Taylor & Francis
Taylor & Francis Group

Inhalation Toxicology

International Forum for Respiratory Research

ISSN: 0895-8378 (Print) 1091-7691 (Online) Journal homepage: http://www.tandfonline.com/loi/iiht20

Ovalbumin-Induced Airway Inflammation and Fibrosis in Mice Also Exposed to Ultrafine Particles

Jerold A. Last, Rachel Ward, Lisa Temple, Kent E. Pinkerton & Nicholas J. Kenyon

To cite this article: Jerold A. Last, Rachel Ward, Lisa Temple, Kent E. Pinkerton & Nicholas J. Kenyon (2004) Ovalbumin-Induced Airway Inflammation and Fibrosis in Mice Also Exposed to Ultrafine Particles, Inhalation Toxicology, 16:2, 93-102, DOI: 10.1080/08958370490265077

To link to this article: https://doi.org/10.1080/08958370490265077

Published online: 19 Oct 2008.

Inhalation Toxicology, 16:93–102, 2004

ISSN: 0895-8378 print / 1091-7691 online
DOI: 10.1080/08958370490265077

Taylor & Francis
healthsciences

Ovalbumin-Induced Airway Inflammation and Fibrosis in Mice Also Exposed to Ultrafine Particles

Jerold A. Last, Rachel Ward, Lisa Temple, Kent E. Pinkerton, Nicholas J. Kenyon

Pulmonary and Critical Care Medicine, School of Medicine, and Center for Health and the Environment, University of California, Davis, California

A murine model of allergen-induced airway inflammation was used to examine the effects of exposure to ultrafine particles ($PM_{2.5}$) on airway inflammation and remodeling. Lung inflammation was measured by quantitative differential evaluation of lung lavage cells. Alterations in lung structure (airway remodeling and fibrosis) were evaluated by quantitative biochemical analysis of microdissected airways and by histological evaluation of stained lung sections. The same total number of cells was observed in lavage fluid from animals exposed for 4 wk to ovalbumin alone or to ovalbumin for 4 wk immediately before or after 6 exposures over a period of 2 wk to 235 ug/m^3 of $PM_{2.5}$. Mice exposed to ovalbumin for 6 wk with concurrent exposure to $PM_{2.5}$ during wk 5–6 had a significant decrease in the total number of cells recovered by lavage as compared with the group exposed to ovalbumin alone. There were no significant differences in the cell differential counts in the lavage fluid from mice exposed to ovalbumin alone as compared with values from mice exposed to ovalbumin and $PM_{2.5}$ under the protocols studied. Airway structural changes (remodeling) were examined by three different quantitative methods. None of the groups exposed to ovalbumin and PM had a significant increase in airway collagen content evaluated biochemically (i.e., total airway collagen) as compared to the matched groups of mice exposed to ovalbumin alone. Airway collagen content evaluated histologically by sirius red staining showed significant increases in all of the animals exposed to ovalbumin, with or without PM, and no apparent difference between the ovalbumin group and mice exposed to PM with ovalbumin. The findings were consistent with an additive, or less than additive, response of mice to exposure to PM and ovalbumin. Air or PM exposure alone for 2 wk did not result in observable goblet cells in the airways, while mice exposed to ovalbumin aerosol alone for 4 wk had about 20–25% goblet cells in their conducting airways. Sequential exposure to ovalbumin and PM (or vice versa) caused significant increases in goblet cells (to about 35% of total cells) in the conducting airways of the exposed mice. We conclude that when mice with allergen-induced airway inflammation induced by ovalbumin are also exposed to $PM_{2.5}$, the lung inflammatory response and airway remodeling may be modified, but that this altered response is dependent upon the sequence of exposure and the duration of exposure to ovalbumin aerosol. At the concentrations of PM tested, we did not see changes in airway fibrosis or airway reactivity for animals exposed to ovalbumin and $PM_{2.5}$ as compared with animals exposed only to ovalbumin aerosol. However, goblet-cell hyperplasia was significantly increased in mice exposed concurrently to ovalbumin and $PM_{2.5}$ as compared with mice exposed to ovalbumin alone.

Received 12 March 2003; sent for revision 22 April 2003; accepted 29 September 2003.

This work was supported in part by grants from the Philip Morris Research Management Group, from the U.S. EPA STAR Program (R829215), and from NIEHS (ES05707). We thank Dale Uyeminami for performing the PM exposures and Christine Huang for technical assistance with the histological and cytological analyses.

Address correspondence to Jerold Last, Toxic Substances Program, 1131 Surge I, University of California, Davis, CA 95616-8723, USA. E-mail: jalast@ucdavis.edu

There have been many epidemiological studies showing statistically significant associations between $PM_{2.5}$ concentration and severity of asthma, as measured by such surrogates as emergency room visits for children (Norris et al., 1999, 2000; Lipsett et al., 1997). Mice exposed to both ovalbumin and diesel exhaust showed enhanced recruitment of eosinophils into the lung, presumably as a consequence of there being increased concentrations of interleukin-5 (IL-5) in their lungs (Takano et al., 1998; Miyabara et al., 1998). Intratracheal instillation of residual oil

fly ash (ROFA) particles increased the number of eosinophils in airways of mice exposed to ovalbumin aerosols, and also amounts of IL-5 and other cytokines in lavage fluid (Gavett et al., 1999). Inhalation of an aerosol prepared from a leachate of ROFA, presumably enriched for metal ions from the fly ash, increased the susceptibility of very young mice to sensitization by inhaled ovalbumin aerosol (Hamada et al., 2000). This finding may reflect the capacity of transition metals to catalyze reactions in the lung that give rise to oxidant stress. Bonner et al. (2000) demonstrated that intratracheal instillation of vanadium pentoxide particles in rats caused airway remodeling and fibrosis.

Exposure to PM containing transition metals such as Fe^{2+} causes oxidant stress in the lung, which may directly or indirectly contribute to the release of mediators and cytokines in the conducting airways. In addition, exposure to PM containing transition metals may cause the normally very tight junctions of the airway epithelium to become more open, resulting in edema and movement of proteins across the epithelium (Hu et al., 1982; Guth et al., 1986). Thus, presentation of an antigen such as ovalbumin to the airways during or immediately after PM exposure might enhance the dose of ovalbumin to the lung at a given exposure concentration. If this putative mechanism is correct, then the temporal sequence of exposure to PM and ovalbumin (PM before, during, or after ovalbumin exposure) could give very different results, even if total exposures to each of these agents were the same. Thus, we designed a set of experiments to test whether the sequence of exposure of mice to the same total amounts of ovalbumin aerosol and PM affected the observed response of the animals.

