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Low-Level Subchronic Exposure to Wood Smoke Exacerbates Inflammatory Responses in Allergic Rats

Yohannes Tesfaigzi,*[,1] Jacob D. McDonald,* Matthew D. Reed,* Shashibhushan P. Singh,* George T. De Sanctis,† Paul R. Eynott,† Fletcher F. Hahn,* Matthew J. Campen,* and Joe L. Mauderly*

**Lovelace Respiratory Research Institute, Albuquerque, New Mexico 87108, and †Sanofi/Aventis Pharmaceuticals, Inc., Bridgewater, New Jersey 08870*

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Epidemiological studies have implicated wood smoke as a risk factor for exacerbating asthma. However, comparisons of findings in animal models with those in humans are currently not possible, because detailed clinically relevant measurements of pulmonary function are not available in animal studies. Brown Norway rats were immunized with ovalbumin and exposed to either filtered air or wood smoke at 1 mg particulate matter/m^3 for 70 days and challenged with allergen during the last 4 days of exposure. Baseline values for dynamic lung compliance were lower while functional residual capacity was increased in rats exposed to wood smoke compared to rats exposed to filtered air. IFN-γ levels were reduced and IL-4 levels increased in the bronchoalveolar lavage fluid and blood plasma, inflammatory lesions in the lungs were 21% greater, and airway mucous cells/mm basal lamina were non-significantly increased in rats exposed to wood smoke compared to controls. Collectively, these studies suggest that the pulmonary function was affected in rats by exposure to wood smoke and this decline was associated with only minor increases in inflammation of the lung. Therefore, this animal model may be useful to elucidate the mechanisms of the decline in pulmonary function caused by environmental pollutants when asthmatics are exposed to allergen.

Key Words: **particulate matter; pulmonary function; exacerbation; mucous cell metaplasia; inflammation.**

Asthma has increased dramatically in prevalence worldwide, reaching epidemic proportions over the past two decades. Epidemiological studies have implicated both a decrease in childhood infections (Riedler *et al.*, 2001; Scrivener *et al.*, 2001) and an increase in environmental pollution as major risk factors in increasing the severity of existing asthma (Gent *et al.*, 2003; Slaughter *et al.*, 2003). Epidemiological studies that directly correlate the increased severity of allergic asthma to environmental factors use measurements based on pulmonary function tests (Nordenhall *et al.*, 2001). The role of pollutants in allergic inflammation, the development of antibodies (such as IgE), and the levels of T cells and their cytokines have been extensively studied in animal models, but few studies report measurements of pulmonary function parameters other than airway resistance. Therefore, analogous comparisons between studies on the role of environmental pollution in exacerbating the asthmatic response in animal models with those from human epidemiological studies have not been feasible to date.

Our previous study reported the effects of subchronic wood smoke exposure on the pulmonary function in naive Brown Norway rats (Tesfaigzi *et al.*, 2002). Exposure conditions in our previous study were designed to resemble conditions in Native American homes in the Jemez Pueblo in New Mexico where primarily pinion pine is used for heating and cooking. In that community, the incidence of childhood asthma is approximately twice the national average. The purpose of our previous study was to determine whether wood smoke alone at concentrations found in homes also affects pulmonary function in rats. Results indicated a small, exposure-related reduction in respiratory function consisting of reduced gas exchange at the alveolar membrane and insignificant reductions in lung size and the uniformity of gas distribution within the lung. Overall, the impact of wood smoke exposure at 1 and 10 mg/m^3 total particulates was minimal and would be considered of little clinical importance. The inflammatory response and histopathological changes, such as increase of mucous cell metaplasia, following 90 days of exposure to wood smoke were essentially insignificant in non-compromised Brown Norway rats. However, several studies have shown that fine particulate matter that is primarily composed of wood smoke, especially in the winter season, does affect the pulmonary function of asthmatic children (Larson and Koenig, 1994; Robin *et al.*, 1996). The purpose of the present study was, therefore, to determine whether exposure to wood smoke would exacerbate allergic airways inflammation and the decline in pulmonary function in sensitized rats when exposed to low allergen concentrations.

MATERIALS AND METHODS

Wood smoke generation and exposures. Wood smoke was generated from a conventional, uncertified wood stove manufactured by Pineridge that has a 0.5 m^3 firebox and a sliding gate air intake damper. The stove was placed in

[1] To whom correspondence should be addressed at Lovelace Respiratory Research Institute, 2425 Ridgecrest Drive, SE, Albuquerque, NM 87108. Fax: (505) 348-4980. E-mail: ytesfaig@lrri.org.

a ~400 m^2 single-room building adjacent to the exposure laboratory. The building was equipped with air conditioning to maintain room temperature between 18 and 32°C during the burn cycle. The stove was operated over a three-phase burn cycle spanning the 6-h exposure period. The fire was started (which initiated exposures) with unprinted/unbleached newspaper and split hardwood (a mix of black and white oak with a ~ 20% moisture content). The phases included kindling (~15–20 min), a high burn rate (4–6 kg of wood for ~90 min), and a low burn rate (4–6 kg of wood for the remainder of the exposure period) controlled by the setting of the air intake damper. For the high burn rate, the sliding damper was open (maximum air intake). During the low burn cycle, the damper was closed to an aperture of approximately 1/8 inches. The flue extended up to a height of ~5 m and terminated within an environmentally controlled enclosure that maintained constant airflow conditions. Smoke was extracted from the top of the flue; routed to the dilution, distribution, and exposure system; and diluted with ambient air that had been scrubbed by passage through high-efficiency particulate air (HEPA) and charcoal-impregnated pre-filters to remove particles and volatile organics.

