Metal Composition of Ambient $PM_{2.5}$ Influences Severity of Allergic Airways Disease in Mice

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Children living in Hettstedt in eastern Germany have been reported to have a higher prevalence of sensitization to common aeroallergens than another cohort living in the neighboring city of Zerbst; these differences correlated with the presence of industrial air pollution. Samples of fine particulate matter (< 2.5 μm aerodynamic diameter; $PM_{2.5}$) collected in Hettstedt in 1999 had several-fold higher levels of zinc, magnesium, lead, copper, and cadmium than samples from Zerbst. To determine if the results from epidemiologic studies could be repeated in an animal model, we administered $PM_{2.5}$ from Hettstedt and Zerbst to ovalbumin-allergic mice. In Balb/c mice, $PM_{2.5}$ from Hettstedt, but not $PM_{2.5}$ from Zerbst or control filter extract, caused a significant increase in immediate responses to ovalbumin challenge when aspirated 2 hr before challenge, but not when aspirated immediately before sensitization 2 weeks earlier. Antigen-specific IgE was increased by Hettstedt $PM_{2.5}$ whether administered before sensitization or challenge. Airway responsiveness to methacholine aerosol and lung inflammatory cell numbers were significantly increased only in allergic mice exposed to Hettstedt $PM_{2.5}$ before challenge. Both Hettstedt and Zerbst $PM_{2.5}$ significantly increased lung injury parameters and proinflammatory cytokines. These results are consistent with epidemiologic findings and show that metal composition of ambient $PM_{2.5}$ influences the severity of allergic respiratory disease. *Key words:* **air pollution, airway hyperresponsiveness, allergic sensitization, asthma, epidemiology, inflammation, metals.** *Environ Health Perspect* **111:1471–1477 (2003). doi:10.1289/ehp.6300 available via** *http://dx.doi.org/* **[Online 27 May 2003]**

Recent cross-sectional epidemiologic studies examined whether regional differences in air pollution could account for differences in prevalence rates of respiratory and allergic diseases in school-age children living in different cities in eastern Germany (Heinrich et al. 1999, 2000, 2002a, 2002b). The city of Hettstedt was strongly impacted by particulate matter (PM) and other air pollutants from industrial (metal mining and smelting) and domestic (burning of brown coal) sources, whereas the city of Zerbst had minimal industrial emissions and was a center of agriculture and administration. After controlling for medical, demographic, and indoor factors, children living in Hettstedt in the early 1990s had a 50% higher lifetime prevalence of allergies, eczema, and bronchitis compared to children from Zerbst and about twice the level of respiratory symptoms including wheeze, shortness of breath, and cough (Heinrich et al. 1999). Sensitization to common aeroallergens (e.g., dust mite, cat, mixed grasses, birch) and specific IgE levels were also significantly greater in children from Hettstedt than in children from Zerbst. These results suggested that exposure to air pollutants, including $PM_{2.5}$ (PM < 2.5 μm aerodynamic diameter), could promote sensitization to common aeroallergens and promote the development or exacerbation of allergic and respiratory diseases. After German reunification, levels of total PM and sulfur dioxide in eastern Germany decreased throughout the 1990s, and regional differences in the prevalence of bronchitis, sinusitis, and frequent colds declined (Heinrich et al. 2000, 2002a). However, the difference in the prevalence of allergies between the two cities continued, despite the convergence of ambient PM concentrations (Heinrich et al. 2002b), suggesting that the composition of Hettstedt PM may also contribute to the higher prevalence of allergies in that area.

In the present study, our objective was to determine if the results observed in these epidemiologic studies could be replicated in an animal model and identify $PM_{2.5}$ composition as a causative factor in the increased prevalence and severity of allergic diseases in Hettstedt compared with Zerbst. We used a well-characterized mouse model of allergic airways disease induced by ovalbumin (OVA) antigen, which displays many features of allergic asthma including increased bronchoalveolar lavage (BAL) eosinophils, T-helper type 2 (Th2) allergic cytokines, and airway hyperresponsiveness to methacholine (Mch) (Gavett et al. 1999). To address the question of whether pollutants increase the prevalence of allergic diseases or only exacerbate responses in previously sensitized subjects, $PM_{2.5}$ from Hettstedt or Zerbst was administered into the airways of mice immediately before the sensitization phase or the challenge phase of the allergic response to OVA. We hypothesized that $PM_{2.5}$ collected from the industrial city of Hettstedt would enhance allergic sensitization, airway responsiveness, and lung inflammation to a greater degree than $PM_{2.5}$ from the nonindustrial and relatively clean city of Zerbst.

Materials and Methods

Collection, recovery, and analyses of $PM_{2.5}$. We collected ambient air $PM_{2.5}$ samples on Teflon filters in 1999 in Zerbst (local health authority at Fischmarkt 2) on 18–21 and 23–28 January, 27 February–4 March, 6–11 March, 17–22 and 24–29 April, 29 May–3 June, and 5–10 June (46 filters), and in Hettstedt (police station at Am Schützenplatz 1) on 13–18 and 20–25 February, 27–31 March, 4–8 and 10–15 April, 15–20 and 23–27 May (39 filters). Each filter sample was collected for 24 hr through a Harvard-Marple impactor (Air Diagnostics, Harrison, ME, USA) with a 50% aerodynamic diameter cutpoint of 2.5 μm. Samples were collected on 37-mm preweighed Anderson Teflon filters with a pore size of 2.0 μm. We equilibrated sample filters at 21 ± 2°C and 35 ± 5% relative humidity for 24 hr before weighing. We used an analytical balance designed for weighing filters with a resolution of 0.1 μg (M5P-000V001; Sartorius, Goettingen, Germany). We calculated ambient $PM_{2.5}$ concentrations from sample weight and air volume.

