effector T cells in the tissue specimen.

Without experimental manipulation, single or serial-day exposures to carbon and ammonium nitrate particles induced expression of interleukin-1 alpha, interleukin-1 beta, and interleukin-12p35 in airway biopsy specimens. Single exposures also induced interleukin-15, interleukin-8, and tumor necrosis factor alpha. Serial-day exposures also induced interleukin-10. In contrast with particle only exposures, combined exposures of carbon and ammonium nitrate particles with ozone resulted in no increase in cytokine expression for the panel of genes evaluated; expression for several cytokines was downregulated in comparison with filtered air control samples. Although it is not known how combined ozone exposure can result in distinct differences in local lung immune responses, there are two potential mechanisms. Ozone exposure may directly affect gene expression by damaging epithelial cells, which are an important source of cytokines. Alternatively, exposure to oxidant stress or injury may trigger cellular pathways that uniformly counteract the transcriptional effects of particulate exposure. The overall viability of the lung tissue samples collected under combined carbon and ammonium nitrate particle with ozone exposure was not directly evaluated in this study. However, we did not observe differences in the quantity or quality of RNA isolated from these samples, suggesting that ozone exposure did not result in substantial tissue necrosis that would affect cellular RNA levels.

Following overnight culture of airway biopsy specimens, cytokine gene expression profiles were retained with allergen stimulation in samples from both single and serial-day exposure to carbon and ammonium nitrate particulates. Cultured lung samples from combined particle and ozone exposure expressed both interleukin-2 and, to a lesser degree, interleukin-1 alpha. Interleukin-2 is a potent growth factor for T cell proliferation, but also has anti-apoptosis effects on neutrophils. The strong interleukin-2 effect observed with combined ozone and particulate exposure was independent of allergen stimulation, suggesting that this may be a late phase modulator of the inflammatory response to air pollutant injury. Single carbon and ammonium nitrate particle exposure differed from serial-day particle exposures in the elevated expression

of interleukin-10. Because interleukin-10 functions primarily as an immunosuppressive cytokine, it may be postulated that this is a mechanism to control the inflammatory events within the lung following chronic air pollutant exposure.

Summary and Conclusions (BN rats)

Four experiments were conducted in animals to correlate with ongoing studies in human volunteers. We found the BN rat can serve as a useful model of allergic airways by treatment with ovalbumin. Repeated aerosol challenge with ovalbumin elicits changes in the airways indicative of airway hyperreactivity, mucous cell hypertrophy and airway inflammation. However, we also found repeated aerosol challenge with ovalbumin leads to a significant influx of inflammatory cells into the lung parenchyma which could potentially obscure effects caused by subsequent particle exposure. Therefore, a single sensitization and aerosol challenge with ovalbumin was implemented in our studies to create an allergic airway to test the effects of particle exposure. We found exposure to aerosolized ammonium nitrate and carbon particles for two days resulted in a significant increase in the levels of OVA-specific serum IgE in BN rats as well as alterations in the epithelial cells lining the conducting airways of the bronchial tree. These findings in BN rats demonstrated a significant particle effect with short-term exposures resulting in increased DNA synthesis in airway epithelial cells. Also noted was a trend for increased numbers of inflammatory cells within the conducting airways, but this change did not attain a level of statistical significance. Subsequent studies demonstrated repeated exposure to ammonium nitrate and carbon particles was associated with a significant elevation in pulmonary mRNA levels for interleukin-4 but no changes in interleukin-5 or eotaxin, all cytokines thought to play critical roles in cell-mediated immune responses of the lungs. Therefore, elevation in the level of mRNA for IL4 may be suggestive of an augmented allergic immune response due to particle exposure. However, continued exposure to these particles was associated with a return to control levels of mRNA for IL-4.

Summary and Conclusions (Humans)

Corollary studies performed at UCSF in human volunteers with a history of asthma demonstrated that lung airway biopsy materials obtained from these volunteers could be processed and tested for the expression of a large panel of cytokine genes. These corollary studies demonstrated experimental manipulation of biopsy specimens in culture resulted in the alteration of gene expression for this panel of cytokines due to exposure to particles alone or in combination with ozone. The cytokines showing the greatest changes following particle exposure included interleukin-1 alpha, interleukin-1 beta and interleukin-12p35. From this pilot project we conclude gene expression gleaned from airway biopsy specimens can provide useful data support observations based on less specific measurements such as bronchoalveolar lavage or pulmonary function testing in human testing of particle-induced effects.

Summary and Conclusions (Animal and Human comparison)

A number of conclusions may be drawn from the findings of our combined animal and human studies. A most unique outcome of these studies has been the opportunity to compare the response in animals and humans to inhaled particles of identical

composition. The use of ammonium nitrate and carbon black in both animal and human exposure studies was based on the ubitiquious nature of these two components in particulate matter of California and the Western United States. Aerosolization of these materials was done in approximately the same ratio with both systems using a nebulizer to deliver particles to the respiratory tract by inhalation.

Although an animal model of allergic airways disease may not allow for the precise duplication of the human asthmatic immune and cellular response, a number of features make this an attractive model. Cellular inflammation of the airways, airway hyperreactivity and increased mucous production are considered hallmarks of an asthmatic condition. However, asthma is also a multi-faceted disease taking on many forms both structurally as well as physiologically.

A critical link observed between our animal model and the human asthmatic rests in the lung airways. Studies in human volunteers demonstrated the significance of particle exposure to alter cytokine gene expression in airway biopsy tissues obtained from these individuals. Studies in the BN rat also demonstrated significant effects in the lungs. These effects were measured in the airways of both humans and animals.

Human airway biopsies contain both epithelial and underlying interstitial cells. Although the panel of cytokines studied in humans resulted in significant elevation of different cytokines than those induced in the BN rat, these findings provide direct evidence of PM-induced effects in the compromised lung airways of both humans and rats. Such findings present the opportunity to further explore how such a response is induced.

In conclusion, these combined studies in animals and humans suggest that exposure to airborne particles over an acute time frame can result in alterations in cell and gene expression within the respiratory system. Such findings may be key to those events responsible for asthma exacerbations due to particle exposure.

Recommendations

The presence of airborne particulate matter in our environment and the health effects associated with exposure to these particles drives in large measure the relevance of why the California Air Resources Board supports endeavors to study the potential mechanisms and causes for such health effects. PM is a major component of air pollution, consisting of a complex mixture of compounds. Ammonium nitrate and carbon represent a significant fraction of the chemical composition of PM for the state of California. Sensitive populations of individuals exist that are more adversely affected by exposure to PM than the general population. These groups include children, the elderly and those with pre-existing cardiopulmonary conditions.

To address specific questions of PM-related health effects on sensitive populations, controlled laboratory experiments which involve both animals and humans are needed. We have demonstrated studies can be conducted in the human clinical setting as well as through animal toxicology protocols to facilitate potentially useful correlations to identify mechanisms that may be involved in adverse health effects due to exposure to PM.

We recommend future studies be designed to evaluate the potential effects of particles of other compositions in both humans and animals. With an animal model of allergic airways disease and human asthmatics, the effects of diesel particulate

emissions or wood smoke, both highly prevalent air pollutants of California could be studied in similar conditions to those used in the current study with ammonium nitrate and carbon black. Due to the increased prevalence of asthma today, future studies should also consider the use of ambient particle exposure using a concentrator system to determine the impact of exposure on airway sensitivity in both animals and humans. Comparative studies which examine both humans and animals provide a mechanism for better determining the relevance of exposure outcomes.

Particle size, particle composition and particle number continue to remain critical unknown entities in the genesis of adverse health outcomes. Both animal and human studies should in the future address these issues. If studies are done in a coordinated and controlled manner for both humans and animals, we will begin to provide critical answers to these issues. Collaborative, multi-institutional endeavors supported by the California Air Resources Board, based on scientifically sound and well coordinated plans, can provide important information that would also be cost effective in better elucidating potential adverse health effects. Controlled human clinical studies coordinated with animal toxicology studies could further address critical issues to assist in establishing guidelines to better safeguard public health.

References

Abbas AK, AH Lichtman, JS Pober. 2000. Cellular and Molecular Immunology. 4th edition. WB Saunders Company: Philadelphia, PA.

Abehsira-Amar O., Gibert M., Joliy M., Thaeze J., Jankovic D. L. (1992) IL-4 plays a dominant role in the differential development of Th0 in Th1 and Th2 cells. J. Immunol. 148:3820-3829.