Exposures were conducted by passing wood smoke or air through whole-body inhalation exposure chambers that were 2 m^3 in size (H2000, Hazleton Systems, Maywood, NJ) at approximately 500 l/min, yielding a material residence time within the chamber of approximately 4 min. The target exposure level was a 6-h average concentration of 1000 μg/m^3 of wood smoke particulate matter (PM). Because the system was on a three-phase burn cycle, the concentrations inside the exposure chambers were cyclical throughout the exposure period.

Exposure atmosphere characterization. The complete exposure atmosphere characterization will be reported elsewhere. In this article we report daily concentrations of PM mass, bulk PM composition (carbon, ion concentration), and several gases that make up the majority of the exposure atmosphere. Exposure atmospheres were monitored daily by sampling PM on 47-mm Pallflex filters (Pall-Gelman, Ann Arbor, MI). Pre- and post-filter weights were measured using a Mettler MT5 microbalance (Mettler, Columbus, OH). A static discharger was used prior to weighing to avoid any interference from electrical charge on the filters. Filter samples were collected hourly from the wood smoke exposure chamber, and one filter sample per day was collected from the air-control chamber. Wood smoke PM is reported as the total PM minus the PM measured in the control chamber. Although filtered, small amounts of PM (0–15 μg/m^3) were measured in the air-control chamber, mainly due to the presence of the rodents (dander, bedding, etc.). We assumed that the rodents' contribution to PM was the same in each exposure chamber; therefore, we subtracted the PM value of the control chambers from the exposure chamber to yield "wood smoke PM."

Carbon monoxide (CO) was determined using a Photoacoustic gas analyzer (Innova 1312, California Analytical Instruments, Irvine, CA). Total nitrogen oxides (NO and NO_2) were measured using a chemiluminescent analyzer (Model 200A NOx Analyzer, Pollution Instruments, San Diego, CA). Both NOx and CO analyzers were calibrated prior to each study using National Institute of Standards and Technology-traceable standards. Techniques for the collection and analysis of SO_2, NO_3^-/SO_4^{-2} and NH_4^+ have been described elsewhere (Chow *et al.*, 1998). Particle size was measured using a 10-stage micro-orifice-uniform deposit impactor (MOUDI, MSP Corp., Minneapolis, MN). The impactor was operated at a flow rate of 30 l per min, providing particle size data from 0.05–10 microns in aerodynamic diameter.

Animals and exposure conditions. Male Brown Norway rats (6 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and housed under specific pathogen-free conditions at the Lovelace Respiratory Research Institute vivarium. This study was approved by the Institutional Animal Care and Use Committee in adherence to the guidelines set forth by the Association for Assessment and Accreditation of Laboratory Animal Care International. Rats were randomly assigned by weight (14 rats in each group) to be exposed to wood smoke for 6 h per day, 7 days per week for 70 days at a concentration of 1 mg PM/m^3 or to filtered air. Following the first day of exposure to wood smoke, both groups of rats were ip injected with a mixture of 10 μg OVA in 2 mg aluminum hydroxide gel adjuvent alum) in a total volume of 0.5 ml of PBS and placed back into the exposure chambers. The immunization was boosted by injecting the rats with the same solution of OVA/Alum 7 days later.

At 67–70 days of exposure to wood smoke or filtered air, rats were exposed for 2 h per day to OVA aerosols at 2 mg/m^3 concentration for four consecutive days after the daily exposure to wood smoke or filtered air. During the study period, each rat was observed twice daily for any clinical signs of abnormality, morbidity, or death. All rats were weighed 1 week prior to the start of the exposure period and again on day 70 following the final exposure (time of necropsy).

Respiratory function tests. The respiratory function of 10 rats per treatment group was measured by plethysmography, as described previously (Harkema *et al.*, 1982; Tesfaigzi *et al.*, 2002). Briefly, rats were anesthetized with halothane and ventilated with a positive end-expiratory pressure (~1 cm H_2O), intubated with orotracheal and esophageal catheters, and placed prone in a heated volume-displacement plethysmograph. The breathing pattern (frequency, tidal volume, and minute volume) and dynamic lung mechanics (dynamic lung compliance and total pulmonary resistance) were measured during stabilized, spontaneous breathing. Single-breath respiratory maneuvers were conducted during brief hyperventilation-induced apnea tests using positive and negative airway pressures. The lung volume at 30 cm H_2O transpulmonary pressure was defined as total lung capacity. Quasistatic inflations and deflations were performed at 5 and 3 ml/s, respectively. Functional residual capacity was measured using a barometric technique. Parameters obtained from these single-breath tests included physiological subdivisions of lung volume, quasistatic pressure-volume curves and compliance, single-breath N_2 washout, and CO diffusing capacity. Forced exhalations from total lung capacity performed at an airway pressure of −50 cm H_2O yielded forced vital capacity and flow-volume curves. Airway reactivity was measured by determining changes in dynamic lung compliance and total pulmonary resistance during step-wise methacholine challenges. Methacholine (Sigma-Aldrich, St. Louis, MO) was dissolved in saline and nebulized using a Buxco nebulizer (Buxco Electronics, Inc., Wilmington, NC) at increasing solution concentrations (0, 6, 12, 25, 50, and 100 mg/ml). Each methacholine exposure lasted 1 min, with 5 min of recovery time between steps. Esophageal pressure was confirmed to return to baseline between each challenge step.