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We recovered $PM_{2.5}$ from filters using a modification of an aqueous extraction technique (Biran et al. 1996). Each filter was wetted with 200 µL 70% ethanol, secured with Teflon rings in a cup with 25 mL deionized distilled water, and sonicated for 30 min while rotating on an orbital shaker. We pooled, lyophilized, and resuspended the extracts of all filters from each location in sterile saline at 2 mg/mL; pooled, lyophilized extract from 43 control blank filters was resuspended in an equivalent volume. Samples were stored at –20°C.

We analyzed Hettstedt, Zerbst, and control filter extracts by inductively coupled plasma (ICP)-mass spectrometry for 26 elements (ELAN 6000; PerkinElmer, Shelton, CT, USA) [U.S. Environmental Protection Agency (U.S. EPA) 2002a], and by ICP-atomic emission spectrometry for sulfate levels (Model Plasma 40; PerkinElmer) (U.S. EPA 2002b). Aqueous and 1 M HCl extracts of pooled, lyophilized samples were analyzed (n = 4 measurements per element, except sulfate, n = 2); the results are reported as mean water or acid extract levels after subtraction of method blank values (nanograms per 100-µg sample). We determined endotoxin levels with a chromogenic limulus amebocyte assay according to the manufacturer's instructions (QCL-1000; BioWhittaker, Walkersville, MD, USA).

Experimental design: exposure to $PM_{2.5}$ and ovalbumin. Young adult (7 weeks old, 18–21 g) female Balb/c mice were obtained from Charles River (Raleigh, NC, USA) and Jackson Laboratories (Balb/cJ; Bar Harbor, ME, USA). To assess inflammatory potency and guide the experimental design of studies examining the effects of $PM_{2.5}$ on allergic responses, we initially examined the ability of filter extracts to induce acute lung injury in normal nonallergic mice. Balb/c mice were dosed with 100 µg Hettstedt or Zerbst filter extract in 50 µL saline, or an equivalent volume of control filter extract (n = 8–9/group), directly into the lung by oropharyngeal aspiration under anesthesia [equivalent to intratracheal instillation in deposition efficiency (Foster et al. 2001)]. Lungs were lavaged 18 hr later, and numbers of inflammatory cells and levels of protein (marker of edema), lactate dehydrogenase (LDH; marker of cell injury), *N*-acetyl-β-D-glucosaminidase (NAG; marker of lysosomal enzyme release), and proinflammatory cytokines were quantified in BAL fluid.

Because of limited sample quantities, we examined the effects of $PM_{2.5}$ on the sensitization phase only in OVA-allergic mice, but effects on the challenge phase were examined in both OVA-allergic and nonallergic mice. To examine effects on the sensitization phase (Figure 1A), Balb/c mice were exposed to 50 µg of Hettstedt or Zerbst $PM_{2.5}$, or an equivalent volume of control filter extract (n = 7–8 mice/sample) by oropharyngeal aspiration 2 hr before sensitization by aspiration of 10 µg OVA (grade V; Sigma, St. Louis, MO, USA). These exposure and sensitization steps were repeated 2 days later, for a total dose of 100 µg filter extract and 20 µg OVA. Fourteen days after the first exposure, we challenged all mice by aspiration of 20 µg OVA and determined immediate airway responses. Responsiveness to Mch aerosol and BAL parameters were measured on days 2 (n = 5/group) and 7 (n = 2–3/group) after OVA challenge. Because of insufficient numbers of mice in groups 7 days after challenge, trends of responses were noted and not statistically analyzed.

To examine effects on the challenge phase (Figure 1B), we sensitized 34 Balb/cJ mice intraperitoneally with 20 µg OVA in 0.2 mL aluminum hydroxide adjuvant (Alhydrogel; Accurate, Westbury, NY, USA), while 15 mice received adjuvant only (nonallergic group). Fourteen days later, mice were exposed to 100 µg of Hettstedt or Zerbst $PM_{2.5}$, or an equivalent volume of control filter extract by oropharyngeal aspiration (n = 10–12 OVA-sensitized and 5 adjuvant-only mice per sample). Two hours later, all mice were challenged by aspiration of 20 µg OVA. We measured immediate responses to OVA challenge in all nonallergic mice (n = 5/group) and half the mice in each allergic group (n = 5–6/group). Responsiveness to Mch aerosol and BAL parameters were measured on days 2 (nonallergic and allergic groups) and 7 (allergic groups) after OVA challenge (n = 5–6/group).