Allakhverdi Z, M Allam, PM Renzi. 2002. Inhibition of antigen-induced eosinophilia and airway hyperresponsiveness by antisense oligonucleotides directed against the common beta chain of IL-3, IL-5, GM-CSF receptors in a rat model of allergic asthma. Am J Respir Crit Care Med. 165(7):1015-21.

Amerio P, A Frezzolini, C Feliciani, R Verdolini, P Teofoli, O De Pita, P Puddu. 2003. Eotaxins and CCR3 receptor in inflammatory and allergic skin diseases: therapeutical implications. Curr Drug Targets Inflamm Allergy 2(1):81-94.

Anjilvel S and B. Asgharian. A multiple-path model of particle deposition in the rat lung. *Fundam. Appl. Toxicol.* 28:41–50 (1995

Amin K, D Ludviksdottir, C Janson, O Nettelbladt, E Bjornsson, G Roomans, G Boman, L Seveus, P Venge. 2000. Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. Am J Respir Crit Care Med 162:2295-2301.

Bandeira-Melo C, K Sugiyama, LJ Woods, PF Weller. 2001. Cutting edge: eotaxin elicits rapid vesicular transport-mediated release of preformed IL-4 from human eosinophils. J Immunol 166(8):4813-4817.

Bellofiore S, JG Martin. 1988. Antigen challenge of sensitized rats increases airway responsiveness to methacholine. J Appl Physiol 65(4):1642-6.

Bischoff SC, G Sellge, A Lorentz, W Sebald, R Raab, MP Manns. 1999. IL-4 enhances proliferation and mediator release in mature human mast cells. Immunology 96:8080-8085.

Bischoff SC, G Sellge. 2002. Mast cell hyperplasia: role of cytokines. Int Arch Allergy Immunol 127(2):118-122.

Bjerke T, M Gaustadnes, S Nielsen, LP Nielsen, PO Schiotz, N Rudiger, CM Reimert, R Dahl, I Christensen, LK Poulsen. 1996. Human blood eosinophils produce and secrete interleukin 4. Respir Med 90(5):271-277.

Brinkman V, CH Heusser. 1993. T Cell-Dependent Differentiation of Human B Cells into IgM, IgG, IgA, or IgE Plasma Cells: High Rate of Antibody Production by IgE Plasma Cells, but Limited Clonal Expansion of IgE Precursors. Cellular Immunology 152:323-332.

Brown DM, K Donaldson. 1996. Wool and grain dusts stimulate TNF secretion by alveolar macrophages in vitro. Occupational and Environmental Medicine 53:387-393.

Brown JR, J Kleimberg, M Marini, G Sun, A Bellini, S Mattoli. 1998. Kinetics of eotaxin expression and its relationship to eosinophil accumulation and activation in bronchial biopsies and bronchoalveolar lavage (BAL) of asthmatic patients after allergen inhalation. Clin Exp Immunol 114(2):137-146.

Bruns HA, U Schindler, MG Kaplan. 2003. Expression of a constitutively active Stat6 in vivo alters lymphocyte homeostasis with distinct effects in T and B cells. J Immunol 170(7):3478-3487.

California Air Resources Board Monitoring and Laboratory Division. Standard operating procedure for analysis of dry deposition samples: Field preparation, mass analysis and extraction procedures. S. O. P. No. MLD 041, Undated

California Air Resources Board Monitoring and Laboratory Division. Standard operating procedure for analyzing anions from ambient air particulate samples from the California acidic dry deposition monitoring network. S. O. P. No. MLD 044, November 12, 1992

Cambrey, J. (Personal communication). Cabot Corporation, Billerica, MA, August 27, 1997

Careau E, J Sirois, EY Bissonnette. 2002. Characterization of lung hyperresponsiveness, inflammation, and alveolar macrophage mediator production in allergy resistant and susceptible rats. Am J Respir Cell Mol Biol 26:579-586.

Castillos M, Borja-Aburto VH, Dockery DW, Gold DR, Loomis D. Airborne coarse particles and mortality. 2000. Inhalation Toxicology: proceedings of the third colloquium on particulate air pollution and human health 12(suppl.1):67-72.

Chow JC, JG Watson, DH Lowenthal, PA Solomon, KL Magliano, SD Ziman, LW Richards. 1993. PM10 and PM2.5 compositions in California's San Joaquin Valley. Atmospheric Environment 26A:3335-3354.

Christian-Ritter KK, LD Hill, EB Hoie, TL Zach. 1994. Effect of interleukin-4 on the synthesis of the third component of complement by pulmonary epithelial cells. Am J Pathol 144(1):171-176.

Clegg, S.L., P. Brimblecombe and A.S. Wexler. The aerosol inorganics model project. <http://www.hpc1.uea.ac.uk/~e770/aim/aim.html> (May 15, 2001)

Clegg, S.L., P. Brimblecombe and A.S. Wexler. A thermodynamic model of the system H^+-NH_4^+-Na^+-SO_4^{2-}-NO_3^--Cl^--H_2O at 298.15K. *J. Phys. Chem.* A 102:2155-2171 (1998)

Cook EB, Stahl JL, Lilly CM, Haley KJ, Sanchez H, Luster AD, Graziano FM, Rothenberg ME. 1998. Epithelial cells are a major cellular source of the chemokine eotaxin in the guinea pig lung. Allergy Asthma Proc 19(1):15-22.

Costello RW, DB Jacoby, GJ Gleich, AD Fryer. 2000. Eosinophils and airway nerves in asthma. Histol Histopathol 15(3):861-868.

Cunningham AF, PG Fallon, M Khan, S Vacheron, H Acha-Orbea, IC MacLennan, AN McKenzie, KM Toellner. 2002. Th2 activities induced during virgin T cell priming in the absence of IL-4, IL-13, and B cells. J Immunol 169(6):2900-2906.

Devouassoux G., Saxon A., Metcalfe D. D., Prussin C., Colomb M. G., Brambilla C., Diaz-Sanchez D. (2002) Chemical constituents of diesel exhaust particles induce IL-4 production and histamine release by human basophils. J Allergy Clin Immunol. 109:847-853.

Diaz-Sanchez D., Tsien A., Fleming J., Saxon A. (1997) Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed-specific IgE and skews cytokine production to a T helper cell 2-type pattern. J Immunol. 158:2406-2413.

Dick CA, P Singh, M Daniels, P Evansky, S Becker, ML Gilmour. 2003. Murine pulmonary inflammatory responses following instillation of size-fractionated ambient particulate matter. J Toxicol Environ Health A 66(23):2193-2207.

Dong W, MJK Selgrade, MI Gilmour. 2003. Systemic administration of bordetella pertussis enhances pulmonary sensitization to house dust mite in juvenile rats. Toxicological Sciences 72:113-121.

Erin EM, TJ Williams, PJ Barnes, TT Hansel. 2002. Eotaxin receptor (CCR3) antagonism in asthma and allergic disease. Curr Drug Targets Inflamm Allergy 1(2):201-214.

Fernvik E, G Peltre, H Senechal, BB Vargaftig. 2002. Effects of birch pollen and traffic particulate matter on Th2 cytokines, immunoglobulin E levels and bronchial hyperresponsiveness in mice. Clin Exp Allergy 32(4):602-611.

Fitch PS, V Brown, BC Schock, M Ennis, MD Shields. 2003. Interleukin-4 and interleukin-4 soluble receptor alpha levels in bronchoalveolar lavage from children with asthma. Ann Allergy Asthma Immunol 90(4):429-433.

Fujii T, S Hayashi, JC Hogg, H Mukae, T Suwa, Y Goto, R Vincent, SF van Eeden. 2002. Interaction of Alveiolar Macrophages and Airway Epithelial Cells Following Exposure to Particulate Matter Produces Mediators that Stimulate the Bone Marrow. Am J Respir Cell Mol Biol 27:34-41.

Fung, K. Particulate carbon speciation by MnO_2 oxidation. *Aerosol Sci. and Technol.* 12:122-127 (1990)

Gauvreau GM, RM Watson, PM O'Byrne. 1999. Kinetics of Allergen-Induced Airway Eosinophilic Cytokine Production and Airway Inflammation. Am J Respir Crit Care Med 160:640-647.

Gavett SH, DJ O'Hearn, X Li, SK Huang, FD Finkelman, M Wills-Karp. 1995. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. J Exp Med 182(5):1527-1536.