Collection of blood and BALF. Following pulmonary function tests, rats were euthanized by injection with a lethal dose of Euthasol (Phenytoin sodium) and bronchoalveolar lavage fluid (BALF) was obtained as previously described (Tesfaigzi *et al.*, 2002). Blood was collected by cardiac puncture into heparnized tubes, separated into cell and plasma fractions, placed in cryotubes, quick frozen in liquid nitrogen, and stored at −80°C until further analysis. The numbers of neutrophils, macrophages, lymphocytes, and eosinophils in the BALF were determined as described (Tesfaigzi *et al.*, 2000).

Detection of cytokines. GROα, IL-1β, and IL-6 were chosen as indicators general inflammation related to neutrophils. IFNγ and IL-4 with IL-10 were selected as indicators of the extent of Th1 or Th2 inflammation, respectively. A standard multiplex assay kit, 8-plex (with eight bead sets) (RCYTO-60K-PMX8, Linco Research, Inc., St. Charles, MO) was used with the Luminex Flowmetrix system (Luminex, Austin, TX) to determine the levels of cytokines in BALF and plasma. Individual bead sets in the 8-plex assay were coupled with monoclonal antibodies to either GRO-α, IL-1β, IL-4, IL-6, IL-10, IL-13, IFN-γ, and TNF-α. The beads were incubated first with diluted standards, BALF, or plasma overnight and then with a detector antibody cocktail for 60 min each at room temperature. After two washes in PBS supplemented with 0.02% Tween 20, 0.1% BSA, and 0.02% NaN_3, the beads were incubated for 30 min with fluorescent dye-conjugated streptavidin. Cytokine levels were measured using a flow cytometer and were analyzed with Flowmetrix software (Luminex). Standard curves for each cytokine were generated on a log-log plot for each assay, and the cytokine concentrations in each sample were calculated from the corresponding curve-fitting equations (Carson and Vignali, 1999). Cytokine levels were measured from standard curve constructed from serial dilutions of

the reference standard provided with the assay kit. The threshold of detection for IFN-γ was 0.7 pg/ml; IL-1β was 1.6 pg/ml; IL-4 was 0.3 pg/ml; IL-6 0.7 pg/ml; IL-10 was 10.3 pg/ml; IL-13 was 4.7 pg/ml; TNFα was 0.9 pg/ml; and GRO-α was 1.2 pg/ml.

Analysis of total and OVA-specific IgE. To determine the sensitization to OVA, total and OVA-specific IgE levels were measured in the serum samples of all rats. Total IgE in serum samples was measured using an enzyme linked immunosorbant assay kit (IgE BD, Pharmingen, San Diego, CA) according to the manufacturer's instructions. OVA-specific IgE was measured with enzyme-linked immunosorbant assay as described (Barrett *et al.*, 2002).

Lymphocyte proliferation assay. Lymphocyte proliferation assays were performed on lung associate lymph nodes (LALNs) and spleens from six rats in each exposure group as an indicator whether exposure to wood smoke not only affects the pulmonary but also the immune system in general. Briefly, immediately after removal of tissues, splenic and LALN cells were prepared, and their proliferative response to Con A, a T cell-specific mitogen, was analyzed as described elsewhere (Singh *et al.*, 2000). Briefly, 2×10^6 cells were cultured in 0.2 ml of complete medium in the presence of various concentrations of Con A in a microtiter plate. Cultures were incubated at 37°C in the presence of 5% CO_2, and cells were harvested after 3 days. Proliferation was assayed by pulsing the culture wells with 0.5 μCi of [^{3}H]Tdr (ICN, Irvine, CA) for 18 h before harvesting.

Histopathologic examinations. Sections (5-μm thick) of the trachea, one section from the larynx, four sections from the nose, and two sections from the left lung were prepared as previously described (Tesfaigzi *et al.*, 2002) and stained with hematoxylin and eosin combined with Alcian Blue (pH 2.5) or Periodic Acid-Schiff (AB/PAS) as described elsewhere (Spicer *et al.*, 1971). Lung lesions, including alveolar septal infiltrates, perivascular infiltrates, and peribronchiolar infiltrates were graded by a pathologist who was blind to exposure conditions. A scale of minimal, mild, and moderate was used as described previously (Barrett *et al.*, 2002). The sum of individual lesion scores was used to generate overall histopathology score for each group. Tissue sections from the lung (generation 5) were stained with AB/PAS and analyzed for mucous cell numbers and intraepithelial stored mucosubstances as described previously (Foster *et al.*, 2003).

Statistical analysis. Data are expressed as mean ± SEM. Differences among groups were examined by ANOVA and *t*-tests using Microsoft Excel software. Differences were considered significant at $p < 0.05$.

RESULTS

Composition of Exposure Atmospheres

Wood smoke contains CO, metals, and hundreds of organic compounds in the gas, semivolatile, and particle phases (McDonald *et al.*, 2000). As indicated previously, the detailed composition of the exposure atmospheres will be reported elsewhere. The vapor phase components of wood smoke in the exposure atmosphere were primarily CO and volatile hydrocarbons, with low to undetectable concentrations of NOx and SO_2 (Table 1). PM in the wood smoke exposure atmospheres had a mass median aerodynamic diameter (MMAD) of ~0.3 μm, and was composed primarily of organic carbon mass with ~5% elemental carbon, and 0.2% metals and associated analytes. Low background concentrations of vapor and PM were detected in the filtered air exposure chamber.