Respiratory responses to antigen and Mch. We used a 12-chamber whole body plethysmograph system (Buxco Electronics, Sharon, CT, USA) to measure immediate airway responses to antigen exposure and airway responsiveness to Mch aerosol in unanesthetized, unrestrained mice (Hamelmann et al. 1997). We determined immediate responses by the change in an index of airflow obstruction (enhanced pause; PenH) measured immediately before and after OVA challenge. PenH is derived from timing of expiration and pressure changes associated with respiration in the plethysmograph chamber (Hamelmann et al. 1997). This index correlates well with lung resistance and reflects changes occurring during bronchoconstriction, although nasal responses and mucus production may also increase PenH. We placed mice in plethysmograph chambers and recorded and averaged baseline values over a 10-min period. Mice were taken out and challenged with OVA antigen by aspiration as described above and then returned to the chambers within 4-7 min of challenge. We then resumed recording and averaged measurements over a 20-min period. The percent change in PenH after exposure to $PM_{2.5}$ was expressed as

[(Post-value – Pre-value)/ Pre-value] × 100%.

We determined responsiveness to Mch aerosol by subtracting baseline values from responses to saline or increasing concentrations of Mch aerosol (0, 4, 8, 16, and 32 mg/mL) and calculating the area under the curve (PenH AUC; PenH-sec) during the recording intervals for each concentration. Since PenH AUC represents the time-integrated change in PenH, it is more representative of changes in responsiveness to Mch than measurement of peak PenH values. After measurement of baseline PenH for 5 min, saline or Mch solution was nebulized through an inlet of the chamber for 1 min followed by drying of the aerosol for 2 min. We continued measurements of PenH and other parameters for an additional 1–8 min after saline or increasing doses of Mch, for a total recording interval of 4, 5, 6, 7, and 11 min (0, 4, 8, 16, and 32 mg Mch/mL, respectively).

Serum OVA-specific IgE and BAL parameters. We determined serum concentrations of OVA-specific IgE by enzyme-linked immunosorbent assay (ELISA). The optical density of wells was converted to arbitrary ELISA units using a sigmoidal standard curve generated by dilutions of a positive control serum (Hylkema et al. 2002) with variable Hill slope and bottom (lowest optical density) set to 0.0 (GraphPad Prism v. 3.0; San Diego, CA, USA).

We lavaged mouse lungs with two aliquots of Ca^{2+}, Mg^{2+}, and phenol red-free Hanks' balanced salt solution (35 mL/kg body weight). Approximately 85% of the total instilled volume was recovered in all treatment groups. The BAL fluid was centrifuged, supernatants were saved for biochemical and cytokine analyses, total cells were counted, and cell differentials were calculated (500 cells/sample). BAL supernatant levels of total protein, albumin, LDH, and NAG are routine measures of lung injury (Henderson et al. 1985) and were carried out as previously described (Gavett et al. 1997).

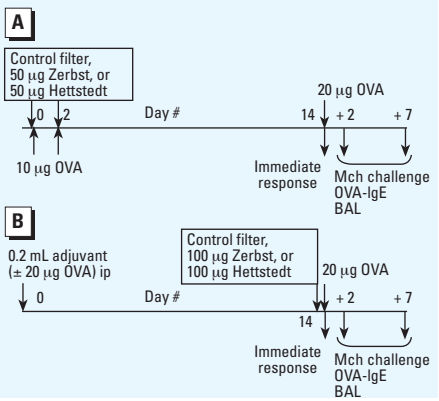

Figure 1. Experimental design for examination of effects of ambient $PM_{2.5}$ on (*A*) sensitization phase and (*B*) challenge phase of allergic response to ovalbumin antigen. ip, intraperitoneal. See "Materials and Methods" for details.

We quantified murine interleukin (IL)-1β, IL-4, IL-5, IL-6, IL-13, macrophage inflammatory protein (MIP)-2, tumor necrosis factor-α (TNF-α), and interferon gamma (IFN-γ) in BAL fluid with sandwich ELISAs according to the manufacturer's instructions (Quantikine M; R&D Systems, Minneapolis, MN, USA). The limit of detection (LOD) was determined by the lowest standard concentration that was within 10% of the back-calculated concentration. Samples with optical density values that indicated a concentration ≤ LOD were assigned the LOD value in the analysis.

Statistical analysis. We analyzed PenH-AUC values by repeated-measures analysis of variance (ANOVA) using SAS software (SAS Institute, Cary, NC, USA). All other parameters were univariate variables and were analyzed by ANOVA, followed by Tukey's multiple range comparison test if the ANOVA was significant overall. Differences were considered significant at $p = 0.05$.

Results

$PM_{2.5}$ concentrations and analyses. The $PM_{2.5}$ concentration on the study days was 17.5 ± 1.2 μg/m^3 (mean ± SEM; range, 5.9–39.0) in Zerbst and 19.2 ± 2.2 μg/m^3 (range, 3.4–63.8) in Hettstedt. A total of 12.07 mg was collected on the 46 Zerbst filters, of which 7.6 mg was recovered after lyophilization (63% yield); 11.04 mg was collected on the 39 Hettstedt filters, of which 6.6 mg was recovered (60% yield). ICP-atomic emission spectrometry analysis of water and 1 M HCl extracts of lyophilized Zerbst and Hettstedt $PM_{2.5}$ showed high levels of total (water plus acid) extract sulfate in both samples (24.7% and 28.9% of sample weight, respectively; Table 1). The majority of sulfate and other species was recovered by water extraction, with little additional components extracted by 1 M HCl. ICP-mass spectrometry analysis showed that sodium, potassium, and zinc were the next most common species, with total concentrations of extract Hettstedt exceeding those of Zerbst by 54, 27, and 82%, respectively. Most minor element concentrations were greater in Hettstedt $PM_{2.5}$ than in Zerbst $PM_{2.5}$; magnesium was 56% greater, lead and copper were 7-fold greater, cadmium was 5-fold greater, and tin and arsenic were 4-fold and 3-fold greater, respectively. Other minor elements were relatively comparable, whereas titanium, vanadium, and nickel were each about 2-fold greater in Zerbst $PM_{2.5}$. Control filter extracts had no sulfate and much lower levels of elements than Zerbst and Hettstedt filter $PM_{2.5}$. Levels of endotoxin in all samples were quite low: 0.183 endotoxin units (EU)/mg (Zerbst), 0.318 EU/mg (Hettstedt), and 0.004 EU/mg (control).