Gavett SH, SL Madison, MA Stevens, DL Costa. 1999. Residual oil fly ash amplifies allergic cytokines, airway responsiveness, and inflammation in mice. Am J Respir Crit Care Med 160(6):1897-1904.

Gent J. F., Triche E. W., Holford T. R., Belanger K., Bracken M. B., Beckett W. S., Leaderer B. P. (2003) Association of low-level ozone and fine particles with respiratory symptoms in children with asthma. Jama. 290:1859-1867.

Guo RF, PA Ward PA, JA Jordan, M Huber-Lang, RL Warner, MM Shi. 1999. Eotaxin Expression in Sephadex-Induced Lung Injury in Rats. American Journal of Pathology 155(6):2001-2008.

Guo RF, AB Lentsch, RL Warner, M Huber-Lang, J Vidya Sarma, T Hlaing, MM Shi, NW Lukacs, PA Ward. 2001. Regulatory effects of eotaxin on acute lung inflammatory injury. The Journal of Immunology 166(8):5208-18.

Haczku A, P Macary, EB Haddad, TJ Huang, DM Kemeny, R Moqbel, KF Chung. 1996. Expression of Th-2 cytokines interleukin-4 and -5 and of Th-1 cytokine interferon-gamma in ovalbumin-exposed sensitized Brown-Norway rats. Immunology 88(2):247-251.

Hakonarson H, Maskeri N, Carter C, Grunstein MM. 1999. Regulation of TH1-andTH2-type cytokine expression and action in atopic asthmatic sensitized airway smooth muscle. The J of Clinical Investigation 103(7)1077-1087.

Han SJ, JH Kim, YJ Noh, HS Chang, CS Kim, KS Kim, SY Ki, CS Park, IY Chung. 1999. Interleukin (IL)-5 Downregulates Tumor Necrosis Factor (TNF)-Induced Eotaxin Messenger RNA (mRNA) Expression in Eosinophils: Induction of Eotaxin MRNA by TNF and IL-5 in Eosinophils. Am J Respir Cell Mol Biol 21:303-310.

Harrington PM, DJ Newton, CMM Williams, JA Hunt, RJ Dearman, I Kimber, JW Coleman, BF Flanagan. 1999. Eotaxin and eotaxin receptor (CCR3) expression in Sephadex particle-induced rat lung inflammation. Int J Exp Path 80:177-185.

Hinners RG, JK Burkart, CL Punte. 1968. Animal inhalation exposure chambers. Arch Environ Health 16(2):194-206.

Holt P. G. (1998) Programming for responsiveness to environmental antigens that trigger allergic respiratory disease in adulthood is initiated during the perinatal period. Environ. Health Perspect. 106 Suppl 3:795-800.

Hsieh C. S., Heimberger A. B., Gold J. S., O'Garra A., Murphy K. M. (1992) Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell receptor transgenic system. Proc. Natl. Acad. Sci. USA. 89:6065-6069.

Humbert M, Corrigan CJ, Kimmitt P, Till SJ, Kay AB, Durham SR. 1997. Relationship between IL-4 and IL-5 mRNA Expression and Disease Severity In Atopic Asthma. Am J respir Crit Care Med 156:704-708.

Institute of Laboratory Animal Resources National Research Council. *Guide for the Care and Use of Laboratory Animals*, 127 p., National Academy Press, Washington, DC, 1996

Isler P, BG de Rochemonteix, F Songeon, N Boehringer, L Nicod. 1999. Interleukin-12 Production by Human Alveolar Macrophages Is Controlled by the Autocrine Production of Interleukin-10. Am J Respir Cell Mol Biol 20:270-278.

Isogai S, Q Hamid, E Minshall, S Miyake, Y Yoshizawa, R Taha, M Toda, JG Martin, A Watanabe. 2000. Interferon-gamma increases IL-12 mRNA expresion and attentuates allergic late-onset airway response in the Brown Norway Rat. Eur Respir J 16(1):22-29.

Isogai S, A Rubin, K Maghni, D Ramos-Barbon, R Taha, Y Yoshizawa, Q Hamid, JG Martin. 2003. The effects of CD8+gammadelta T cells on late allergic airway responses and airway inflammation in rats. J Allergy Clin Immunol 112(3):547-555.

Jimenez LA, EM Drost, PS Gilmour, I Rahman, F Antonicelli, H Ritchie, W MacNee, K Donaldson. 2002. PM_{10}-exposed macrophages stimulate a proinflammatory response in lung epithelial cells via TNF-α. Am J Physiol Lung Cell Mol Physiol 282:L237-L248.

Jinquan T, HH Jacobi, C Jing, A Millner, E Sten, L Hviid, L Anting, LP Ryder, C Glue, PS Skov, E Jarman, K Lamberth, HJ Malling, LK Poulsen. 2003. CCR3 expression induced by IL-2 and IL-4 functioning as a death receptor for B cells. J Immunol 171(4):1722-1731.

Justice JP, J Crosby, MT Borchers, A Tomkinson, JJ Lee, NA Lee. 2002. CD4+ T cell-dependent airway mucus production occurs in response to IL-5 expression in lung. Am J Physiol Lung Cell Mol Physiol 282:L1066-L1074.

Kanehiro A, M Lahn, MJ Makela, A Dakhama, M Fujita, A Joetham, RJ Mason, W born, EW Gelfand. 2001. Tumor necrosis factor-α negatively regulates airway hyperresponsiveness through $\gamma\delta$ T cells. Am J Respir Crit Care Med 164:2229-2238.

Karulin AY, MD Hesse, HC Yip, PV Lehmann. 2002. Indirect IL-4 pathway in type 1 immunity. J Immunol 168(2):545-553.

Kelly-Welch, AE, EM Hanson, MR Boothby, AD Keegan. 2003. Interleukin-4 and Interleukin-13 Signaling Connections Maps. Science 300:1527-1528.

Kim J, AC Merry, JA Nemzek, GL Bolgos, J Siddiqui, DG Remick. 2001. Eotaxin represents the principal eosinophil chemoattractant in a novel murine asthma model induced by house dust containing cockroach allergens. The Journal of Immunology 167(5):2808-2815.

Kleinman, M.T. (Personal communication). University of California at Irvine, May 2, 2001

Kleinman, M.T., C. Bufalino, R. Rasmussen, D. Hyde, D.K. Bhalla and W.J. Mautz. Toxicity of chemical components of ambient fine particulate matter (PM 2.5) inhaled by aged rats. *J. Appl. Toxicol.* 20:357-364 (2000)

Kobayashi M, M Ito, K Sano, M Koyama. 2000. Granulomatous and cytokine responses to pulmonary Cryptococcus neoformans in two strains of rats. Mycopathologia 151:121-130.

Lahn M, A Kanehio, K Takeda, A Joetham, J Schwarze, G Kohler, R O'Brien, EW Gelfand, W Born. 1999. Negative regulation of airway responsiveness that is dependent on $\gamma\delta$ T cells and independent of $\alpha\beta$ cells. Nature Medicine. 5(10):1150-1156.

Lambert AL, W Dong, DW Winsett, MJK Selgrade, MI Gilmour. 1999. Residual oil fly ash exposure enhances allergic sensitization to house dust mite. Toxicology and Applied Pharmacology 158:269-277.

Lambert AL, W Dong, MJK Selgrade, MI Gilmour. 2000. Enhanced allergic sensitization by residual oil fly ash particles is mediated by soluble metal constituents. Toxicology and Applied Pharmacology 165:84-93.

Lilly CM, H Nakamura, H Kesselman, C Nagler-Anderson, K Asano, EA Garcia-Zepeda, ME Rothenberg, JM Drazen, AD Luster. 1997. Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids. J Clin Invest 99(7):1767-1773.

Lovik M, AK Hogseth, PI Gaarder, R Hagemann, I Eide. 1997. Diesel exhaust particles and carbon black have adjuvant activity on the local lymph node response and systemic IgE production to ovalbumin. Toxicology 121:165-178.

MacFarland HN. 1983. Designs and operational characteristics of inhalation exposure equipment – a review. Fundam Appl toxicol 3:603-613.

Masuda A, Y Yoshikai, K Aiba, T Matsuguchi. 2002. Th2 cytokine production from mast cells is directly induced by lipopolysaccharide and distinctly regulated by c-Jun N-terminal kinase and p38 pathways. J Immunol 169(7):3801-3810.