TABLE 1
Particle and Gas Composition in Hardwood Smoke Exposure Atmospheres

	Units	Air	Wood Smoke
Particle			
Particle mass	μg/m^3	6.4 ± 6.9	1041.1 ± 123.5
Wood smoke particle mass[a]	μg/m^3		1034.7
Elemental carbon	μg/m^3	0.2 ± 0.1	42.6 ± 2.7
Organic carbon mass	μg/m^3	2.8 ± 0.2	907.7 ± 29.5
Sum of elements	μg/m^3	0.04 ± 0.03	2.21 ± 0.18
Particle size			
Mass median aerodynamic diameter	μm ± SD	NQ	0.36 ± 2.1
Gases			
Nitrogen monoxide	ppm	0.0 ± 0.0	0.0 ± 0.0
Nitrogen dioxide	ppm	0.0 ± 0.0	0.0 ± 0.0
Sulfur dioxide	ppm	ND	ND
Carbon monoxide	ppm	0.2 ± 0.0	13.0 ± 1.6
Total vapor hydrocarbon	ppm	0.1 ± 0.4	3.1 ± 0.5

Note. GSD = Standard Deviation, ND = nondetectable, NQ = not quantified.
[a]Total particle mass − background particle mass measured in sham chamber.

Body Weight and Respiratory Function

No differences in clinical signs or body weight were observed among rats exposed to wood smoke compared to those exposed to filtered air (Table 2). Significant differences for only a few parameters of lung function were observed between rats exposed to wood smoke and controls (Table 2). In rats exposed to wood smoke, the dynamic lung compliance (C_{dyn}) before methacholine challenge was 45% lower compared to rats receiving only filtered air. A decreasing trend for C_{dyn} over the increasing concentrations of methacholine challenge was observed for both groups. However, the reduction in C_{dyn} following methacholine challenge was greater

TABLE 2
Respiratory Function Results

	Air	Wood smoke
Body weight (g)	298 ± 27	296 ± 16
DLCO (ml/min/mmHg)	0.24 ± 0.04	0.24 ± 0.03
DLCO/body weight (DLCO/kg)	0.82 ± 0.14	0.80 ± 0.11
Forced vital capacity (ml)	16.4 ± 2.2	16.5 ± 2.1
Functional residual capacity (ml)	4.8 ± 0.5	5.3 ± 0.3[a]
Vital capacity (ml)	16.6 ± 1.8	16.5 ± 2.5
Forced expiratory volume in 0.1 s	59 ± 11	54 ± 7
Total pulmonary resistance ($cmH_20/ml/s$)	0.08 ± 0.04	0.08 ± 0.02
Quasistatic lung compliance (ml/cmH_2O)	1.1 ± 0.3	1.1 ± 0.2
Dynamic lung compliance	0.64 ± 0.29	0.35 ± 0.05[a]

[a]Significantly different from air-exposed group.

in the air-exposed group. This may be explained by the already lower baseline values in the smoke-exposed group (Fig. 1). Thus, at the highest dose of methacholine, the C_{dyn} in controls was similar to the baseline value of the smoke-exposed group. No significant differences in total pulmonary resistance were observed between groups before or after methacholine challenge.

Functional residual capacity was increased 10% in rats exposed to wood smoke ($p = 0.02$). Forced expiratory parameters were not significantly altered. The forced vital capacity was slightly larger in smoke-exposed rats and the forced expired volume in 0.1 s ($FEV_{0.1}$) was slightly smaller, resulting in an insignificant 10% reduction of volume-normalized $FEV_{0.1}$. Similar values for the CO diffusing capacity in the two groups indicated that alveolar-capillary gas exchange was not significantly affected by the exposure.

BALF Cell Differentials and Cytokines

No difference was observed in the numbers of lymphocytes, eosinophils, and neutrophils recovered from BALF between the two groups. We observed an increase in the number of macrophages recovered from BALF of rats exposed to wood smoke compared to those exposed to filtered air. However, this did not reach statistical significance (Fig. 2 A).

From the cytokines measured in the BALF and plasma of rats exposed to air or wood smoke, major differences were observed in the levels of GRO-α, IFN-γ, IL-1β, IL-4, and IL-6. TNF-α, IL-13, and IL-10 were not detected or detected at very low levels in the samples in either group (Figs. 2B and 2C). In the control group, the levels of GRO-α in the BALF were approximately half of what was detected in the plasma. IFN-γ levels were similar in the BALF and plasma in both groups of rats and exposure to wood smoke reduced INF-γ levels in both samples. Approximately 130 pg/ml of IL-1β was detected in the BALF, but this cytokine was not detected in the plasma of air-exposed controls. Exposure to wood smoke significantly reduced IL-1β levels in the BALF and this cytokine was undetected in the plasma samples from both groups of rats. While IL-4 levels were increased significantly from non-detectable levels in the BALF of wood smoke-exposed rats, this cytokine remained undetected in the plasma of both rat groups. Similar levels of IL-6 were detected in the BALF and plasma of control rats and while a non-significant increase was found in the plasma, IL-6 decreased to undetectable levels in the BALF of wood smoked-exposed rats.