Induction of acute lung injury by Hettstedt $PM_{2.5}$. To assess inflammatory potency and guide the experimental design of studies examining the effects of $PM_{2.5}$ on allergic responses, we initially examined the ability of filter extracts to induce acute lung injury in normal, nonallergic Balb/c mice. Acute lung injury was assessed 18 hr after aspiration of 100 μg of Hettstedt, Zerbst, or control filter extracts (Table 2). Hettstedt $PM_{2.5}$ significantly increased BAL protein and NAG levels in comparison with control filter extract, indicating increased epithelial permeability and lysosomal enzyme release. Zerbst $PM_{2.5}$ did not cause any increases in biochemical indices of lung injury; Zerbst-exposed mice had even lower values of LDH than mice exposed to control filter extract. Neutrophils accounted for about 33 and 30% of total lavaged cells in Hettstedt-exposed and Zerbst-exposed mice, respectively, compared with 5% in mice exposed to control filter extract, although these increases did not reach statistical significance due to a high degree of variability in all groups. Proinflammatory cytokines including IL-1β, IL-6, and MIP-2 were increased about 2-fold in Hettstedt-exposed mice in comparison with mice exposed to Zerbst extract and control filter extract, although these differences were also not statistically significant. These data indicated that although some parameters of acute lung injury were increased in Hettstedt-exposed mice, the 100-μg dose of $PM_{2.5}$ was not overtly toxic and would be suitable for testing the ability to enhance sensitization and allergic responses when administered at either the sensitization phase or the challenge phase of the response to OVA antigen.

Respiratory tract responses to OVA challenge and Mch aerosol. As expected, there was no detectable immediate response to OVA antigen in nonallergic mice exposed to control filter extract before challenge (Figure 2A), and neither Hettstedt nor Zerbst $PM_{2.5}$ administered before OVA challenge had any effect in nonallergic mice. In allergic mice, Hettstedt $PM_{2.5}$ administered before challenge increased PenH 190% compared to baseline values,

Table 1. Mean levels of water- and acid-extractable elements in $PM_{2.5}$ recovered from filters after subtraction of method blank values (nanograms/100 μg sample).

Compound or element	Control filters H_2O extract	Control filters 1 M HCl extract	Hettstedt $PM_{2.5}$ H_2O extract	Hettstedt $PM_{2.5}$ 1 M HCl extract	Zerbst $PM_{2.5}$ H_2O extract	Zerbst $PM_{2.5}$ 1 M HCl extract	Ratio Hettstedt/Zerbst (H_2O + 1 M HCl)
Sulfate	0.0[a]	0.0[a]	27803.5	1095.4[a]	23051.0	1605.7[a]	1.17
Sodium	466.7	52.1	1267.8	140.7	889.9	26.4	1.54
Calcium	1643.6[a]	0.0[a]	1018.3[a]	0.0[a]	294.8[a]	0.0[a]	3.45
Potassium	111.1[a]	2.1[a]	816.9	50.6[a]	669.7	14.8[a]	1.27
Zinc	164.3	12.9	654.6	81.3	390.7	14.6	1.82
Magnesium	34.3	0.0	157.8	6.2	105.4	0.0	1.56
Lead	0.0[b]	0.0	124.2	46.2	16.0	7.9	7.12
Copper	9.9	0.0	118.1	12.5	17.9	0.0	7.31
Titanium	6.8[b]	6.3	82.3	38.3	187.6	26.5	0.56
Aluminum	41.1	9.3	60.0	11.7	68.4	0.0	1.05
Iron	0.0[a]	0.0[a]	35.5[a]	21.2[a]	56.1[a]	5.5[a]	0.92
Vanadium	27.2	0.0[a]	17.2	0.0[a]	34.5	0.0[a]	0.50
Manganese	1.4[c]	0.0[a]	12.7	0.2[b]	13.3	0.4[b]	0.94
Cadmium	0.3[b]	0.0[a]	10.2	0.4[b]	2.0	0.0[a]	5.20
Strontium	12.2	0.3[c]	7.8	0.3[c]	2.7	0.0[c]	2.98
Nickel	4.9	0.1[c]	6.2	0.5[c]	12.8	0.2[c]	0.52
Barium	1.7[c]	0.0[a]	6.0	0.3[b]	5.9	0.3[b]	1.01
Arsenic	0.0[a]	0.0[a]	5.7	0.0[a]	2.1[c]	0.0[a]	2.74
Antimony	1.6	0.0[a]	2.6	0.3[b]	2.0	0.1[a]	1.36
Molybdenum	1.1	0.0[a]	2.3	0.1[b]	3.1	0.1[b]	0.75
Tin	0.0[a]	0.0[a]	1.6[c]	2.9	0.7[b]	0.4[b]	4.44

[a]Below LOD or failed quality control check (accuracy not within 25%). [b]Semiquantitative (accuracy within 25% but not within 10%). [c]Detected level is < 3 × LOD.