Mercer, T. T., M. I. Tillary and G. J. Newton. A multi-stage low flow rate cascade impactor. *Aerosol Sci.* 1:9-15 (1970)

Michielsen CPPC, A Leusink-Muis, JG Vos, N Bloksma. 2001. Hexachlorobenzene-induced eosinophilic and granulomatous lung inflammation is associated with in vivo airways hyperresponsiveness in the brown Norway rat. Toxicology and Applied Pharmacology 172:11-20.

Minshall EM, RJ Dandurand, Q Hamid, DH Eidelman. 1998. Local cytokine messenger ribonucleic acid expression and in vitro allergic late phase responses in Brown-Norway rats. Eur Respir J 11(3):630-635.

Mishima H, M Hojo, A Watanabe, QA Hamid, JG Martin. 1998. Cd4+ T cells can induce airway hyperresponsiveness to allergen challenge in the brown Norway rat. Am J Respir Crit Care Med 158:1863-1870.

Miyamasu M, Y Misaki, M Yamaguchi, K Yamamoto, Y Morita, K Matsushima, T Nakajima, K Hirai. 2000. Regulation of human eotaxin generation by Th1-/Th2-derived cytokines. Int Arch Allergy Immunol 122 Suppl 1:54-58.

Molet S, D Ramos-Barbon, JG Martin, Q Hamid. 1999. Adoptively transferred late allergic response is inhibited by IL-4, but not IL-5, antisense oligonucleotide. J Allergy Clin Immunol 104(1):205-214.

Mountford AP, PS Coulson, AW Cheever, A Sher, RA Wilson, TA Wynn. 1999. Interleukin-12 can directly induce T-helper 1 responses in interferon-gamma (IFN-gamma) receptor-deficient mice, but requires IFN-gamma signalling to downregulate T-helper 2 responses. Immunology 97(4):588-594.

Nakamura H, AD Luster, H Tateno, S Jedrzkiewicz, G Tamura, KJ Haley, EA Garcia-Zepeda, K Yamaguchi, C Lilly. 2001. IL-4 differentially regulates eotaxin and MCP-4 in lung epithelium and circulating mononuclear cells. Am J Physiol Lung Cell Mol Physiol 281:L1288-L1302.

Neuhaus-Steinmetz U., Ulffhausen F., Herz U., Renz H. (2000) Priming of Allergic Immune Responses by Repeated Ozone Exposure in Mice. Am. J. Respir. Cell Mol. Biol. 23:228-233.

Oberdorster G, Gelein RM, Ferin J, Weiss B. 2001. Association of particulate air pollution and acute mortality: involvement of ultrafine particles? Inhal Toxicol 7(1):111-24.

Ogawa K, O Kaminuma, H Kikkawa, A Nakata, M Asahina, RW Egan, K Akiyama, A Mori. 2002. Transient contribution of mast cells to pulmonary eosinophilia but not to hyper-responsiveness. Clin Exp Allergy 32(1):140-148.

Ohtsuka R, K Doi, S Itagaki. 1997. Histological characteristics of respiratory system in brown Norway rat. Exp Anim 46(2):127-133.

Okumura S, J Kashiwakura, H Tomita, K Matsumoto, T Nakajima, H Saito, Y Okayama. 2003. Identification of specific gene expression profiles in human mast cells mediated by Toll-like receptor 4 and FcεRI. Blood 102:2547-2554.

Ormstad H, PI Gaarder, BV Johansen, M Lovik. 1998. Airborne house dust elicits a local lymph node reaction and has an adjuvant effect on specific IgE production in the mouse. Toxicology 129:227-236.

Osebold J. W., Zee Y. C., Gershwin L. J. (1988) Enhancement of allergic lung sensitization in mice by ozone inhalation. Proc Soc Exp Biol Med. 188:259-264.

Ostro BD, Lipsett M, Mann J, Braxton-Owens H, White M. 2001. Air pollution and exacerbation of asthma in African-American children in Los Angeles. Epidemiology 12:200-8.

Ozols, J. Amino acid analysis in M. P. Deutscher, Ed., *Guide to Protein Purification, Methods in Enzymology*, Vol. 182, pp. 587-601, Academic Press, San Diego, CA, 1990

Palmans ELS, JC Kips, RA Pauwels. 2000. Prolonged allergen exposure induces structural airway changes in sensitized rats. Am J Respir Crit Care Med 161:627-635.

Penttinen P., Timonen K. L., Tiittanen P., Mirme A., Ruuskanen J., Pekkanen J. (2001) Ultrafine particles in urban air and respiratory health among adult asthmatics. Eur Respir J. 17:428-435.

Pinkerton KE, Green FHY, Saiki C, Vallyathan V, Plopper CG, Gopal V, Hung D, Bahne EB, Lin SS, Menache MG, Schenker MB. 2000. Distribution of Particulate matter and Tisue remodeling in the Human Lung. Environmental Health Perspectives 108(11):1063-1069.

Pinkerton, K.E., A.R. Buckpitt, D.M. Hyde and C.G. Plopper. Mechanisms of particulate toxicity: Effects on the respiratory system. Final report for California Air Resources Board Contract Number 96-310, December 2000

Plopper CG, Fanucchi MV. 2000. Do urban enviromental pollutants exascerbate childhood lung diseases? Environmental Health Perspectives 108(6):A252-A253.

Reibman J., Hsu Y., Chen L. C., Bleck B., Gordon T. (2003) Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter. Am J Respir Cell Mol Biol. 28:648-654.

Reiter R, K Pfeffer. 2002. Impaired germinal centre formation and humoral immune response in the absence of CD28 and interleukin-4. Immunology 106(2):222-228.

Roberts ES, JH Richards, R Jaskot, KL Dreher. 2003. Oxidative stress mediates air pollution particle-induced acute lung injury and molecular pathology. Inhal Toxicol 15(13):1327-1346.

Rush JS, PD Hodgkin. 2001. B cells activated via CD40 and IL-4 undergo a division burst but require continued stimulation to maintain division, survival and differentiation. Eur J Immunol 31(4):1150-1159.

Salmon M, DA Walsh, H Koto, PJ Barnes, KF Chung. 1999. Repeated allergen exposure of sensitized brown Norway rats induces airway cell DNA synthesis and remodeling. Eur Respir J 14:633-641.

Salvi S, A Semper, A Blomberg, J Holloway, Z Jaffar, A Papi, L Teran, R Polosa, F Kelly, T Sandstrom, S Holgate, A Frew. 1999. Interleukin-5 Production by Human Airway Epithelial Cells. Am J Respir Cell Mol Biol 20:984-991.

Sanmugalingham D, E De Vries, R Gauntlett, FA Symon, P Bradding, AJ Wardlaw. 2000. Interleukin-5 enhances eosinophil adhesion to bronchial epithelial cells. Clin Exp Allergy 30(2)255-263.

Sapienza T, Du T, Eidelman DH, Wang NS, Martin JG. 1991. Structural changes in the airways of sensitized brown Norway rats after antigen challenge. Am Rev Respir Dis 144:423-427.

Scannell C., Chen L., Aris R. M., Tager I., Christian D., Ferrando R., Welch B., Kelly T., Balmes J. R. (1996) Greater ozone-induced inflammatory responses in subjects with asthma. Am. J. Respir. Crit. Care Med. 154:24-29.

Scheerens J, SB van Gessel, FP Nijkamp, G Folkerts. 2002. Eotaxin protein levels and airway pathology in a mouse model for allergic asthma. Eur J Pharmacol 453(1):111-117.

Schelegle ES, LJ Gershwin, LA Miller, MV Fanucchi, LS Van Winkle, JP Gerriets, WF Walby, AM Omlor, AR Buckpitt, BK Tarkington, VJ Wong, JP Joad, KE Pinkerton, R Wu, MJ Evans, DM Hyde, CG Plopper. 2001. Allergic asthma induced in rhesus monkeys by house dust mite (dermatophagoides farinae). American Journal of Pathology 158:333-341.

Schneider T, D van Velzen, R Moqbel, AC Issekutz. 1997. Kinetics and quantitation of eosinophil and neutrophil recruitment to allergic lung inflammation in a brown Norway rat model. Am J Respir Cell Mol Biol 17:702-712.