We have previously observed that there is a significant increase in collagen content, which can be localized to the airway compartment, in the lungs of previously sensitized BALB/*c* mice exposed for 4 wk to ovalbumin aerosol (Kenyon et al., 2003a). We have also observed a characteristic temporal pattern of inflammatory-cell recruitment to the lung, as measured by differential cell counts of lavage fluid, and increased airway reactivity to methacholine challenge in mice exposed to ovalbumin aerosol (Kenyon et al., 2003a). Thus, for the present study, mice were exposed to ovalbumin for 4 wk either before or after 6 exposures to PM over a period of 2 wk, or to ovalbumin aerosol for 6 wk with concurrent exposure to PM during wk 1–6. We analyzed (1) lung inflammation, as measured by quantitative evaluation of cells recovered by lung lavage, (2) alterations in lung structure (airway remodeling), as measured by quantitative biochemical analysis of microdissected airways or a semiquantitative histological staining score for collagen (Kenyon et al., 2003b), and for goblet cell content, and (3) lung function, measured as Penh values by whole-body plethysmography after bronchoprovocation testing with methacholine aerosols.

EXPERIMENTAL METHODS

Animals

Specific pathogen-free BALB/*c* mice aged about 6 wk (16–20 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA). Animals were housed until used in filtered Bio-Clean facilities in the Animal Resources Center at our facility on a 12-h light, 12-h dark cycle. The mice were provided standard feed (Purina rat chow) and water ad libitum for several days prior to any experimental procedures to acclimatize to their new surroundings. All procedures were performed under an IUCAC-approved protocol.

Ovalbumin Aerosol Exposures

Procedures for sensitization and exposure of mice to ovalbumin aerosol have been described in detail elsewhere (Kenyon et al., 2003a). Briefly, mice were systemically sensitized to ovalbumin by 2 intraperitoneal (ip) injections, administered on days 1 and 15 of an experiment, of 10 μg/0.1 ml of chicken egg albumin (ovalbumin, grade V, 98% pure, Sigma, St. Louis, MO). Alum was used as an adjuvant. Exposures to ovalbumin aerosol, 10 ml of a 10 mg/ml (1%) solution, were begun on day 28 after the first ip injection of ovalbumin. Mice were exposed for up to 60 min 3 times per week for the duration of a given experiment.

PM Exposures

Mice were exposed to PM in a special facility at the Center for Health and the Environment on the UC Davis campus. A diffusion flame system is used to generate an aerosol of soot and iron oxide (Yang et al., 2001; Zhou et al., 2003). The primary fuel is ethylene. Iron is introduced by passing ethylene over liquid iron pentacarbonyl. The aerosol emission from the flame is diluted by secondary air to a level that can be used in animal exposure studies. The system is designed to operate at a constant soot production rate, while the iron loading can be varied from 0 to 50 $\mu g/m^{-3}$ in the diluted postflame gases. The impact of the iron on soot production is counteracted by the addition of acetylene to the fuel. The flame system is capable of consistently producing steady concentrations of soot and iron for delivery to animals, without the confounding presence of toxic gaseous compounds. Although the amount of soot is maintained at a fixed mass loading, the size distribution of the aerosol is affected by iron addition. When iron is added, the concentration of larger particles is diminished and a greater portion of the aerosol resides in the very small size range around 80–110 nm. The iron appears as iron oxide crystals, often spatially segregated from the soot (quasi-graphitic carbon) itself. Note that while we call the material $PM_{2.5}$ for consistency with environmental usage, the ultrafine particles we are studying are actually more appropriately designated as $PM_{0.1}$. The maximum total aerosol mass (particle) concentration we can generate in the exposure chambers is about

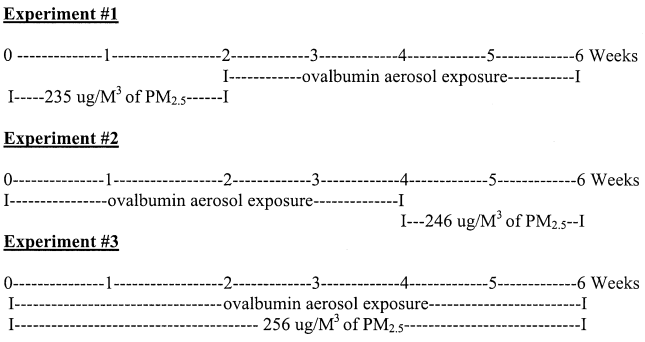

FIG. 1. Exposure protocols used in experiments 1–3 presented in this article. Actual measured concentrations (mean ± SD) of $PM_{2.5}$ were 235 ± 14 (experiment 1), 246 ± 24 (experiment 2), and 256 ± 67 (experiment 3).

0.26 ± 0.01 mg/m^3. About 200 μg/m^3 of this is soot, and the remainder is iron oxide. Particle number is monitored in real time with a 488-nm laser system. CO concentrations in the exposure chambers are <0.8 ppm, and NO_x levels are <0.4 ppm. Polycyclic aromatic hydrocarbon concentrations are beneath the detection limit of gas chromatography/mass spectrometry (GC/MS) analysis. The elemental to organic carbon ratio (EC/OC) of the soot in the experiments reported herein (mean ± SEM) was 0.53 ± 0.10, $n = 5$.

Mice were exposed for 4 h/day, 3 days/wk, to 250 μg/m^3 (nominal concentration) of PM. Three independent experiments with separate durations of exposure and analyses were performed, as illustrated in Figure 1 (actual measured concentrations of PM in each exposure are given in the figure). A 2-wk PM exposure preceded 4 wk of ovalbumin aerosol exposure in the first experiment (experiment 1), and followed the ovalbumin exposure in the second experiment (experiment 2). PM exposures were timed so as to maintain the initiation of ovalbumin aerosol exposure on day 28 after the initiation of systemic sensitization. In the third experiment (experiment 3), ovalbumin aerosol was administered for 6 wk with simultaneous exposure to PM (6 wk). Control animals were treated identically as PM exposed and/or ovalbumin exposed, except the exposure chambers contained only filtered air.

Lung Isolation and Fixation

Mice were killed with an overdose of pentobarbital/dilantin 1 h after cessation of the last aerosol exposure, their tracheas were cannulated, and their lungs were lavaged twice with 1-ml portions of phosphate-buffered saline, pH 7.6 (PBS). The pooled lavage fluid was centrifuged in a bench-top unit at 1200 rpm for 10 min and the resulting pellet was again suspended in PBS; 100 μl of this suspension was processed using a cytocentrifuge, and was then stained with Diff-Quik (International Reagent Corp, Kobe, Japan), to determine differential cell counts. Lungs were fixed at 30 cm pressure with 1% paraformaldehyde for at least 24 h, then either embedded in paraffin for subsequent histological evaluation or stored in 70% ethanol for dissection and biochemical analysis of airways.

Tissue Staining and Evaluation of Fibrosis

After fixation, the left lung was placed in 70% ethanol and prepared for paraffin embedding in a standard fashion. Lung sections of 5 μm thickness were made with special attention to cutting through the larger lobar bronchi in parallel. Sections were baked at 37°C overnight prior to staining. Lung sections were treated with xylene and alcohol to remove paraffin and rehydrate, then stained with Alcian blue–periodic acid–Schiff (PAS) and counterstained with hematoxylin, or were stained with 0.1% Picro-sirius red stain F3BA (Pfaltz and Bauer, CT)–0.5% picric acid for 30 min. Light microscopy was used for analysis of the slides. A grading system of 0–4 was established to evaluate fibrosis (Kenyon et al., 2003b), and the airways were scored under 200× power. The grading scale used was: (0, no peribronchial sirius red stain; 1, evident sirius red stain, but not consistently increased in all airways; 2, slight, consistent increase in sirius red depth of stain; 3, increased, uniform stain depth throughout the airways; 4, dramatically increased depth of stain in all airways).