Table 2. Acute lung injury 18 hr after aspiration of 100-μg filter extract.[a]

	Control	Zerbst	Hettstedt
Macrophages (10^4)	10.10 ± 1.47	6.90 ± 0.94	8.59 ± 1.52
Neutrophils (10^4)	0.50 ± 0.18	3.82 ± 1.79	4.80 ± 2.20
Lymphocytes (10^4)	0.12 ± 0.03	0.07 ± 0.02	0.10 ± 0.02
Protein (μg/mL)	177 ± 7	194 ± 10	256 ± 32*
LDH (U/L)	62 ± 11	33 ± 3*	47 ± 8
NAG (U/L)	1.6 ± 0.2	2.4 ± 0.3	2.9 ± 0.5*
IL-6 (pg/mL)	15.6 ± 0.0	17.0 ± 1.4	33.2 ± 9.8
IL-1β (pg/mL)	7.8 ± 0.0	10.0 ± 2.2	17.1 ± 5.1
MIP-2 (pg/mL)	7.8 ± 0.0	8.1 ± 0.3	14.5 ± 3.6

[a]Values shown are mean ± SEM of acute lung injury parameters in BAL fluid of normal nonallergic mice ($n = 8$–9, except cytokines, $n = 6$). *$p < 0.05$ versus control filter extract value.

while Zerbst $PM_{2.5}$ and control filter extract increased PenH 120% and 44%, respectively (Figure 2B). Of these three groups, only the response to Hettstedt $PM_{2.5}$ was significant compared to responses in nonallergic mice. In allergic mice administered $PM_{2.5}$ samples or control filter extract before OVA sensitization, there were no differences in immediate responses to OVA (Figure 2C).

Two days after OVA challenge, respiratory responses to Mch aerosol were not significantly different among nonallergic groups exposed to control filter extract or Hettstedt or Zerbst $PM_{2.5}$ before the challenge phase (Figure 3A). Similarly, there were no differences among allergic groups exposed prior to the sensitization phase 2 days (Figure 3B) or 7 days (not shown) after challenge. However, Hettstedt $PM_{2.5}$ administered to allergic mice before challenge caused a significant increase in Mch responsiveness 2 days after challenge in comparison with allergic mice exposed to Zerbst $PM_{2.5}$ or control filter extract and nonallergic mice exposed to Hettstedt $PM_{2.5}$ (Figure 3C). The Mch responsiveness in allergic Hettstedt $PM_{2.5}$-exposed mice was still higher 7 days after challenge, but the difference was not statistically significant (Figure 3D).

Effect of treatments on OVA-specific IgE. Particles from Hettstedt increased sensitization to OVA allergen, as determined by serum levels of OVA-specific IgE collected 2 days after OVA challenge, when administered at either the sensitization phase or the challenge phase of OVA exposure, in comparison with nonallergic mice exposed to Hettstedt $PM_{2.5}$ and in comparison with allergic mice exposed to Zerbst $PM_{2.5}$ (Figure 4). By 7 days after challenge, there were no significant differences among allergic groups exposed before OVA sensitization or challenge (data not shown).

BAL cells, proteins, and cytokines. Nonallergic and allergic mice exposed to Hettstedt or Zerbst $PM_{2.5}$ at the challenge phase tended to have decreased BAL macrophage numbers 2 days after OVA challenge compared with mice exposed to control filter extract, but these differences were not statistically significant (Figure 5A). Hettstedt and Zerbst $PM_{2.5}$ increased BAL neutrophil numbers about 3-fold (though not significantly) in nonallergic mice exposed before challenge compared with control filter extract; in allergic mice exposed before challenge, only Hettstedt $PM_{2.5}$ significantly increased BAL neutrophil numbers compared with control filter extract (Figure 5B). Eosinophil numbers in allergic mice exposed to Hettstedt $PM_{2.5}$ before challenge were significantly increased 3-fold compared with allergic mice exposed both to control filter extract and to Zerbst $PM_{2.5}$ and compared with nonallergic mice exposed to Hettstedt $PM_{2.5}$ (Figure 5C). There were no significant differences in lymphocyte numbers among the exposure groups (Figure 5D). Exposure to $PM_{2.5}$ or control filter extract before sensitization had no effect on BAL inflammatory cells in allergic mice (Figure 5B–D). By 7 days after challenge, inflammation had generally subsided in all allergic groups, and there were no significant differences in BAL cell numbers among any groups exposed before sensitization or challenge (not shown).