Schuster M, T Tschernis, N Krug, R Pabst. 2001. Lymphocytes migrate from the blook into the bronchoalveolar lavage and lung parenchyma in the asthma model of the brown Norway rat. Am J Respir Crit Care Med 161(2):558-566.
Schwarze J, E Hamelmann, G Cieslewicz, A Tomkinson, A Joetham, K Bradley, EW Gelfand. 1998. Local treatment with IL-12 is an effective inhibitor of airway hyperresponsiveness and lung eosinophilia after airway challenge in sensitized mice. J Allergy Clin Immunol 102(1):86-93.
Shahana S, C Kampf, GM Roomans. 2002. Effects of the cationic protein poly-L-arginine on airway epithelial cells in vitro. Mediators Inflamm. 11(3):141-148.
Shang X-Z, B-C Chiu, V Stolberg, NW Lukacs, SL Kunkel, HS Murphy, SW Chensue. 2002. Eosinophil recruitment in type-2 hypersensitivity pulmonary granulomas. American Journal of Pathology 161(1):257-266.
Shinagawa K, A Trifilieff, GP Anderson. 2003. Involvement of CCR#-reactive chemokines in eosinophil survival. Int Arch Allergy Immunol 130(2):150-157.
Shirakawa T., Enomoto T., Shimazu S., Hopkin J. M. (1997) The inverse association between tuberculin responses and atopic disorder. Science. 275:77-79.
Shukla A, C Timblin, K BeruBe, T Gordon, W McKinney, K Driscoll, P Vacek, BT Mossman. 2000. Inhaled Particulate Matter Causes Expression of Nuclear Factor (NF)-κB-Related Genes and Oxidant-Dependent NF-κB Activation In Vitro. Am J Respir Cell Mol Biol 23:182-187.
Singh P, M Daniels, DW Winsett, J Richards, D Doerfler, G Hatch, KB Adler, MI Gilmour. 2002. Phenotypic comparison of allergic airway responses to house dust mite in three rat strains. Am J Physiol Lung Cell Mol Physiol 284:L588-L598.
Steerenberg PA, JA Dormans, CC van Doorn, S Middendorp, JF Vos, H van Loveren. 1999. A pollen model in the rat for testing adjuvant activity of air pollution components. Inhal Toxicol 11(12):1109-22.
Stenton GR, M Ulanova, RE Dery, S Merani, MK Kim, M Gilchrist, L Puttagunta, S Musat-Marcu, D James, AD Schreiber, AD Befus. 2002. Inhibition of allergic inflammation in the airways using aerosolized antisense to SYK kinase. J Immunol 169(2):1028-1036.
Striz I, T Mio, Y Adachi, P Heires, RA Robbins, JR Spurzem, MJ Illig, DJ Romberger, SI Rennard. 1999b. IL-4 induces ICAM-1 expression in human bronchial epithelial cells and potentiates TNF-a. Am J Physiol 277:L58-L64.
Striz I, T Mio, Y Adachi, RA Robbins, DJ Romberger, SI Rennard. 1999a. IL-4 and IL-13 stimulate human bronchial epithelial cells to release IL-8. Inflammation 23(6):545-555.
Svensson L, B Lilliehook, R Larsson, A Bucht. 2003. γδ T cells contribute to the systemic immunoglobulin E response and local B-cell reactivity in allergic eosinophilic airway inflammation. Immunology 108:98-108.
Swain S. L., Weinberg A. d., English M., Huston G. (1990) IL-4 directs the development of Th2-like helper effectors. J. Immunol. 148:3820-3829.
Takeda K, T Tanaka, W Shi, M Matsumoto, M Minami, S Kashiwamura, K Nakanichi, N Yoshida, T Kishimoto, S Akira. 1996. Essential role of Stat6 in IL-4 signalling. Nature 380(6575):627-630.

Tangye SG, A Ferguson, DT Avery, CS Ma, PD Hodgkin. 2002. Isotype switching by human B cells is division-associated and regulated by cytokines. J Immunol 169(8):4298-4306.

Teague, S. V., H. C. Yeh and G. J. Newton. Fabrication and use of krypton-85 aerosol discharge devices. *Health Phys.* 35:392-395 (1978)

Teran LM, MP Carroll, JK Shute, ST Holgate. 1999. Interleukin 5 Release into Asthmatic Airways 4 and 24 Hours After Endobronchial Allergen Challenge: Its Relationship with Eosinophil Recruitment. Cytokine 11(7):518-522.

Timblin C, K BeruBe, A Churg, K Driscoll, T Gordon, D Hemenway, E Walsh, AB Cumins, P Vacek, B Mossman. 1998. Ambient particulate matter causes activation of the c-jun kinase/stress-activated protein kinase cascade and DNA synthesis in lung epithelial cells. Cancer Res 58(20):4543-4547.

Trautman A, P Schmid-Grendelmeier, K Kruger, R Crameri, M Akdis, A Akkaya, EB Brocker, K Blaser, CA Akdis. 2002. T cells and eosinophils cooperate in the induction of broncial epithelial cell apoptosis in asthma. J Allergy Clin Immunol 109(2):329-337.

Ulrich MM, GM Alink, P Kumarathasan, R Vincent, AJ Boere, FR Cassee. 2002. Health effects and time course of particulate matter on the cardiopulmonary system in rats with lung inflammation. J Toxicol Environ Health A 65(20):1571-1595.

van der Velden VHJ, BAE Naber, AF Wierenga-Wolf, R Debets, HFJ Savelkoul, SE Overbeck, HC Hoogsteden, MA Versnel. 1998. Interleukin 4 Receptors on Human Bronchial Epithelial Cells. An In Vivo and In Vitro Analysis of Expression and Function. Cytokine 10(10):803-813.

von Klot S., Wolke G., Tuch T., Heinrich J., Dockery D. W., Schwartz J., Kreyling W. G., Wichmann H. E., Peters A. (2002) Increased asthma medication use in association with ambient fine and ultrafine particles. Eur Respir J. 20:691-702.

Walters DM, PN Breysse, B Schofield, M Wills-Karp. 2002. Complement Factor 3 Mediates Particulate Matter-Induced Airway Hyperresponsiveness. Am J Respir Cell Mol Biol 27:413-418.

Wan GH, CS Li, RH Lin. 2000. Airborne endotoxin and the development of airway antigen-specific allergic responses. Clin Exp Allergy 30(3):426-432.

Wynn TA, R Morawetz, T Scharton-Kersten, S Hieny, HC 3rd Morse, R Kuhn, W Muller, AW Cheever, A Sher. 1997. Analysis of granuloma formation in double cyto-kine deficient mice reveals a central role for IL-10 in polarizing both T helper cell 1- and T helper cell 2-type cytokine responses in vivo. J Immunol 159(10):5014-5023.

Yamada T, D Zhu, A Saxon, K Zhang. 2002. CD45 Controls Interleukin-4-mediated IgE Class Switch Recombination in Human B Cells through Its Function as a Janus Kinase Phosphatase. The Journal of Biological Chemistry 277(32):28830-28835.

Ying S., Humbert M., Barkans J., Corrigan C. J., Pfister R., Menz G., Larche M., Robinson D. S., Durham S. R., Kay A. B. (1997) Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. J Immunol. 158:3539-3544.

Zhang J, LJ Lathbury, LA Salamonsen. 2000. Expression of the Chemokine Eotaxin and Its Receptor, CCR3, in Human Endometrium. Biology of Reproduction 62:404-411.

Zhou Y, C Zhong, IM Kennedy, VJ Leppert, KE Pinkerton. 2003. Oxidative stress and NFκB activation in the lungs of rats: a synergistic interaction between soot and iron particles. Toxicology and Applied Pharmacology 190:157-169.