Preparation of Airway Homogenates for Assay

Lobar bronchi and daughter airways were prepared and isolated under a dissecting microscope. Lungs were blunt dissected using two sets of forceps to obtain a preparation of the larger airways separated from adhering parenchyma and attached to the remaining stub of trachea after cannulation. The tracheal stub was discarded prior to assay. The dissected airways were stored at 4°C in 70% ethanol until assayed (fixed lungs). For hydroxyproline and total protein assays, half of the airway preparation (from the right lung) was used. The airway was homogenized in water with a Branson sonifier 250 (VWR Scientific, San Francisco, CA, duty cycle 40, output control 4) until the tissue was completely homogenized.

Protein Assay

The Micro BCA protein assay reagent kit (Pierce, Rockford, IL) was used, following the manufacturer's instructions. Standard curves using bovine serum albumin were linear between 0 and 20 ug.

Hydroxyproline Assay

The colorimetric method of Woessner (1961) was used. Aliquots of 50 and 100 μl of the neutralized hydrolysate (6 *N* HCl, 24 h, 121°C) were routinely assayed, but smaller and larger aliquots were used when necessary to adjust samples to add a constant amount of protein to each vial for hydrolysis. Standard curves were linear between 0 and 32 μg hydroxyproline.

Physiological Studies

Because we wanted to observe the evolution of any lesions we might observe in a cohort of animals over time, we were constrained to do noninvasive physiological studies. Recently, the measurement of a calculated parameter, the so-called "enhanced pause" (Penh), has become a widely used measurement for airway reactivity that may be used in conjunction with methacholine aerosol exposure to do bronchoprovocation testing in unrestrained small animals (Hamelmann et al., 1997; Lin, 2001). Increased values of Penh are considered to be a valid surrogate measurement for airway hyperreactivity, although the mechanistic basis for changes in Penh values is controversial (Hantos & Brusasco, 2002; Lennart et al., 2002). Details of these methods as we perform them using a commercially available apparatus (Buxco, Troy, NY) are described elsewhere (Kenyon et al., 2003a). It should be stressed that all Penh measurements reported in this article (and in all of our work) reflect comparisons between different experimental groups where Penh is measured under identical conditions in the Buxco apparatus. Chamber gas conditions are identical for all measurements. Thus, whether we are actually measuring airway resistance, functional residual capacity, tidal volume, or alterations in ventilation pattern or timing, we are quantifying alterations in lung function between the groups.

Statistical Analysis of Data

Instat 2.0 (GraphPad Software, San Diego, CA) was used for data analysis. For nonparametric analysis of data, analysis of variance (ANOVA) with appropriate correction (Tukey's multiple comparison test) for multiple comparisons and, where appropriate, unequal standard deviations between groups were used.

RESULTS

We performed experiments with three different protocols, (1) PM preceding or (2) PM following exposure to ovalbumin aerosol, or (3) PM simultaneously with ovalbumin aerosol.

Experiment 1, PM Exposure for 2 wk Followed by Ovalbumin Aerosol for 4 wk

The normal lung lavage from a healthy mouse contains more than 90% alveolar macrophages (Hamada et al., 2000), and our observations were consistent with this finding. Control mice exposed only to filtered air contained 319,500 ± 74,250 total cells per lavage sample, of which 92 ± 2% were pulmonary alveolar macrophages. Similar cell numbers were found in the group exposed to PM alone for 2 wk, 309,000 ± 76,800. After a period of 4 wk (12 exposures to ovalbumin aerosol), total cell number recovered by lavage from these BALB/*c* mice was 237,200 ± 31,700 (Figure 2A). Of these cells, 38 ± 1% were lymphocytes, 52 ± 2% were macrophages, 6.2 ± 2% were neutrophils, and 4 ± 2% were eosinophils (Table 1). There was no significant difference between the total cell number or in the cell differential counts in the lavage fluid from mice exposed to ovalbumin alone and those exposed to PM followed by ovalbumin, 220,500 ± 43,000.

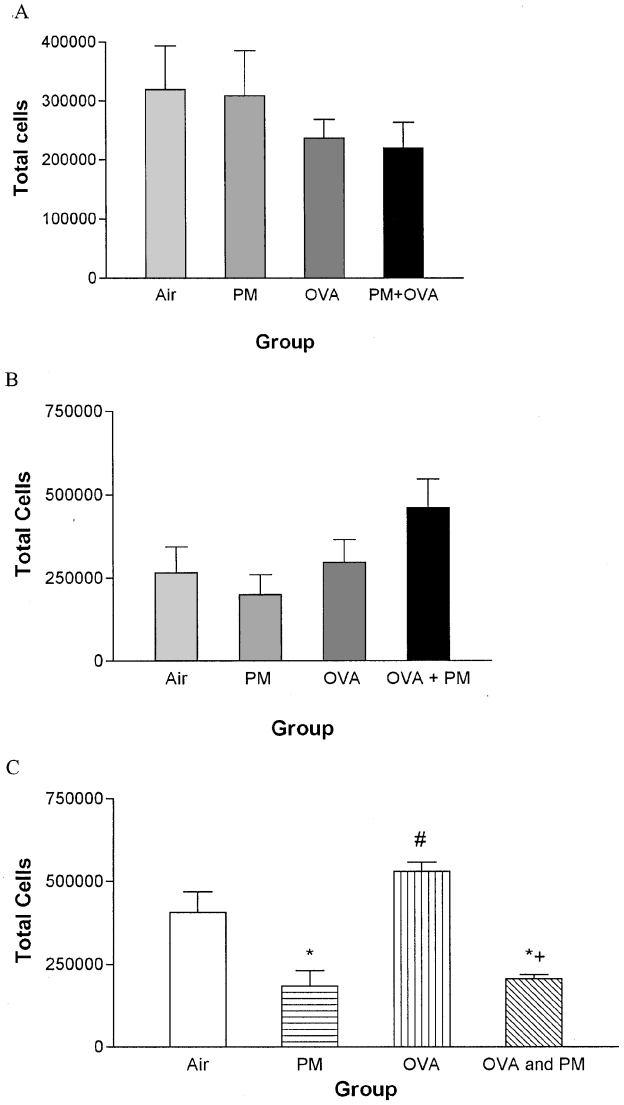

FIG. 2. Total cells recovered by lung lavage from mice exposed to ovalbumin aerosols with and without additional exposure to $PM_{2.5}$. Data are mean values ± SEM; n, the number of mice examined in each group, varied between 4 and 8 in these experiments. In total, 5 fields (200× magnification) were evaluated from the cytocentrifuge slides from each animal, and converted to total cells in the lavage fluid by multiplication by a factor of 900. (A) $PM_{2.5}$ exposure for 2 wk followed by ovalbumin exposure for 4 wk. (B) Ovalbumin for 4 wk followed by $PM_{2.5}$ exposure for 2 wk. (C) Simultaneous exposure to ovalbumin and $PM_{2.5}$ for 6 wk. Asterisk indicates significantly different ($p < .05$) from the air group; #, significantly different ($p < .001$) from the PM-alone group; +, significantly different ($p < .001$) from the ovalbumin-alone group.