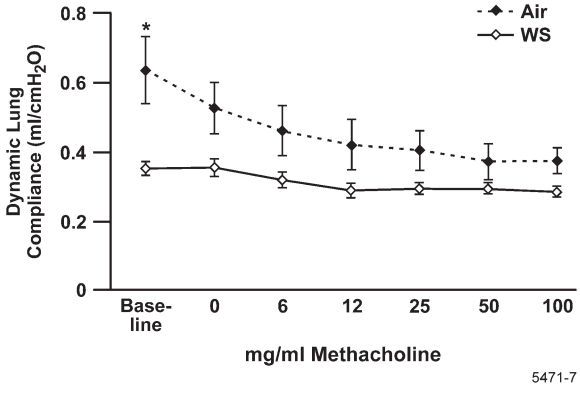

FIG. 1. Dynamic lung compliance over increasing concentrations of methacholine challenge in sensitized rats exposed to wood smoke or filtered air and challenged with allergen for the last four days of exposure. The baseline C_{dyn} was significantly lower in rats exposed to wood smoke. This difference between wood smoke and air-exposed rats diminished over increasing concentrations of methacholine challenge. Group mean ± standard error from the mean is shown in the figure. *Significant difference from wood smoke-exposed group ($p < 0.05$).

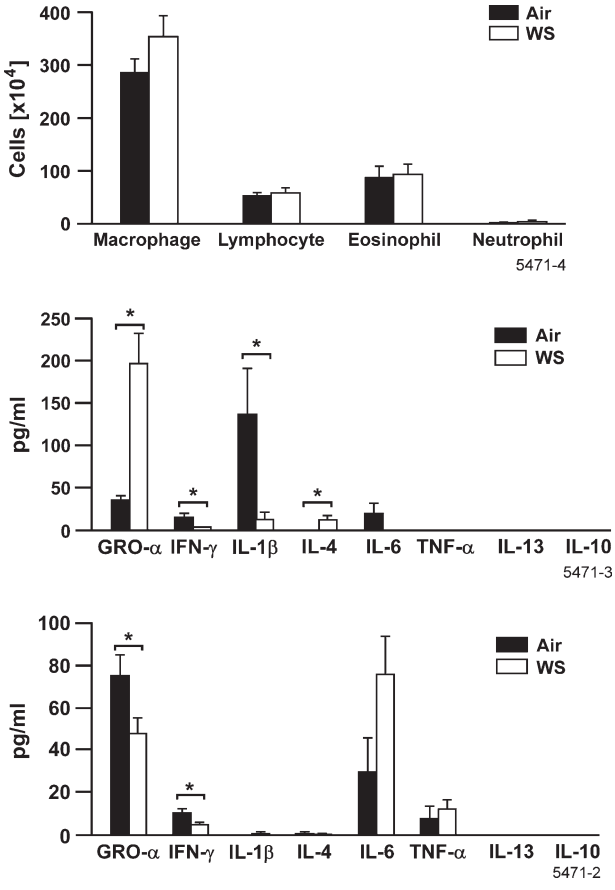

FIG. 2. (A) Number of macrophages, lymphocytes, eosinophils, and neutrophils in the BALF of rats exposed to air or wood smoke at 1 mg/m^3. Group mean ± standard error from the mean is shown in the figure. (B) Levels of GRO-α, IFN-γ, IL-1β, IL-4, IL-6, TNF-α, IL-13, and IL-10 in the BALF or in the plasma (C) of rats exposed to filtered air or wood smoke. Bars show group mean ± standard error from the mean. Cytokine levels between air and wood smoke-exposed rats were compared by Student's *t*-tests. Group mean ± standard error from mean is shown in the figure. *Significant difference from air control ($p < 0.05$).

Lymphocyte Proliferation Assay and Total IgE in Plasma

T lymphocytes isolated from both the lung-associated lymph nodes and the spleen were not affected in their proliferative response to Con A by exposure to wood smoke (Fig. 3A).

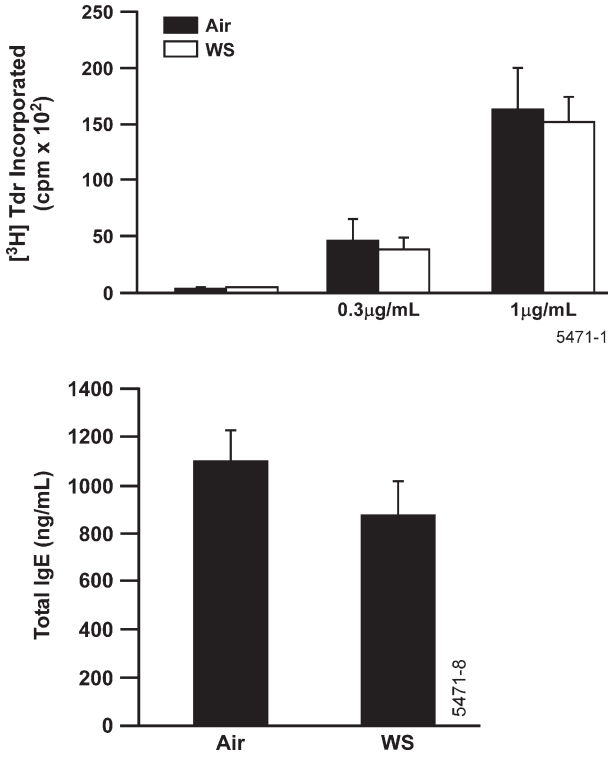

FIG. 3. (A) T lymphocytes isolated from the lung associated lymph nodes and the spleen of rats exposed to air or wood smoke and their proliferative response to Con A at 0.3 and 1 μg/ml. Proliferation of T cells was quantified by their incorporation of tritiated thymidine. Group mean ± standard error from mean is shown in the figure. (B) Total IgE levels in blood plasma of asthmatic rats exposed to air or wood smoke. Group mean ± standard error from the mean is shown in the figure.

Although not statistically significant, there was a trend toward reduced total serum IgE levels in rats exposed to wood smoke compared to those exposed to air (Fig. 3B). Similarly, a statistically nonsignificant reduction in OVA-specific IgE was observed in the wood smoke-exposed rats compared to air controls (data not shown).