Glossary of Terms, Abbreviations, and Symbols

AB/PAS	alcian blue/periodic acid Schiff
ANOVA	analysis of variance
BADJ	bronchiole alveolar duct junction
BAL	bronchoalveolar lavage
BN	Brown Norway
BrdU	bromodeoxyuridine
BV	blood vessel
BW	body weight
C	carbon
C	degree centigrade
CCR3	CC chemokine receptor
CEM	combined eosinophil/mast cell
cm	centimeter
DNA	deoxynuclei acid
EC200RL	effective concentration to double lung resistance
EDTA	ethylene-diamine-tetra-acetic acid
EtD-1	ethidium-1-homodimer
FA	filtered air
FcεR	Fc (antibody) epsilon receptor
g	gram
GM-CSF	granulocyte-monocyte-colony stimulating factor
H&E	hematoxylin and eosin
IgE	immunoglobulin E
IL	interleukin
IP	intraperitoneal
^{85}Kr	krypton 85
m^3	cubic meter
m^3/min	cubic meter/minute
Mch	methacholine
mg	milligram
MHCII	major histocompatiblity antigen, class II
mRNA	messenger ribonucleic acid
MMAD	mass median aerodynamic diameter
MT	Masson's trichrome
N	normal
N/C	not sensitized/challenged
NH_4CO_3	ammonium nitrate
NIH	National Institutes of Health
nm	nanometer
OVA	ovalbumin
PBS	phosphate buffered saline
PFA	polyfluorinated acetate
PFT	pulmonary function test
PM	particulate matter

PMA	phorbol myristate acetate
Resp/min	respirations/minute
RT-PCR	reverse transcriptase-polychain reaction
S	sensitized
S/C	sensitized/challenged
SE	standard error
TB/AD	terminal bronchiole/alveolar duct
Th	T-helper
TNF α	tumor necrosis factor alpha
wk	week
δ INF	interferon gamma
δΔ-T	gamma delta T lymphocytes
σ g	sigma g
µm	micrometer
µg/ml	microgram/milliliter
WBC	white blood cells

Appendix

UC San Francisco Human Asthmatic Subjects Protocol

Materials and Methods

Design:

This project consisted of two separate controlled human exposure experiments. Experiment One utilized 15 subjects and Experiment Two utilized 10 subjects. All subjects for both experiments were individuals with mild to moderate asthma. The exposure conditions were: Experiment One: separate single exposures to each of Filtered-Air (FA); carbon and ammonium-nitrate particles at a total concentration of 300 $\mu g/m^3$ (P); P and O_3 at a concentration of 0.2 ppm (PO): Experiment Two: separate single exposures to FA; and P; and three serial-day exposures to P (P-3). The duration of all the exposures was 4 h, during which subjects completed 4 x 30 min exercise periods, separated by 4 x 30 min rest periods.

For both experiments, each subject attended the laboratory for one characterization session, and subsequently for three or four exposure and bronchoscopy sessions. The characterization session was used to collect physical and pulmonary characteristics, and to familiarize each subject with the procedures of the experiment. Each of the experiments utilized a repeated measures design, with each subject completing each condition within the experiment. The order of the experimental conditions was counterbalanced/randomized within each experiment.

Controls:

For both experiments, a control exposure condition of FA was used. To allow recovery from preceding sessions, a minimum of three weeks separated each of the exposure conditions within each experiment.

Independent Variables:

The independent variables were:

The exposure conditions:

1) FA; single exposure.
2) P; at 300 $\mu g/m^3$; single exposure.
3) PO; at 300 $\mu g/m^3$ and 0.2 ppm; single exposure.
4) P-3; at 300 $\mu g/m^3$; three serial-day exposures.

Dependent Variables:

The dependent variables measured were:

1) Cell distribution; in Bfx and BAL; total and differential cell counts (macrophages, lymphocytes, neutrophils, eosinophils, epithelial cells, squamous cells).
2) Protein/cytokines: in Bfx and BAL; Total protein, IL-6, IL-8, CRP.
3) Gene expression: in Bfx, BAL, and epithelial cells; IL-6, IL-8, IL-10, HIN-1, TFF-3.
4) Spirometric pulmonary function: FVC, FEV_1, FEF_{25-75}.

5) Airway inflammation grading (visual).
6) Symptoms (general and respiratory).
7) Heart-rate variability.

Subjects:

All subjects were informed of the risks of the experiment and provided informed consent prior to participation. The procedures for this experiment were approved by the University of California, San Francisco, Institutional Review Board, Committee on Human Research.

All subjects completed a medical history questionnaire, were current non-smokers, had no history of excessive smoking, and had no serious health problems. Female subjects were not pregnant throughout the project. Subjects had no respiratory-tract illness in the three weeks preceeding, or during, each session. Subjects were characterized by physical characteristics, spirometric pulmonary function, non-specific airway reactivity, and allergy skin test.

Experiment One:

The 15 subjects had mild to moderate asthma, and were otherwise healthy. Asthma status was determined using the guidelines of the National Asthma Education Program (National Asthma Education Program Expert Panel, 1997). All subjects had non-specific airway reactivity of < 10 mg/ml methacholine. Subjects were characterized by physical, pulmonary, allergy, and medication characteristics (Table 1.).

Experiment Two:

The 10 subjects had mild to moderate asthma, and were otherwise healthy. Asthma status was determined using the guidelines of the National Asthma Education Program (National Asthma Education Program Expert Panel, 1997). All subjects had non-specific airway reactivity of < 10 mg/ml methacholine. Subjects were characterized by physical, pulmonary, allergy, and medication characteristics (Table 2.).

Controls and Medications:

Subjects abstained from caffeine for 8 h prior to each session, and were instructed not to take any medication with known or potential, anti-inflammatory or broncho-active properties, for specific periods, before or during the exposure and bronchoscopy periods. Subjects abstained from all inhaled steroids for a minimum of two weeks prior to all testing sessions.

Equipment and Procedures:

Laboratory:

All sessions, excluding bronchoscopy (Refer to Bronchoscopy section), were conducted in the Human Exposure Laboratory at the Lung Biology Center, San Francisco General Hospital Campus, University of California San Francisco.

Spirometric Pulmonary Function:

Spirometry for the determination of indices of pulmonary function; FVC, FEV_1, FEF_{25-75}, FEF_{75}, was conducted using a dry, rolling-seal spirometer (Anderson

Instruments; Spirotech Division, Model No. S400), using standardized procedures (Crapo *et al.*, 1995). Spirometry was conducted for subject characterization, non-specific airway reactivity, immediately pre- and post-exposure, and pre-bronchoscopy.

Non-Specific Airway Reactivity:

Non-specific airway reactivity was determined by the FEV_1 response to inhalation of nebulized (Devilbiss, Model No. 646) phosphate-buffered saline (PBS) and doubling concentrations of methacholine in PBS (0.313, 0.625, 1.25, 2.5, 5.0, 10.0 mg ml) delivered via a dosimeter (Rosenthal) at the rate of 0.01 ml breath (Kranner *et al.*, 1994). Non-specific airway reactivity was determined for subject characterization.

Allergy Skin Test:

Epicutaneous skin-prick testing with nine local aeroallergens (DP plus *aspergillus fumigatus*, birch mix, Chinese elm, cat, dog, mountain cedar, mugwort sage, olive tree, perennial rye) and controls of saline/50% glycerol and histamine were performed on the volar forearm to determine atopic status. Sensitivity was be defined as a >2 x 2 mm skin wheal response.

Exposure Chamber:

The exposure sessions were conducted in a custom-built steel and glass exposure chamber (Nor-Lake Inc., Model No. W00327-3R), which is 2.5 m x 2.5 m x 2.4 m in size, and has an average airflow rate of 300 ft^3 min. The chamber air supply is sourced from ambient air, which is filtered by passing through purifying (Purafil Model No. 6239), and high efficiency particle (Aeropac Model No.53 HEPA 95) filters. The filtered air is dehumidified by passing through a drier (Cargocaire Engineering Corp.). HC-575), and the air temperature is decreased with a chilled-water coil. Subsequently, temperature and humidity are increased with steam (Nortec Model No. NHMC-050), to obtain the pre-set temperature (20 ^{O}C) and relative humidity (50%) conditions in the chamber. The temperature and relative humidity inside the chamber are monitored (LabView) and controlled throughout the exposures (Johnson Controls, Model No. DSC 8500).

Particle Generation and Measurement:

The carbon and ammonium nitrate particles were generated using a solution of 2% carbon and 2% ammonium nitrate and series of five nebulizers (McGraw Respiratory Therapy), using compressed medical grade air. The outlet from the nebulizers went directly into the inlet duct of the exposure chamber.

The total particle concentration was measured at the subjects breathing zone using a filter (Pallflex; 0.22 µm), sampling at 14 l/min. The filter mass was determined pre- and post-sampling (Micro-systems). Particle concentration samples were collected for the complete 30 min of each exposure (Table 4. Table 5.).

Ozone Generation and Measurement:

The O_3 was produced using compressed O_2 (balance argon) and a corona-discharge O_3 generator (Model T 408; Polymetrics, San Jose, CA). The O_3 concentration was measured at the subjects breathing zone using an ultraviolet-light

photometer (Model 1008 PC; Dasibi). The O_3 concentration was maintained at 0.2 ppm by adjusting the voltage of the generator.

Exposures:

All exposures were of 4 hr duration.