Collagen content of microdissected airways was analyzed from the exposed mice and from their age-matched control

TABLE 1
Differential cell content in lavage of mice exposed to ovalbumin ± $PM_{2.5}$

Exposure group	Macrophages	Lymphocytes
Expt. 1: Air	92 ± 2	—
PM	94 ± 1	—
Ovalbumin	52 ± 2	38 ± 1
PM + Ovalbumin	59 ± 2	36 ± 2
Expt. 2: Air	91 ± 3	—
PM	94 ± 1	—
Ovalbumin	57 ± 3	33 ± 4
Ovalbumin + PM	54 ± 4	36 ± 4
Expt. 3: Air	96 ± 1	—
PM	98 ± 0.8	—
Ovalbumin	37 ± 3	48 ± 5
Ovalbumin + PM	50 ± 2	46 ± 2

Note. Differential cell count in lung lavage from mice exposed to ovalbumin ± PM. Data are presented as mean value (percent of total cells counted in five fields) ± SEM.

mice exposed only to filtered air. Figure 3A illustrates the airway collagen content in mice exposed to ovalbumin aerosol, ±235 $\mu g/m^3$ of PM, and their appropriate controls. Control animals exposed only to filtered air had 21.2±0.4 μg hydroxyproline ($n = 4$) in their airways. The animals exposed to PM alone had 22.1 ± 1.0 μg of airway collagen. There was a significant increase, to 27.4 ± 0.7 μg hydroxyproline, in the collagen content of the airways from the mice exposed to ovalbumin. There was no significant difference between the airway collagen content of the mice exposed to the sequence of PM followed by ovalbumin, 25.3 ± 1.8 as compared with the mice exposed to ovalbumin alone.

We also evaluated airway collagen content by histology, using sirius red staining to visualize collagen and a semiquantitative scoring system for comparison of the different experimental groups. As shown in Figure 4A, there was a significantly greater amount of collagen as graded histologically in the airways of the mice exposed to ovalbumin, or to PM followed by ovalbumin, as compared to the air controls. Exposure to PM alone did not significantly alter the collagen staining of the airways.

We examined the overall process of airway remodeling by examining whether there were goblet cells apparent in the airway epithelium of mice in the test groups in this experiment. There were no apparent goblet cells (positive for staining with Alcian blue–periodic acid–Schiff stain) in either the group exposed to air or the animals exposed to PM alone (Table 2). However, about 23% of the total epithelial cells in the large bronchi of the mice exposed to ovalbumin and 35% in the mice exposed to PM followed by ovalbumin stained as goblet cells, indicating extensive remodeling of the airway epithelium had occurred in this experiment. The combined exposure group had

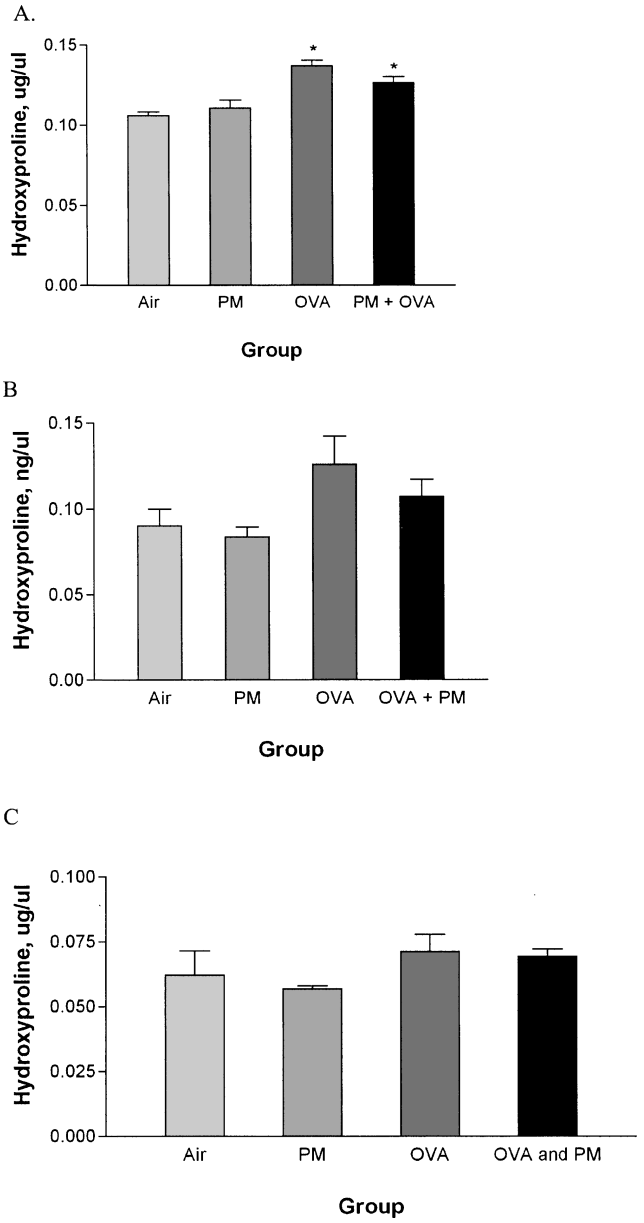

FIG. 3. Airway collagen content from mice exposed to ovalbumin aerosol three times per week for the indicated number of weeks, with or without additional exposure to $PM_{2.5}$. Aliquots of microdissected airways were hydrolyzed and analyzed by a colorimetric method for their content of 4-hydroxyproline. Data are presented as μg 4-hydroxyproline/μl of sample of right lung ± SEM for lungs from 4–6 mice per group. The numerical values plotted may be converted to total μg 4-hydroxyproline found per right lung by multiplication by a factor of 200. (A) $PM_{2.5}$ exposure for 2 wk, followed by ovalbumin exposure for 4 wk. (B) Ovalbumin for 4 wk, followed by $PM_{2.5}$ exposure for 2 wk. (C) Simultaneous exposure to ovalbumin and $PM_{2.5}$ for 6 wk. Asterisk indicates significantly different ($p < .01$) from the air group and also significantly different ($p < .05$) from the PM-alone group.