Histopathology

Examination of H&E–stained tissues sections taken from the epiglottis, larynx, and trachea revealed minimal to mild signs of inflammation in both exposure groups. No inflammatory lesions were present in the nasal tissues (data not shown). In the lung, inflammatory lesions were characterized by widely scattered foci of monocytes, macrophages, lymphocytes, eosinophils, and occasional neutrophils. Often, these lesions, filled perivascular spaces surrounding venules, together with thickened alveolar septa, and filled alveolar lumens (Fig. 4). Eosinophils were loosely scattered in the adventitial tissues around the veins, venules, and larger airways of all rats in both groups. The average severity for eosinophils (sum of severity grade/number of rats) for the wood smoke-exposed group was 21% greater than that observed in the air-exposed control group

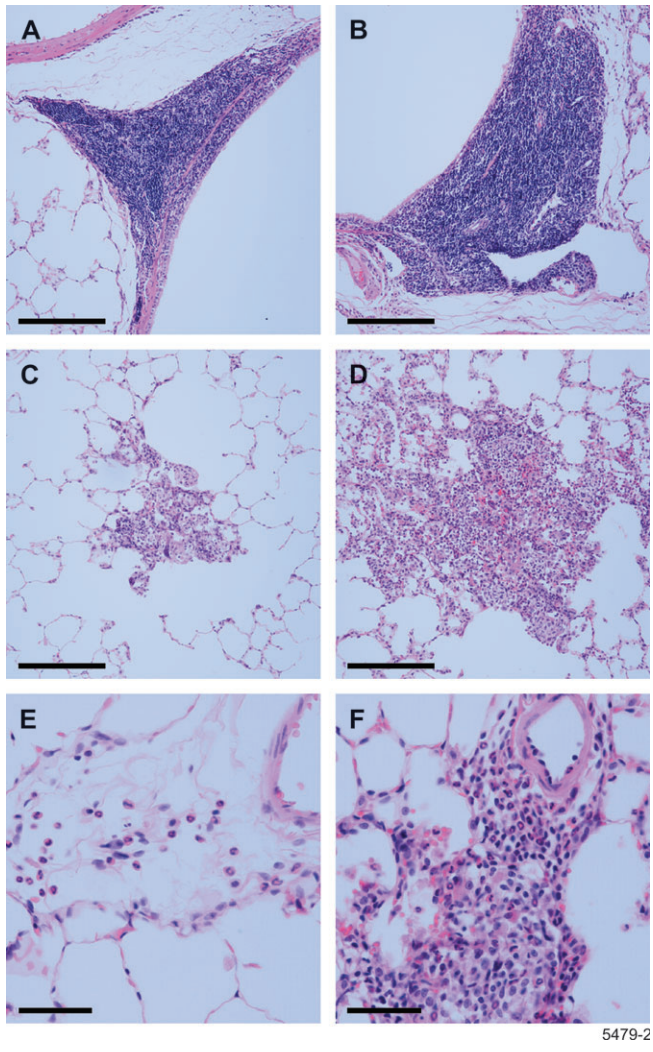

FIG. 4. Photomicrographs of lung tissues showing the lymphoid hyperplasia (A and B), granulomatous lesions (C and D), and eosinophilic inflammation (E and F) in rats exposed to filtered air (A, C, E) or wood smoke (B, D, F). Bar represents 100 μm in each photomicrograph.

(see Table 3, and representative photomicrographs in Fig. 4). A statistically non-significant increase in the amounts of intraepithelial stored mucosubstances and in the number of mucus-producing cells per mm basal lamina were observed around the epithelial lining of rats exposed to wood smoke compared to air-exposed controls (Figs. 5A, 5B, 5C, and 5D).

DISCUSSION

This study shows that allergic rats exposed repeatedly to a wood smoke atmosphere have reduced pulmonary function upon antigen challenge when compared to sensitized rats maintained in filtered air. However, exposure to wood smoke only minimally exacerbated the allergen-induced inflammatory response.

TABLE 3
Microscopic Observations in the Lungs with Average Severity Grade

Exposure	Air	Wood smoke
Granulomatous pneumonia[a]	1.1	2.1
Eosiniphils, peribronchial	1.4	1.7
Lymphoid hyperplasia, peribronchial	1.5	1.9

Note. Average grade = sum of all grades/number of rats examined.
[a]Granulomatous pneumonia includes all associated inflammatory lesions.

Overall, findings in this study are consistent with epidemiological evidence of reduced pulmonary function in asthmatics who live in areas where environmental concentrations of wood smoke particles are elevated (Koenig *et al.*, 1993; Larson and Koenig, 1994; Robin *et al.*, 1996). While the exposure concentrations in this study are relatively high for outdoor concentrations for wood smoke particles, they are well within the indoor concentrations in homes where people use wood stoves for heating and cooking in developing countries (Ezzati and Kammen 2001a,b, 2002). Therefore, this animal model, together with further optimization of the described exposure parameters, and additional time points post exposure for evaluation may be useful to understand the mechanisms underlying the reduction of pulmonary function by environmental wood smoke exposure in asthmatics.

Wood Smoke Characterization

The wood smoke PM in this study was readily respirable by rats. The MMAD of 0.36 μm was similar to the size of PM reported for pine, oak, and eucalyptus smoke (Kamens *et al.,* 1984; Kleeman *et al.*, 1999). In contrast, although the different wood, stove, and dilution system used in our previous wood smoke study produced a similar MMAD (0.5 μm), one-third of the PM mass was greater than 5 μm MMAD, a size beyond the respirable range for rats (Miller, 2000). In the present study, using a first-stage dilution immediately at the flue extraction point and a different dilution-cooling profile, kept nearly all of the PM mass under 1.0 μm, and thus within the respirable range for rats.