Exercise and Pulmonary Ventilation:

During each exposure, exercise was utilized to induce mouth breathing and to increase minute ventilation. The exercise consisted of 4 x 30 min periods of either walking/running on a treadmill (Cybex, Model No. T400) or pedaling a cycle-ergometer (Monark, Model No. 90818e). The exercise intensity was adjusted for each subject to a target expired minute ventilation of 25 l/min/m^2 body surface area. During exercise, expired ventilation was calculated from tidal volume and breathing frequency measured using a pneumotachograph (Fleisch, Model No. 3) at the 10- and 20-min interval of each 30 min exercise period. Following each 30 min exercise period, subjects were seated at rest for 4 x 30 min periods.

Bronchoscopy:

Due to the subjects in both experiments having asthma, 30 min before the bronchoscopy each subject underwent a standard inhaled nebulized albuterol procedure to minimize the chance of bronchoconstriction during the bronchoscopy.

The bronchoscopies were conducted in a dedicated room at San Francisco General Hospital. Vital signs were measured pre- and post-bronchoscopy. Throughout the procedure, intravenous access was maintained, and arterial hemoglobin:oxygen percent saturation, the electrocardiograph, and blood pressure were monitored. Atropine, to decrease airway secretions, and if required, midazolam, to maintain subject comfort, was administered intravenously. The posterior pharynx was anesthetized using a 1% lidocaine spray, and 4% lidocaine-soaked cotton-tipped plegets applied to the mucosa over the ninth cranial nerve. Supplemental oxygen was delivered via a nasal cannula at 2 l/min. The bronchoscope (Pentax, Model No. FB 18x), tipped with lidocaine jelly, was introduced through the mouth, and the larynx and airways were anesthetized using 1% lidocaine solution as required. The bronchoscope was directed and wedged into the right middle lobe orifice (2 x 50 ml lavage), and subsequently into the lingula (1 x 50 ml lavage). The lavages were conducted using 0.9% saline heated to 37^{O}C. The first 15 ml of lavage fluid returned was designated as the bronchial fraction (Bfx). The lavage fluids were immediately centrifuged at 200 g for 15 minutes (Girofuge model No. 1805), and the supernatant separated and recentrifuged at 1800 g for 15 minutes to remove any cellular debris. The supernatant was then frozen and stored at –80 ^{O}C for biochemical analysis.

Following the lavages, five epithelial brushing were conducted by passing a specialized cytology brush (Mill-Rose Laoroatories, Inc., Model No. 149; working diameter: 1.6 mm), against the airway wall. This technique collects samples that are >95 % epithelial cells. Following brushing, 6-8 endobronchial biopsies were obtained from multiple sites of the airway bifurcations within the right middle lobe and carina using spiked forceps (Pentax Precision Instrument Corporation). The bronchoscopy was conducted 18 h post-exposure.

Cell Counts:

Total cells were counted in un-spun aliquots of Bfx and BAL using a hemacytometer (Fisher Scientific, Cat. No. 0267110). Differential cell counts were conducted on slides prepared using a cytocentrifuge at 200 g for 5 min (Shandon Southern Products Ltd., Model No. Cytospin 2) and stained in Diff-Quik (Diff-Quik, Baxter, Cat. No. B4132-1). All differential leukocytes cell counts were expressed as a percentage of total leukocytes (macrophages, lymphocytes, neutrophils, eosinophils), and differential epithelial cell counts were expressed as a percentage of total leucocytes + epithelial cells. Two readers each performed all cell counts in duplicate.

Protein Assays:

In both the Bfx and BAL supernatant, protein levels were determined for total protein (Pierce BCA), IL-6 and IL-8 (ELISA; R&D Systems), and CRP (Diagnostics Automation Inc.).

Gene Expression:

Total RNA will be extracted from both the Bfx and BAL cell pellets, and the epithelial cell pellet (Agilent), and used for determining the gene expression for IL-6, IL-8, IL-10, HIN-1, TFF-3 using quantative Real-Time RT-PCR (Applied Biosystems, TaqMan). The RNA purity and quality was checked for all samples (Agilent RNA 6000 Nano assay). This procedure allows isolation of 20-30 μg of total RNA per sample.

For RT RT-PCR 100 ng of total RNA was used as a template for single-strand complimentary DNA (cDNA) synthesis using gene-specific reverse transcription (RT) primers and Reverse Transcriptase (Superscript II ™). The cDNA was pre-amplified by PCR using Advantage Klentaq DNA polymerase and a mixture of gene-specific primers (TM primers) that are located internal to the RT primers (2 minutes at 94 °C, followed by 15-25 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 70 °C for 45 seconds). Samples were processed in parallel without RT to evaluate DNA content and assess RNA quality.

Equal volumes of pre-amplified cDNA were mixed with forward and reverse Taqman® primers (TM primers) along with a gene-specific fluorescence-labeled Taqman® probe. Samples were analyzed using 40 cycles of PCR with real-time fluorescence measurement using an Applied Biosystems 2700 Sequence Detector. The mean number of cycles to threshold (C_T) of fluorescence detection was calculated for each sample and the results normalized to the mean C_T of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for each sample tested. The results were expressed as a fold-increase (or decrease) in cDNA abundance as calculated by the following formula: Fold increase (or decrease) = 2 exp (C_T target – C_T GAPDH), where C_T equals the mean of triplicate measurements. The greater the fold-increase, the greater the expression of the specific gene.

Heart Rate Variability:

The HRV data were collected immediately pre-exposure, and during the final 25-min of each of the exposure conditions. Five standardized electrode sites that allow for recording of two channels, as well as a ground lead, were identified on the subject's chest wall. The sites were, if required, carefully shaved to remove any chest hair that may interfere with electrode adhesion and vigorously cleaned with the skin preparation pads. The Holter monitor (Forest Medical: Trillium Model 3000), leads were attached in the proper locations, according to the manufacturers instructions. If there was any difficulty in placing the electrodes or any variation from the standard positions due to bony irregularities, skin irritation, or other aspects of the subject's anatomy, a descriptive note was made.

Once the Holter monitor leads were connected, the subject's seated blood pressure was measured. The subject then performed the following maneuvers during the 25-minute Holter monitoring session (6).

1) Five minutes of rest while supine (respiratory rate and three supine blood pressures will be measured).
2) Five minutes of standing (standing blood pressure will be measured three times after allowing for two minutes of equilibration).
3) Five minutes of indoor exercise (marching in place at a pace comfortable to the participant).
4) Five minutes of recovery while supine (respiratory rate again recorded).
5) Twenty ten-second respiratory cycles (five second inhalation followed by five second exhalation).

The Holter monitor flash card was removed and the data downloaded from the flash card to a desktop computer. Files were named according to study subject ID number, then saved onto the computer's hard drive in the ".mcd" format. The files were scanned and edited for mislabeled beats. The number of episodes, if present, of ventricular tachycardia, ventricular fibrillation and episodes of bradyarrhythmia/asystole was calculated for each monitoring session.

The HRV analysis was performed using the manufactures computer software (Forest Medical: Trillium 3000 Holter monitor). The two principle types of HRV parameters, time-domain parameters and frequency-domain parameters, were analyzed. In the HRV analysis, only sinus R-R intervals were used. Artifacts, ectopy (both supraventricular and ventricular), and uninterpretable complexes were not considered for analysis. Also, intervals whose duration is <80% or >120% of that of the running R-R average were excluded to eliminate intervals related to premature supraventricular complexes and ventricular arrests (defined as R-R intervals >2 seconds). Prolonged intervals after a short interval were excluded as partial compensatory pauses.

Time-Domain Analysis: In time-domain analysis, one of the principal metrics employed is the standard deviation of the sinus R-R intervals (termed the heart period) over time. This method will use the following variables:

1) The SDANN gives the standard deviation of the means of heart periods derived from successive 5-minute blocks. The R-R intervals of normal sinus rhythm beats are averaged within each 5-minute block, then the standard deviation of the 5-minute averages is derived. The SDANN is relatively resistant to beat misclassification, which can occur with artifact or ectopy. It is also the HRV variable best able to demonstrate the circadian variations in HRV.

2) The SD gives the mean of the standard deviations of heart periods derived from successive 5-minute blocks. It is, in essence, the reverse of the SDANN. Here, the standard deviations of the R-R intervals from each 5-minute block are averaged. Therefore, rather than being sensitive to heart period variations over longer period of time (like circadian rhythms) the SD is sensitive to heart period variations over short (5 minute) periods of time.