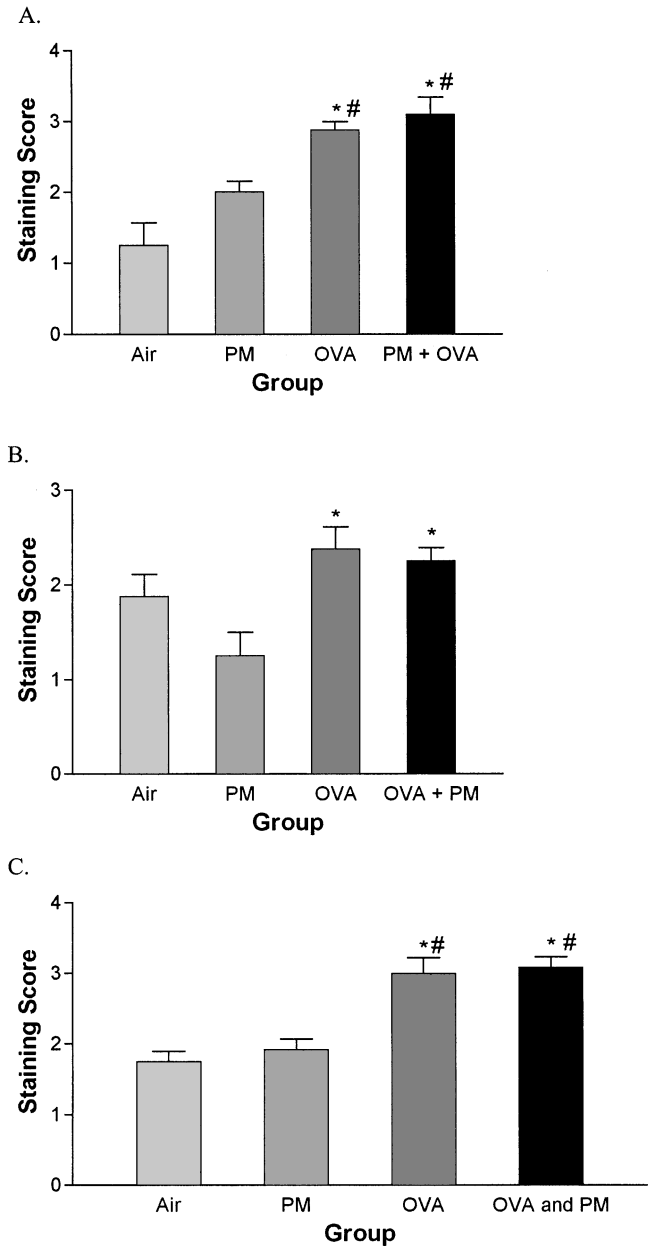

FIG. 4. Airway collagen content, as evaluated by sirius red staining of tissue sections, for the same animals as described in Figure 3. (A) $PM_{2.5}$ exposure for 2 wk, followed by ovalbumin exposure for 4 wk. (B) Ovalbumin for 4 wk, followed by $PM_{2.5}$ exposure for 2 wk. (C) Simultaneous exposure to ovalbumin and $PM_{2.5}$ for 6 wk. Asterisk indicates significantly different ($p < .001$) from the air group; #, significantly different ($p < .001$) from the PM-alone group.

significantly more goblet cells than did the group exposed to ovalbumin alone.

We measured airway reactivity after methacholine challenge in these animals, immediately prior to their sacrifice. As shown in Figure 5A, there were no significant differences in Penh

TABLE 2
Percent goblet cells in conducting airways of mice exposed to ovalbumin ± $PM_{2.5}$

Exposure group	Goblet cells in airways, percent
Expt. 1: Air	0 ± 0
PM × 2 wk	0 ± 0
Ovalbumin × 4 wk	23.0 ± 1.9
PM, then ovalbumin	34.7 ± 2.5[a]
Expt. 2: Air	0 ± 0
PM × 2 wk	0 ± 0
Ovalbumin × 4 wk	22.5 ± 0.7
Ovalbumin, then PM	34.1 ± 3.6[a]
Expt. 3: Air	0 ± 0
PM × 6 wk	0 ± 0
Ovalbumin × 6 wk	51.2 ± 3.0
Ovalbumin with PM × 6 wk	60.8 ± 3.6[b]

Note. Goblet cell count in conducting airways from mice exposed to ovalbumin ± ozone. Data are presented as mean value ± SEM with the total number of weeks exposed to ovalbumin (3 exposures per week) or PM (3 exposures per week) indicated. The percent of goblet cells is the actual number of goblet cells counted in one representative daughter airway with a length of at least 100 basal epithelial cells, visualized at 200× magnification, divided by the total cell number counted (goblet cells + basal epithelial cells). All values for ovalbumin and for ovalbumin + PM are significantly different ($p < .001$) from groups exposed to either air or PM alone.

[a]Significant difference for ovalbumin compared with ovalbumin + PM, $p < .001$.

[b]Significant difference for ovalbumin compared with ovalbumin + PM, $p < .05$.

values between the mice in any of the experimental groups, although there is an apparent trend toward higher values in the group exposed to PM then ovalbumin.

Experiment 2, Ovalbumin Aerosol for 4 wk Followed by PM Exposure for 2 wk

In our second set of experiments, we reversed the order of exposure so that the mice received 4 wk of exposure to ovalbumin prior to exposure to PM for 2 wk. Because of this difference in experimental design, the group exposed to ovalbumin alone had a recovery period of 2 wk breathing filtered air prior to their being killed and analyzed. In this experiment (Figure 2B) we found comparable values to the first experiment, 266,600 ± 77,700 cells per lavage sample, in the control animals exposed only to filtered air, 201,200 ± 59,800 in animals exposed to PM alone, and 297,000 ± 68,300 in the group exposed to ovalbumin alone, a value that was not significantly different from the controls. In the latter group, 33 ± 4% were lymphocytes, 57 ± 3% were macrophages, 4 ± 1% were neutrophils, and 5 ± 3% were eosinophils (Table 1). The apparent difference between the total cell number in the lavage fluid from mice exposed to ovalbumin and those exposed to the combination of ovalbumin