The PM component of wood smoke was composed of primarily organic material (OM) and small amounts of elemental carbon and metals and associated analytes. The portions of OM in the PM were slightly higher than in our previously reported inhalation study (Tesfaigzi *et al.*, 2002), but these concentrations were within the range of what has been reported for wood smoke (McDonald *et al.*, 2000). Individual chemical species within the OM are not reported here as they were for the previous study. However, there are key differences in the composition of the OM that are expected based on the differences between the composition of oak and pinion pine, which were used for the previous study. Several studies have contrasted the composition of smoke produced from oak and pine species (Fine *et al.*, 2002; Hawthorne *et al.*, 1992; McDonald *et al.*, 2000; Schauer *et al.*, 2001). Key contrasts in the composition of oak and pine are the presence of resin acids and the absence of dimethoxylated lignan polymers in

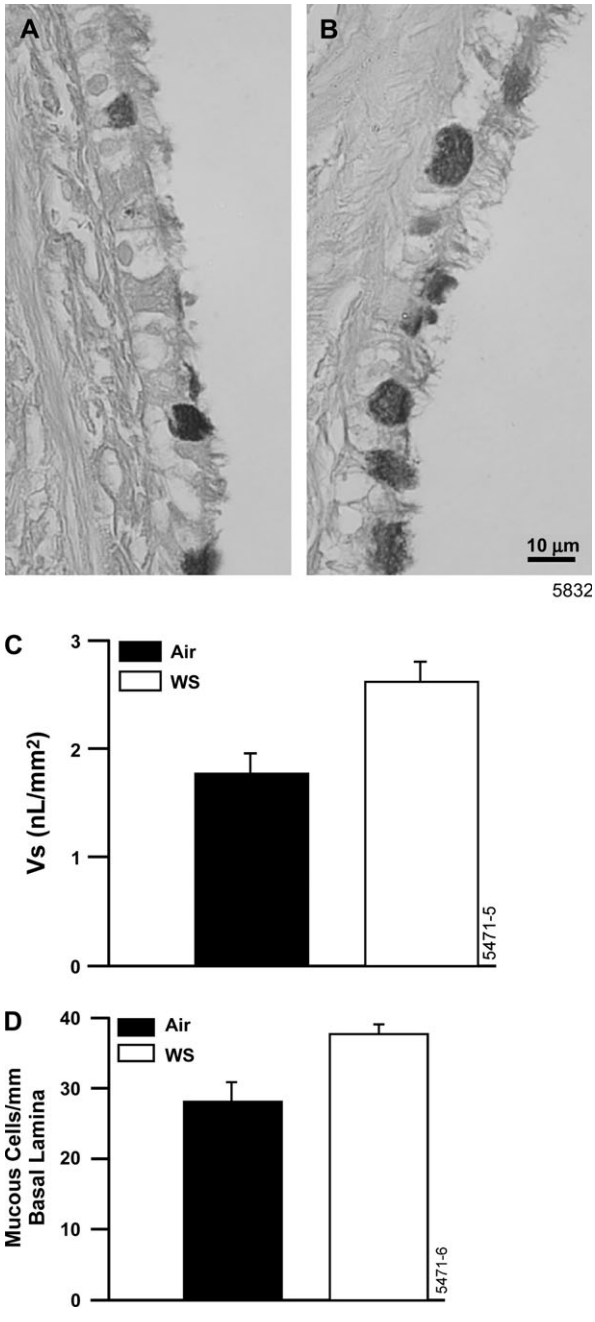

FIG. 5. (A) Representative micrograph of airway epithelial mucous cells at airway generation 5 of rats exposed to air (a) or wood smoke (b). Tissue sections were stained with AB/PAS as described in Materials and Methods. The volume of intraepithelial stored mucosubstances (B) and the number of mucous cells/mm basal lamina (C) in air or wood smoke-exposed rats are shown. Group mean ± standard error from the mean was compared using Student's *t*-tests. *Significant difference from air control ($p < 0.05$).

pine. Thus, resin acids were not present in the current study exposure atmosphere, and that of the previous study did not contain large amounts of dimethoxylated phenol compounds. Despite these key differences in composition between oak and pine, the wood smoke in the exposure atmospheres in both studies was similar in composition to what has been reported for environmental measurements (Fine *et al.*, 2002).

Health Effects

Exposure to wood smoke did not significantly affect the airway resistance response to methacholine challenge; the differences that were observed in C_{dyn} could be a direct result of exposure to the allergen. Because lung function was not measured in rats that were not sensitized to allergen, we cannot conclude that the decrease in pulmonary function was directly due to wood smoke, as opposed to an interaction between wood smoke and the allergen.