3) The root mean square successive differences (r-MSSD) takes the square root of the mean of the summed squared differences between successive normal R-R intervals. This measure looks at beat-to-beat variability rather than 5-minute variability (SD) or longer-term variability (SDANN). For this reason, it is particularly sensitive to the misclassification of beats. Because the vagus nerve is responsible for very short-term variations in heart period, the r-MSSD reflects vagal tone.

4) The RR50 totals the number of times during the monitoring period that the difference between two adjacent normal R-R intervals exceeds 50 milliseconds. This is the time-domain variable most sensitive to beat misclassification.

5) The %RR50 (or pNN50) is the percent of the total number of successive normal R-R intervals during the recording period that differ by more than 50 milliseconds. It is essentially the RR50 normalized by the total number of R-R intervals and expressed as a percentage.

Frequency-Domain Analysis: This HRV analysis approach, also referred to as power spectral analysis, separates the heart rate signal into its frequency components, then quantifies those components in terms of their relative intensity, or power. By separating high-frequency components from low-frequency components, power spectrum analysis delineates parasympathetic and sympathetic effects. The high-frequency components (0.15-0.40 Hz) related to respiratory sinus arrhythmia represent parasympathetic neural activity. Low-frequency components (0.04-0.15 Hz) represent mostly sympathetic neural activity, though may reflect some parasympathetic input as well.

Airway Grading:

During each bronchoscopy the visual airway inflammation in the airways was graded by the bronchoscopist on a scale of: 0 = Normal, 1 = Mildly inflamed, 2 = Moderately inflamed, 3 = Severely inflamed.

Symptoms:

Subject self-graded symptoms of; anxiety, chest discomfort or chest tightness, chest pain on deep inspiration, cough, eye irritation, headache, nasal irritation, nausea, phlegm or sputum production, shortness of breath, throat irritation, wheezing; were graded on a scale of; 0 = None, 1 = Minimal (symptom is barely noticeable), 2 = Mild (symptom is present but not annoying), 3 = Moderate (symptom is somewhat annoying), 4 = Severe (symptom is very annoying and/or limits performance. Symptoms were graded immediately pre- and post-exposure.

Statistical Analysis:

The sample size of 15 subjects for each experiment was calculated to achieve >95% power, at alpha = 0.05 to detect a 15% absolute (approx 100% relative) increase in neutrophils in BAL following zinc oxide fume exposure (Kuschner et al., 1997).

The data for the majority of dependent variables in this project were not normally distributed, therefore, non-parametric methods were utilized. For the paired comparisons with-in each of the three experiments, both with-in and between, the exposure conditions, the Wilcoxon Signed-Rank test was used. For the unpaired comparisons across the three experiments the Mann-Whitney test was used. For all analyses differences were assigned as statistically significant at an alpha of <0.05.

Heart Rate Variability:

The analytical strategy first involved generating descriptive statistics for all variables in the data-set to examine variable distributions and to check for outliers. Several of the expected outcomes from the time-domain analysis, including SDNN, and RR50 were utilized. These outcomes were log-normally distributed among the population (5,6). For the most part, the time-domain measures of HRV will be analyzed as continuous variables. However, we also explored the data using logistic regression procedures after dividing certain dependent variables into binary outcomes. The outcomes from the frequency-domain analysis, such as total power, low or high frequency power, or the LF/HF ratio were treated as continuous variables and analyzed in a manner similar to the time-domain outcomes. Our analysis provided adequate control of the major potential confounders that occur both on a daily basis and consistently over the study period. However, we attempted to minimize other potential confounders and effect modifiers by restrictive eligibility criteria and by the use of controlled exposure. Relevant Pearson correlation coefficients (r) among all the predictor variables were calculated and measures of HRV were plotted against exposure variables.

Table 1. Experiment One: Individual subjects physical, spirometric pulmonary function, airway responsiveness, allergen, and medication characteristics

Subj.	Gender	Age (yr)	Height (cm)	Mass (kg)	FVC (l)	FEV_1 (l)	FEV_1/FVC (%)	NSAR PC_{20} (mg ml)	Allergen	Medication
1	M	45	183	82	5.07	3.95	78	10	Birch	AH
2	F	26	157	59	3.77	3.49	93	10	Mite	AH
3	M	32	175	73	4.56	3.53	77	1	Dog	SB
4	F	21	168	64	3.90	3.03	80	1	Olive	SB
5	M	44	173	68	3.88	2.21	57	0.25	Cedar	SB
6	M	34	170	80	4.09	3.29	80	1	Mite	SB
7	F	41	168	61	3.27	2.64	81	8	Cat	IS
8	F	29	160	88	3.19	2.38	75	2	Mite	SB, LB
9	F	36	165	132	2.52	2.04	81	2	Rye	SB, LB, IS
10	M	54	165	77	3.87	2.52	65	0.25	Olive	LA, NS, AH Nil
11	F	41	163	80	2.21	1.83	83	0.25	Olive	SB, LB, IS, NS, AH
12	F	51	165	88	2.27	1.82	80	0.25	Dog	Nil
13	F	41	168	127	3.18	2.66	84	4	Mite	SB, NS
14	F	32	185	75	3.40	2.73	80	1	Olive	SB
15	M	43	175	68	4.92	3.90	79	1	Elm	SB, AH, DE
Mean	(F=9)	38.0	169.3	81.5	3.61	2.80	78.2	2.9		

± SD	(M=6)	9.1	7.8	21.5	0.87	0.71	8.2	3.6

Abbreviations: FVC = forced vital capacity; FEV_1 = forced expired volume in 1 s; NSAR PC_{20} = non-specific airway reactivity, methacholine provocative concentration at whichFEV_1 decreased 20%, maximum dose = 10 mg ml; Allergen = allergen to which the subject had the largest response at skin test; SB: short term bronchodilator; LB: long term bronchodilator, IS: inhaled steroid; LA: leukotriene antagonist; NS: nasal steroid; AH: antihistamines; DE: decongestant/expectorant.

Table 1. Experiment One: Individual subjects physical, spirometric pulmonary function, airway responsiveness, allergen, and medication characteristics

Subj.	Gender	Age (yr)	Height (cm)	Mass (kg)	FVC (l)	FEV_1 (l)	FEV_1/FVC (%)	NSAR PC_{20} (mg ml)	Allergen	Medication
1	M	45	183	82	5.07	3.95	78	10	Birch	AH
2	F	21	168	64	3.90	3.03	80	1	Olive	SB
3	M	44	173	68	3.88	2.21	57	0.25	Cedar	SB
4	M	34	170	80	4.09	3.29	80	1	Mite	SB
5	F	29	160	88	3.19	2.38	75	2	Mite	SB, LB
6	F	36	165	132	2.52	2.04	81	2	Rye	SB, LB, IS LA, NS, AH
7	M	54	165	77	3.87	2.52	65	0.25	Olive	Nil
8	F	41	163	80	2.21	1.83	83	0.25	Olive	SB, LB, IS, NS, AH
9	F	51	165	88	2.27	1.82	80	0.25	Dog	Nil
10	F	41	168	127	3.18	2.66	84	4	Mite	SB, NS
Mean ± SD	(F=6) (M=4)	39.6 10.0	168.0 6.4	88.6 22.9	3.42 0.91	2.57 0.68	76.3 8.6	2.2 3.2		

Abbreviations: FVC = forced vital capacity; FEV_1 = forced expired volume in 1 s; NSAR PC_{20} = non-specific airway reactivity, methacholine provocative concentration at whichFEV_1 decreased 20%, maximum dose = 10 mg ml; Allergen = allergen to which the subject had the largest response at skin test; SB: short term bronchodilator; LB: long term bronchodilator, IS: inhaled steroid; LA: leukotriene antagonist; NS: nasal steroid; AH: antihistamines; DE: decongestant/expectorant.

Table 4. Experiment One: Exposure Particle Concentrations

	Exposure Condition		
	FA	P	PO
[Particle] (?g/m^3)	--	283	237
±	--	44	49

Values are mean ± SD. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles; PO = P + ozone [0.2 ppm].

Table 5. Experiment Two: Exposure Particle Concentrations

	Exposure Condition				
	FA	P	P-3 Exp.-1	Exp.-2	Exp.-3
[Particle] (?g/m^3)	--	288	271	274	255
±	--	44	42	67	52

Values are mean ± SD. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles; P-3 = P x 3 serial-day exposures; Exp.-1 = Exposure-1; Exp.-2 = Exposure-2; Exp.-3 = Exposure-3.