In comparison to the effects of Hettstedt and Zerbst $PM_{2.5}$ on BAL cell numbers, the effects on BAL biochemical indices of lung injury 2 days after OVA challenge showed a different response pattern (Figure 6). Both Hettstedt and Zerbst $PM_{2.5}$ administered before challenge in allergic mice caused similar significant increases in protein, LDH, and

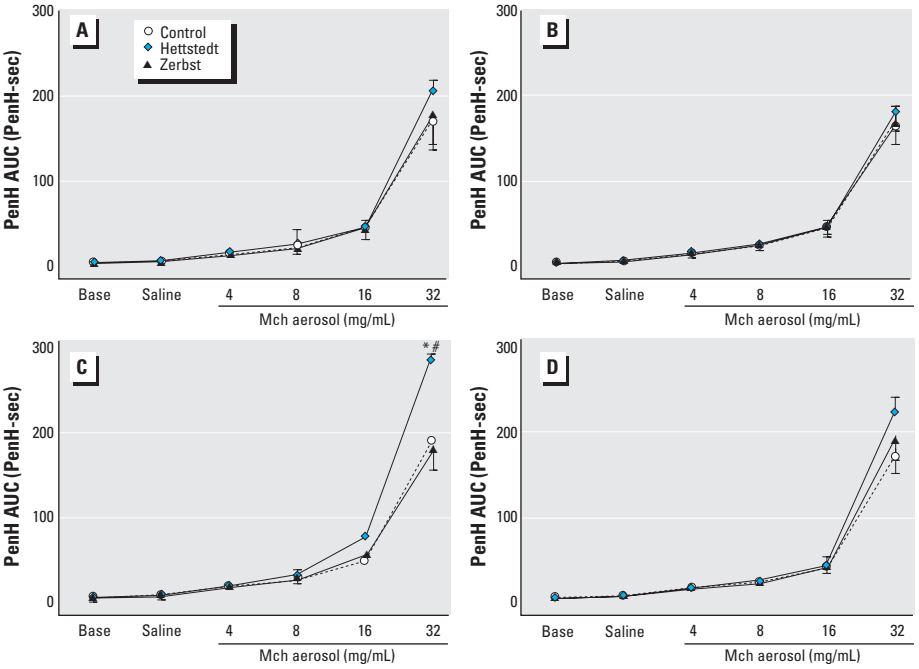

Figure 3. Airway responsiveness to aerosolized Mch measured (*A*) 2 days after OVA challenge in nonallergic mice exposed to control filter extract or 100 µg Zerbst or Hettstedt $PM_{2.5}$ at challenge phase of allergic response to OVA; (*B*) 2 days after OVA challenge in allergic mice exposed at sensitization phase; (*C*) 2 days after OVA challenge in allergic mice exposed at challenge phase; and (*D*) 7 days after OVA challenge in allergic mice exposed at challenge phase. Data shown are mean and SEM.
*$p < 0.05$ versus nonallergic mice exposed to Hettstedt filter extract. #$p < 0.05$ versus allergic mice exposed to Zerbst $PM_{2.5}$ or control filter extract.

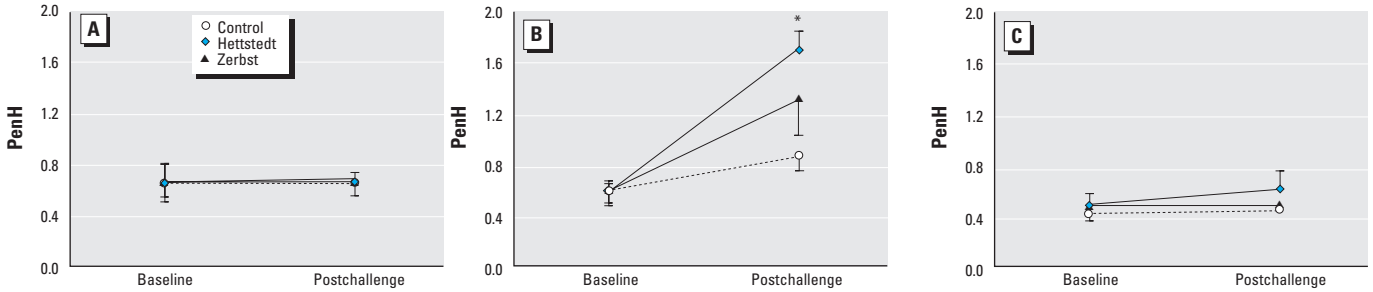

Figure 2. Immediate airway responses to OVA challenge (increase in PenH from baseline to postchallenge value). (*A*) Responses in nonallergic mice exposed to control filter extract or 100 µg Zerbst or Hettstedt $PM_{2.5}$ at challenge phase of response to OVA (2 hr before OVA challenge). (*B*) Responses in allergic mice exposed at challenge phase. (*C*) Responses in allergic mice exposed at sensitization phase. Data shown are mean and SEM.
*$p < 0.05$ versus nonallergic mice exposed to Hettstedt filter extract.

NAG in comparison to corresponding nonallergic groups. Aspiration of either Hettstedt or Zerbst $PM_{2.5}$ before challenge also caused a significant increase in LDH in comparison to allergic mice exposed to control filter extract, while only Hettstedt $PM_{2.5}$ caused a significant increase in NAG compared with control extract. Similar results were seen with albumin (not shown). Administration of filter extracts before sensitization had no effect on biochemical indices 2 days after challenge. By 7 days after challenge, biochemical indices of lung injury had returned to control levels, and there were no significant differences among any allergic groups exposed before sensitization or challenge (not shown).