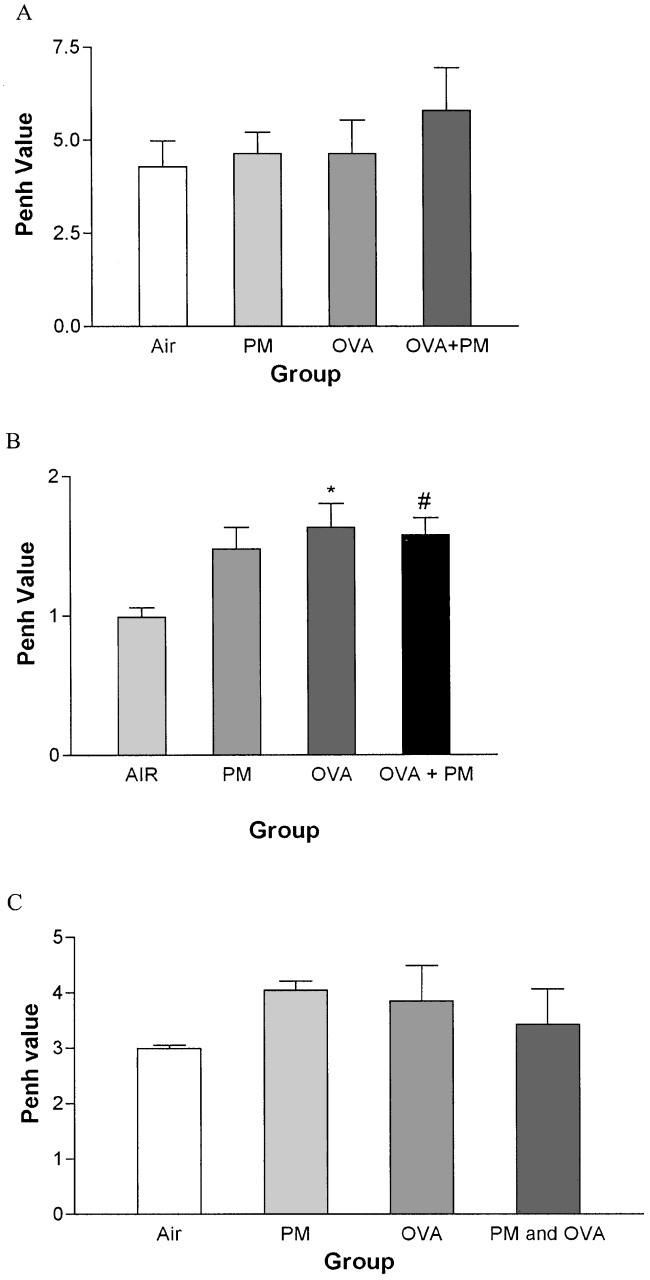

FIG. 5. Airway responsiveness after methacholine challenge, as evaluated by the calculated parameter Penh, for the mice studied in Figures 2–4. All data are from animals sequentially challenged with 1, 2, 5, and 10 mg/ml of methacholine. Data are presented as mean values ± SEM for the response to 5 mg/ml of methacholine; *n*, the number of mice examined in each group, varied between 4 and 8 in these experiments. (A) $PM_{2.5}$ exposure for 2 wk, followed by ovalbumin exposure for 4 wk. (B) Ovalbumin for 4 wk, followed by $PM_{2.5}$ exposure for 2 wk. (C) Simultaneous exposure to ovalbumin and $PM_{2.5}$ for 6 wk. Asterisk indicates significantly different from air, $p < .01$; #, significantly different from air, $p < .05$.

and PM, 461,000 ± 86,000, was not significant. Nor were the cell differentials significantly different between mice exposed to ovalbumin or to the sequence of ovalbumin followed by PM. For the combined exposure group we found (values for PM alone are given in parentheses) the following: 36 ± 4% lymphocytes (5 ± 3%), 54 ± 4% macrophages (94 ± 1), 4 ± 1% (1 ± 1%) neutrophils, and 6 ± 1% eosinophils (0%).

In this experiment there was no significant increase in the airway collagen content in the mice exposed to ovalbumin as compared with those exposed only to air, even though a trend toward this result was apparent (Figure 3B). There were no significant differences in airway collagen content between mice exposed to ovalbumin alone, to PM alone, to air alone, or to ovalbumin combined with PM. However, significant differences were observed in airway collagen content evaluated histologically between the mice exposed to air and the groups exposed to ovalbumin alone or to ovalbumin followed by PM (Figure 4B). It should be noted that in this experiment (cf. Figure 1), there was a 2-wk interval between cessation of exposure to ovalbumin aerosol and sacrifice of the mice for analysis.

There were no apparent goblet cells (positive for staining with Alcian blue–periodic acid–Schiff stain) in either the group exposed to air or the animals exposed to PM alone (Table 2). However, about 22% of the total epithelial cells in the large bronchi of the mice exposed to ovalbumin and 34% in the mice exposed to PM followed by ovalbumin stained as goblet cells, again indicating extensive remodeling of the airway epithelium had occurred in this experiment. The combined exposure group had significantly more goblet cells than did the group exposed to ovalbumin alone.

As shown in Figure 5B, there were significant differences in Penh values between the mice in the group exposed to filtered air and the groups exposed to ovalbumin alone or to ovalbumin followed by PM. A trend toward increased Penh values in the group exposed to PM alone is also apparent.

Experiment 3, Ovalbumin Aerosol for 6 wk Concurrently With PM Exposure

In our third set of experiments, mice were exposed concurrently to PM and ovalbumin. In this experiment (Figure 2C) we found 406,400 ± 62,500 cells per lavage sample in the control animals exposed to air, and 530,800 ± 27,000 in the group exposed for 6 wk to ovalbumin alone, a value that was not significantly different than the controls. Of the total lavageable cells in the animals exposed to ovalbumin, 48 ± 5% were lymphocytes and 37 ± 3% were macrophages (Table 1); the remainder were mainly eosinophils. There were significantly more total cells in the lavage fluid from mice exposed to ovalbumin than those exposed to the combination of ovalbumin and PM ($p = .001$). The cell differentials were similar between mice exposed to ovalbumin or to the combination of ovalbumin and PM. For the combined exposure to ovalbumin and PM for 6 wk, we found 46 ± 2% were lymphocytes and 50 ± 2% were macrophages.

With simultaneous exposure to ovalbumin and PM, there was a significantly higher percentage of lymphocytes (and a significantly lower percentage of macrophages) in the lavage fluid than in the animals exposed to PM before or after ovalbumin (Table 1). Mice exposed to PM alone for 6 wk had 98 ± 0.8% macrophages in their lavage fluid, similar to the filtered air controls (96 ± 1%), and significantly fewer ($p < .05$) total cells (185,200 ± 46,500) in their lavage.

Consistent with our results in the previous two experiments, we found no goblet cells in airways from control animals that had breathed only room air, nor did we see goblet cells in the airways from mice exposed to PM alone. Mice exposed to ovalbumin alone 6 wk had about 51% goblet cells (Table 2), while the animals exposed to ovalbumin + PM simultaneously had a significantly higher percentage of goblet cells, 61%.

Figure 3C illustrates the collagen content, determined by chemical analysis, of isolated airway tissue from mice exposed to PM and ovalbumin simultaneously. There was no significant difference in the amount of collagen in the airways of the animals exposed to ovalbumin as compared to the sensitized control animals breathing filtered air, despite the apparent trend illustrated in the figure. Nor was there a significant difference in airway collagen content between any of the other groups in the study. Noteworthy is the lack of any apparent difference between airway collagen content in mice exposed to ovalbumin aerosol with and without accompanying particulate matter. However, when we evaluated collagen content on sirius red-stained sections, as shown in Figure 4C, we found significantly more collagen than in either the mice exposed to air or the mice exposed only to PM in both the group exposed to ovalbumin and the group exposed to ovalbumin and PM. There was no significant difference in response between the latter two groups.

There were no significant differences between Penh values between any of the experimental groups tested in this experiment (Figure 5C).