The values for total quasistatic lung compliance, vital capacity, functional residual capacity, forced vital capacity, FEV at 0.1 s appeared to be similar in this study compared to what we had reported for non-immunized rats exposed to wood smoke in our previous study (Tesfaigzi *et al.*, 2002). However, pulmonary resistance was lower in sensitized rats in the present study and was not affected by exposure to wood smoke. The reason for lower pulmonary resistance values in the present study is not clear but may have been due to the positive pressure that was applied during measurements. C_{dyn} in air-exposed rats was similar in both our previous and present studies, indicating that sensitization and challenge with low levels of allergen alone did not affect C_{dyn}. However, while C_{dyn} was significantly increased by exposure to wood smoke in our previous study, the baseline value for C_{dyn} was significantly decreased by exposure to wood smoke in the present study, where rats were sensitized and challenged with an allergen. These findings suggest that the observed differences in the effect of wood smoke on C_{dyn} were likely related to the allergen challenge combined with wood smoke exposures. Allergen challenge is known to reduce C_{dyn} (Kanehiro *et al.*, 2001), and our observations suggest that wood smoke may augment such decreases by enhancing perivascular inflammation and mild edema. The decrease of C_{dyn} is enhanced when surfactant protein A is absent (Yang *et al.*, 2002), and exposure to wood smoke may also have altered surfactant protein function (Nieman *et al.*, 1995) and, thereby, further reduced C_{dyn}.

While statistically significant, the reduction in the levels of the Th1 cytokine, IFN-γ, and the increase in the Th2 cytokine, IL-4, in the BALF of rats exposed to wood smoke compared to those exposed to filtered air were minimal. The presence of eosinophilic inflammation in the lung tissue was increased by only 21% in rats exposed to wood smoke compared to air-exposed controls. Taken together, observations suggest that exposure to wood smoke had only minor effects in enhancing allergen-induced reduction in Th1 cytokines and a shift toward a Th2 phenotype, which is a hallmark of asthma. This conclusion is further supported by the fact that the observed increase in IL-4 did not translate in a significant increase in either total or OVA-specific IgE in the plasma and did not result in the production of IL-13, another important Th2 cytokine in asthma. The lack of increases in IL-13 and IgE may be due to the short exposure period to allergen. The allergic response in the rats will be increased in future studies by increasing the allergen concentration to determine whether exacerbation by wood smoke exposure will be more obvious.

GRO-α is a CXC chemokine, which has a number of biological effects on various cell types (Persson-Dajotoy *et al.*, 2003). The major role of GRO-α is to attract and activate leukocytes, causing transendothelial migration of leukocytes, stimulating degranulation. Therefore, increased inflammation in the lung may be caused by the induction of GRO-α and other chemokines. The four-fold induced levels of GRO-α in the BALF directly mimic the findings from human studies where exposure of healthy volunteers to diluted diesel exhaust induces the expression of GRO-α in the bronchial epithelium (Salvi *et al.*, 2000). These findings further support the hypothesis that exposure of rats to wood smoke may be a useful animal model for studying the underlying mechanisms of pulmonary inflammation in humans exposed to environmental PM. The presence of low levels of TNF-α and IL-6 in plasma and BALF indicate that immunization and exposure to low-level allergen caused an immune response in rats irrespective of wood smoke exposure.

The cytokines of IL-1β and IL-6 are primarily produced by circulating macrophages, particularly when human subjects are exposed to high levels of environmental matter (PM10) (van Eeden *et al.*, 2001). Although van Eeden *et al.* (2001) did not identify whether exposed individuals were asthmatics or not, our findings that exposure to wood smoke resulted in an insignificant but noticeable increase in IL-6 levels in the blood plasma of rats are consistent with their study (van Eeden *et al.*, 2001). In our study, IL-1β levels in BALF were significantly decreased in sensitized and allergen-challenged rats exposed to wood smoke. Because there was no effect on the activation of T cells from associated lymph nodes or spleen, wood smoke exposure may have affected macrophages, which may be the primary producers of IL-6, IL-1β, and TNF-α.

The mild inflammation in the upper airways may have been a result of the allergen challenge and was not exacerbated by exposure to wood smoke. However, the severity of lesions in the lung was mildly increased in rats exposed to wood smoke compared to air-exposed controls. This mild increase in inflammation was associated with a statistically non-significant increase in the number of mucus-producing cells lining the bronchial epithelium and in the amount of intraepithelial stored mucosubstances in rats exposed to wood smoke compared to controls. Similar findings were reported for increased MUC5AC expression in rats exposed to wood smoke (Bhattacharyya *et al.*, 2004). In our study, the increased levels

of mucosubstances were not associated with increased production of the known inducers of mucin expression, such as IL-1β and IL-13. It is possible that there is a direct effect of the PM in wood smoke in inducing expression of MUC5AC. Expression of MUC5AC in human airway epithelia can occur via production of oxygen radicals (Fischer and Voynow, 2002). Oxygen radicals produced by cigarette smoke directly activate the transcription factors AP1 and JNK and cause activation of the MUC5AC promoter (Gensch *et al.*, 2004). Similarly, wood smoke may induce the production of MUC5AC by directly affecting epithelial cells in the absence of cytokines known to induce mucin production.

In summary, the observations in this study show that exposure of allergic rats to wood smoke only minimally enhances the overall inflammatory responses to allergens in the lung. This exacerbation of airway inflammation may cause deficits in pulmonary function at the time of exposure to an allergen. However, our studies do not exclude the possibility that such effects are unique to wood smoke, but may be also caused by particulate matter in general. Rats will be exposed to particulate matter similar to that contained in wood smoke to fully examine the effect of wood smoke on allergic inflammation. Future studies will also evaluate additional time points post exposure to wood smoke and various doses of allergen and will include nonallergic controls. Several studies have shown that environmental pollution, including diesel exhaust, enhances airway responsiveness in asthmatic subjects (Nordenhall *et al.*, 2001). Therefore, this rat model may be useful for elucidating the mechanisms underlying how environmental pollution enhances inflammation and the decline of pulmonary function in subjects with asthma.

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