Allergic groups exposed before challenge tended to have slightly increased levels of BAL Th2 cytokines (IL-4, IL-5, IL-13) in comparison with nonallergic groups 2 days after challenge, but these increases were rather small (Figure 7A and B; IL-4 not shown). Hettstedt and Zerbst $PM_{2.5}$ significantly increased IL-5 in allergic mice compared to similarly exposed nonallergic mice, and Zerbst $PM_{2.5}$ also increased IL-13, but these levels were not significantly increased compared with levels in allergic mice exposed to control filter extract. In contrast, levels of pro-inflammatory cytokines (TNF-α, IFN-γ) in allergic mice were increased 6- to 8-fold by exposure to either Hettstedt or Zerbst $PM_{2.5}$ particles before challenge compared with control filter extract (Figure 7C and D). Hettstedt $PM_{2.5}$ also tended to increase TNF-α and IFN-γ in nonallergic mice, but these levels were not significantly different from levels in nonallergic mice exposed to control filter extract.

Discussion

The results of this study show that ambient $PM_{2.5}$ with high levels of toxic and transition metals caused significant increases in a number of parameters of allergic lung disease in mice relative to $PM_{2.5}$ with lower levels of metals. The increase in lung inflammation and airway responsiveness in allergic mice exposed to $PM_{2.5}$ from Hettstedt relative to Zerbst provides coherence with previous epidemiologic findings in children (Heinrich et al. 1999) and points to $PM_{2.5}$ as a significant factor in allergic respiratory diseases associated with air pollution. A similar linkage between epidemiologic findings and toxicologic effects has been reported recently in studies of PM_{10} (PM < 10 μm aerodynamic diameter) pollution of the Utah Valley, where hospital admissions for bronchiolitis and asthma correlated strongly with PM_{10} levels associated with the operation of a local steel mill (Pope 1989). Extracts of PM_{10} filter samples collected when the steel mill was open had higher concentrations of metals and caused significantly greater increases in lung injury and inflammation in healthy nonallergic rats (Dye et al. 2001) and humans (Ghio and Devlin 2001) compared with samples collected when the plant was closed. The present study extends this linkage to show that ambient $PM_{2.5}$ composition is a critical factor in the enhancement of allergic respiratory disease.

The relative differences between Hettstedt and Zerbst in levels of transition metals and other elements shown here for $PM_{2.5}$ collected in 1999 are generally consistent with those found in 1992 and 1994, when fallen dust samples from Hettstedt were shown to have greater concentrations of lead, cadmium, chromium, nickel, and arsenic than samples from Zerbst (Heinrich et al. 1999). Despite the closure of several industries in Hettstedt and the convergence of particle mass levels during the 1990s, regional differences in PM elemental composition persist (Heinrich et al. 2002a), perhaps as a result of the reopening of industries in Hettstedt, persisting industrial emissions, resuspension of contaminated dust from slagheap, and geographical characteristics of the region, as Hettstedt is in a valley surrounded by hills, whereas Zerbst is located in a fairly level county (Heinrich et al. 1999, 2002a). Although Zerbst has a predominant agricultural economy, endotoxin levels present in both Zerbst and Hettstedt $PM_{2.5}$ samples were very low, indicating that endotoxin was not responsible for the observed enhancement of allergic airway responses.

Total sulfate levels were about 17% higher in Hettstedt $PM_{2.5}$ than in Zerbst $PM_{2.5}$ (Table 1), suggesting that differences in sulfate levels were probably not responsible for differences in pulmonary responses. In contrast, concentrations of many other transition and toxic metals, including zinc, lead, copper, cadmium, tin, and arsenic were several-fold greater in Hettstedt $PM_{2.5}$ than in Zerbst $PM_{2.5}$. Several of these metals have been associated with effects on airway inflammation and physiologic responses in both animal models and humans exposed to various samples of particulate matter (Dye et al. 1999; Ghio et al. 2002). In studies of two residual

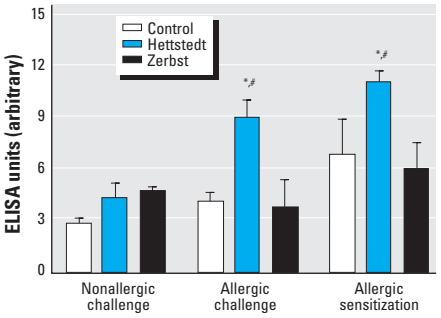

Figure 4. OVA-specific serum IgE collected 2 days after OVA challenge in allergic mice exposed to control filter extract or 100 μg Zerbst or Hettstedt $PM_{2.5}$ before sensitization or challenge or in nonallergic mice exposed before challenge. The sigmoidal standard curve used to calculate sample ELISA unit values fit optical density values of positive control serum dilutions with $R^2 = 0.9995$. Values shown are mean and SEM ($n = 5$–6).

*$p < 0.05$ versus nonallergic mice exposed to Hettstedt $PM_{2.5}$. #$p < 0.05$ versus allergic mice exposed to Zerbst $PM_{2.5}$.