DISCUSSION

We analyzed (1) cells recovered by lung lavage as an index of lung inflammation, (2) collagen content of microdissected airways as an index of airway fibrosis, and (3) pulmonary function as airway reactivity (Penh) after bronchoprovocation challenge in unrestrained animals. We hypothesized that various temporal combinations of PM and ovalbumin might give additive or antagonistic responses, depending upon the assay chosen. We further hypothesized that such a duality of response might be due to the potential for the inflammatory response to either be antagonized or not affected by a combination of existing and de novo allergen-induced airway inflammation and whatever effects exposure to $PM_{2.5}$ might itself elicit on macrophages and other cells resident in the lungs of exposed animals.

We observed the same total number of cells in lavage fluid from animals exposed to ovalbumin alone or to the combinations of ovalbumin and PM regardless of whether the mice were exposed to PM before, during, or after the ovalbumin. Exposure to PM alone was associated with high percentages of macrophages in the lavage fluid, comparable to values seen in animals exposed only to filtered air.

In experiment 1, where animals were sacrificed immediately after the cessation of 4 wk of ovalbumin exposure, a high relative percentage (38%) of lymphocytes was seen in the ovalbumin-alone exposure group, while 36% lymphocytes were observed in the combined exposure group. Airway collagen content was similar in both groups. In experiment 2, where animals were sacrificed 2 wk after the cessation of their 4 wk of ovalbumin exposure, a similar (33%) percentage of lymphocytes was seen in the ovalbumin-alone exposure group, with about 36% lymphocytes in the combined exposure groups. Airway collagen content was similar in the ovalbumin-alone groups and in all of the combined exposure groups.

In experiment 3, where animals were sacrificed immediately after the cessation of 6 wk of ovalbumin exposure, a significantly higher percentage of lymphocytes was observed in both exposure groups (48% in ovalbumin alone, 46% in the combined exposure group). This finding may reflect the different protocol in experiment 3, where mice were exposed to ovalbumin for a total of 6 wk rather than the 4 wk of ovalbumin exposure used in the first two experiments. Airway collagen content was similar in both the group exposed to ovalbumin and the group exposed to ovalbumin and PM, whether evaluated biochemically or histologically. There was no obvious correlation across all three experiments between the percentage of lymphocytes found in lung lavage fluid and airway collagen content.

In our previous studies, we reported that BALB/*c* mice demonstrate significant increases in accumulation of airway collagen after 4 wk of exposure to ovalbumin aerosol under conditions identical to those used in the current study (Kenyon et al., 2003a; Last et al., 2003). We therefore measured the collagen content of microdissected airways from mice exposed to ovalbumin aerosol, with and without PM. Consistent with our earlier observations, mice exposed to ovalbumin for 4 wk exhibited a significant increase in their airway collagen content (Figure 3A) or a trend toward increased collagen content (Figures 3B and 3C). There was no significant effect of 2 wk of exposure to PM alone, before or after the exposure to ovalbumin aerosol, on airway collagen content. We also examined airway collagen content in mice exposed simultaneously to PM and ovalbumin aerosol. In these experiments, the apparent increase in airway collagen content in mice exposed to ovalbumin aerosol was not significantly greater than in those exposed to air (Figure 3C). None of the combinations showed a significant increase in airway collagen content with combined exposure as compared to ovalbumin exposure alone. When we quantified airway collagen content by sirius red staining, we found significant increases in all of the groups exposed to ovalbumin, either alone or with PM exposure before, during or after

ovalbumin exposure. Again, there were no significant differences between the groups exposed to ovalbumin alone or to ovalbumin with PM. These results are consistent with an additive (or less than additive) response to combined simultaneous exposure to ovalbumin and PM. Thus, by the quantitative (or semiquantitative) evaluation of airway fibrosis, an important marker of lung structure, we conclude that exposure of BALB/*c* mice to 235–256 $\mu g/m^3$ of $PM_{2.5}$ before, during, or after exposure to ovalbumin aerosol causes additive effects (or less than additive effects) when airway collagen content is measured.

We examined one other aspect of airway remodeling in the lungs of the mice in these experiments, goblet-cell hyperplasia in the airway epithelium (Table 2). Mice exposed only to filtered air or to PM alone had no detectable goblet cells. We consistently found (all three experiments) increased goblet cells in mice exposed to ovalbumin aerosol and significantly more goblet cells in the matched mice exposed to ovalbumin and PM. These results demonstrate increased goblet-cell hyperplasia in mice exposed to ovalbumin and PM as compared to ovalbumin alone. We would suggest that exposure to PM before, during, or after exposure to ovalbumin might be affecting the inflammatory cell populations present in the airways during the exposure, and therefore might affect the final epithelial cell populations due to altered cytokine signaling in the lungs.

Quantification of functional change, as measured by airway reactivity to methacholine challenge, gave different results for the different exposure protocols studied. The only significant changes observed in Penh values were in experiment 2, when ovalbumin exposure was followed by exposure to PM. In that case, response to ovalbumin followed by PM gave results consistent with an additive response as compared to response to ovalbumin alone. It is noteworthy that at the histological level we were able to appreciate significant changes in epithelial goblet-cell content and airway submucosal collagen content, the latter a change that we did not observe when we evaluated whole airway preparations removed from the lungs by microdissection. This speaks to the focal nature of the changes we are observing, which perhaps accounts for the apparent lack of functional change in the animals in two of the three experiments we performed.

Over the last several years there has been a great deal of interest in the possible role of environmental factors in the increased prevalence of asthma and other allergic conditions reportedly occurring worldwide, at least in the developed countries. Particulate air pollution, especially smaller respirable fractions like $PM_{2.5}$, can influence allergic reactions during both the sensitization and provocation phases. Insoluble PM can act as an adjuvant during sensitization, and can contribute to oxidative stress and to inflammation in the respiratory tract during provocation (Granum & Lovik, 2002). For example, Gavett et al. (1999) have demonstrated increased airway hyperresponsiveness and increased cytokine levels and numbers of eosinophils in lung lavage fluid from ovalbumin-sensitized mice intratracheally treated with fly ash. Epidemiological studies have shown an association between ambient $PM_{2.5}$ levels and emergency room visits for children with asthma (Norris et al., 1999; Granum & Lovik, 2002). Inhalation of diesel exhaust increases eosinophil recruitment and airway hyperresponsiveness in ovalbumin-sensitized mice (Takano et al., 1998), as well as airway remodeling (increased epithelial goblet cells) and specific immunoglobulin E (IgE) in the serum (Miyabara et al., 1998) and increased IL-5 in the lung (Ichinose et al., 1998). Ambient PM can itself cause airway inflammation and hyperresponsiveness in naive mice (Walters et al., 2001). Our studies seemingly demonstrate, at least with the form of $PM_{2.5}$ and exposure conditions we studied, that PM exposure does not directly exacerbate the development of antigen-induced airway inflammation in a well-studied model in the mouse. It should be emphasized that the $PM_{2.5}$ used in this study consisted of a combination of soot and iron particles, and that insofar as having metal ions that can cause oxidative stress in a $PM_{2.5}$ preparation makes it relevant to atmospheric $PM_{2.5}$, between ultrafine particles should be biologically active. Our experiments clearly do not give a definitive answer to the question of whether exposure to environmental $PM_{2.5}$, a complex mixture of components, might give different results. Our results do, however, underscore the issue of whether all $PM_{2.5}$ are equivalent for regulatory purposes, independent of their chemical composition and physical state, as is implied by the setting of ambient air quality standards on this basis. If indeed all PM is the same in terms of health effects on the lung, then the results of our experiments might indeed have validity for extrapolation to complex mixtures.

In summary, we had several goals in the present study. One such goal was to answer the question as to whether exposure of mice with preexisting or developing allergen-induced airway inflammation, an animal model for asthma, to $PM_{2.5}$ caused responses that were additive or were more or less than additive. Based upon the epidemiological literature suggesting exacerbation of asthma in human populations exposed to $PM_{2.5}$, we anticipated finding potentiation of the lung response to ovalbumin in the mice also inhaling $PM_{2.5}$. Perhaps not surprisingly, our results in the present study indicate that the answer to this question depends on the assay chosen. Based upon examination of airway remodeling as percentage of goblet cells present, we would say that response to ovalbumin with PM is greater than the sum of response to ovalbumin alone and to PM alone. This observation may reflect the process of ongoing airway repair rather than structural or functional airway damage, as do the other reported assays. Based upon measurements of airway inflammatory cells (Table 1 and Figure 2), airway reactivity (Figure 5), or airway collagen content (Figures 3 and 4), response to ovalbumin with PM is the same or less than the response to ovalbumin alone; that is, responses are additive or less than additive. Further studies are necessary to determine whether these findings accurately reflect the results that would be found for exposure to complex mixtures of

$PM_{2.5}$ under controlled conditions in other animal models of asthma.

REFERENCES

Bonner, J. C., Rice, A. B., Moomaw, C. R., and Morgan, D. L. 2000. Airway fibrosis in rats induced by vanadium pentoxide. *Am. J. Physiol. Lung Cell Mol. Physiol.* 278:L209–L216.

Gavett, S. H., Madison, S. L., Stevens, M. A., and Costa, D. L. 1999. Residual oil fly ash amplifies allergic cytokines, airway responsiveness, and inflammation in mice. *Am. J. Respir. Crit. Care Med.* 160:1897–1904.

Granum, B., and Lovik, M. 2002. The effect of particles on allergic immune responses. *Toxicol. Sci.* 65:7–17.

Guth, D. J., Warren, D. L., and Last, J. A. 1986. Comparative sensitivity measurements of lung damage made by bronchoalveolar lavage after short-term exposure of rats to ozone. *Toxicology* 40:131–143.

Hamada, K., Goldsmith, C., Goldman, A., and Kobzik, L. 2000. Resistance of very young mice to inhaled allergen sensitization is overcome by coexposure to an air pollutant aerosol. *Am. J. Respir. Crit. Care Med.* 161:1285–1293.

Hamelmann, E., Schwarze, J., Takeda, K., Oshiba, A. Larsen, G. L., and Irvin, C. G. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156:766–775.

Hantos, Z., and Brusasco, V. 2002. Assessment of respiratory mechanics in small animals: The simpler the better? *J. Appl. Physiol.* 93:1196–1197.

Hu, P. C., Miller, F. J., Daniels, M. J., Hatch, G. E., Graham, J. A., Gardner, D. E., and Selgrade, M. K. 1982. Protein accumulation in lung lavage fluid following ozone exposure. *Environ. Res.* 29:377–388.

Ichinose, T., Takano, H., Miyabara, Y., and Sagai, M. 1998. Long-term exposure to diesel exhaust enhances antigen-induced eosinophilic inflammation and epithelial damage in the murine airway. *Toxicol. Sci.* 44:70–79.

Kenyon, N. J., Ward, R. W., and Last, J. A. 2003a. Airway fibrosis in a mouse model of airway inflammation. *Toxicol. Appl. Pharmacol.* 186:90–100.

Kenyon, N. J., Ward, R. W., McGrew, G., and Last, J. A. 2003b. TGF-B1 causes airway fibrosis and increased collagen I and III mRNA in mice. *Thorax* 58:772–777.

Last, J. A., Ward, R., Temple, L., and Kenyon, N. J. 2003. Ovalbumin-induced airway inflammation and fibrosis in mice also exposed to ozone. *Inhal. Toxicol.*

Lennart, K., Lundblad, A., Irvin C. G., Adler, A., and Bates, J. H. T. 2002. A reevaluation of the validity of unrestrained plethysmography in mice. *J. Appl. Physiol.* 93:1198-1207.

Lin, C.-C. 2001. Noninvasive method to measure airway obstruction in nonanesthetized allergen-sensitized and challenged mice. *Respiration* 68:178–185.

Lipsett, M., Hurley, S., and Ostro, B. 1997. Air pollution and emergency room visits for asthma in Santa Clara County, California. *Environ. Health Perspect.* 105:216–222.

Miyabara, Y., Takano, H., Ichinose, T., Lim, H.-B., and Sagai, M. 1998. Diesel exhaust enhances allergic airway inflammation and hyperresponsiveness in mice. *Am. J. Respir. Crit. Care Med.* 157:1138–1144.

Norris, G., YoungPong, S. N., Koenig, J. Q., Larson, T. V., Sheppard, L., and Stout, J. W. 1999). An association between fine particles and asthma emergency department visits for children in Seattle. *Environ. Health Perspect.* 107:489–493.

Norris, G., Larson, T., Koenig, J., Claiborn, C., Sheppard, L., and Finn, D. 2000. Asthma aggravation, combustion, and stagnant air. *Thorax* 55:466–470.

Takano, H., Ichinose, T., Miyabara, Y., Shibuya, T., Lim, H.-B., Yoshikawa, T., and Sagai, M. 1998. Inhalation of diesel exhaust enhances allergen-related eosinophil recruitment and airway hyperresponsiveness in mice. *Toxicol. Appl. Pharmacol.* 150:328–337.

Walters, D. M., Breysse, P. N., and Wills-Karp, M. 2001. Ambient urban Baltimore particulate-induced airway hyperresponsiveness and inflammation in mice. *Am. J. Respir. Crit. Care. Med.* 164:1438–1443.

Woessner, J. F., Jr. 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch. Biochem. Biophys.* 93:440–447.

Yang, G., Teague, S., Pinkerton, K., and Kennedy, I. M. 2001. Synthesis of an ultrafine iron and soot aerosol for the evaluation of particle toxicity. *Aerosol. Sci. Technol.* 35:759–766.

Zhou, Y.-M., Zhong, C.-Y., Kennedy, I. M., Leppert, V. J., and Pinkerton, K. 2003. Oxidative stress and NF-κB activation in the lungs of rats: A synergistic interaction between soot and iron particles. *Toxicol. Appl. Pharmacol.* 190:157–169.