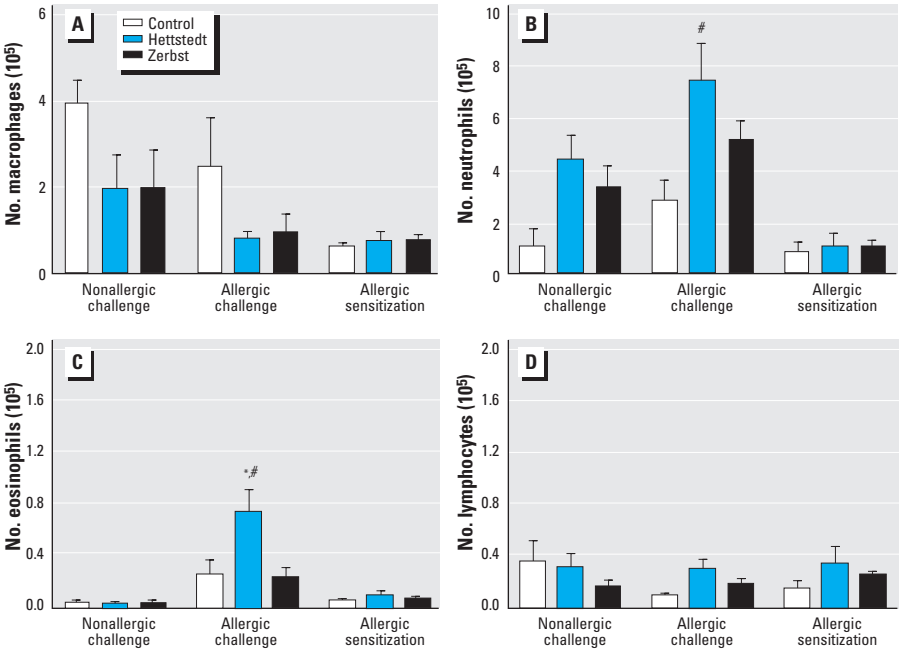

Figure 5. Numbers of (*A*) BAL alveolar macrophages, (*B*) neutrophils, (*C*) eosinophils, and (*D*) lymphocytes recovered 2 days after OVA challenge in nonallergic mice exposed at challenge phase, allergic mice exposed at challenge phase, and allergic mice exposed at sensitization phase. Data shown are mean and SEM.

*$p < 0.05$ versus nonallergic mice exposed to Hettstedt $PM_{2.5}$. #$p < 0.05$ versus allergic mice exposed at challenge phase to control filter extract and to Zerbst $PM_{2.5}$.

oil fly ash (ROFA) samples with different sulfate and metal compositions, the sample with higher zinc caused significantly greater lung inflammation and airway hyperresponsiveness in rats than the sample with higher sulfate, nickel, and vanadium (Gavett et al. 1997). Accordingly, the relationships of zinc, nickel, and vanadium content with pulmonary effects observed in normal, nonallergic rats (Gavett et al. 1997) are consistent with those observed in this study. In a Brown-Norway rat model of house dust mite allergy, administration of ROFA or its soluble metal constituents before sensitization increased serum house dust mite-specific IgE levels, immediate allergic responses, and numbers of BAL eosinophils (Lambert et al. 1999, 2000). Taken together, these studies suggest that metal composition of ambient air $PM_{2.5}$ has a strong influence on subsequent allergic responses.

The role of air pollution in the increased incidence and severity of allergic diseases has become the focus of increasing attention (Patton and Lopez 2002; Salvi 2001). A central question is whether exposure to certain air pollutants increases the incidence of allergic disease (i.e., sensitization) or only exacerbates allergic and respiratory responses in previously existing disease. Many studies have shown that high levels of common ambient air pollutants can exacerbate preexisting allergic asthma (Peden 2001). Acute elevations in ozone and PM_{10} have been shown to exacerbate asthmatic symptoms (Ostro et al. 2001) and to correlate with emergency room and hospital admissions for asthma attacks (Chew et al. 1999). It is less well known whether pollutants can enhance sensitization to allergens or increase the incidence of asthma. The fact that levels of common outdoor air pollutants such as PM_{10}, ozone, and sulfur dioxide have declined considerably in the United States (U.S. EPA 2002c) and most Western countries over the past 20 years suggests that these air pollutants cannot be responsible for the concurrent rise in asthma and allergic diseases. However, it is possible that physical and chemical properties of PM have changed over this period while particle mass has steadily decreased. Some animal and human studies indicate that increased sensitization is possible under certain conditions. Pulmonary administration of ROFA in rats before sensitization with house dust mite antigen increases subsequent allergic responses to antigen challenge, including specific IgE, immediate bronchoconstrictive responses, and BAL eosinophils and proteins (Lambert et al. 1999). Similar results have been seen in rats coadministered grass pollen and diesel exhaust particles (Steerenberg et al. 1999) and guinea pigs exposed to diesel exhaust and ovalbumin (Kobayashi 2000). Volunteer subjects exposed to diesel particles and a novel antigen (keyhole limpet hemocyanin) developed higher levels of antigen-specific IgE and the Th2 pro-allergic cytokine IL-4 than subjects exposed to diesel particles or antigen alone (Diaz-Sanchez et al. 1999).

Mice exposed to Hettstedt $PM_{2.5}$ 2 hr before OVA challenge had the most significant changes in respiratory tract responsiveness and pulmonary inflammation measured 2 days after challenge, whereas Zerbst $PM_{2.5}$ had no significant physiologic responses and fewer inflammatory effects. The effects observed in Hettstedt $PM_{2.5}$-exposed mice 2 days after challenge were transient: airway responsiveness and BAL cell numbers diminished to control levels by 7 days after challenge. These results suggest that repeated exposures are necessary to produce persistent pulmonary effects. Unlike other types of $PM_{2.5}$ such as ROFA (Lambert et al. 1999; 2000) and diesel exhaust particles (Diaz-Sanchez et al. 1999; Kobayashi 2000), neither Hettstedt nor Zerbst $PM_{2.5}$ administered before sensitization enhanced allergic responses, with the exception that Hettstedt $PM_{2.5}$ significantly increased serum

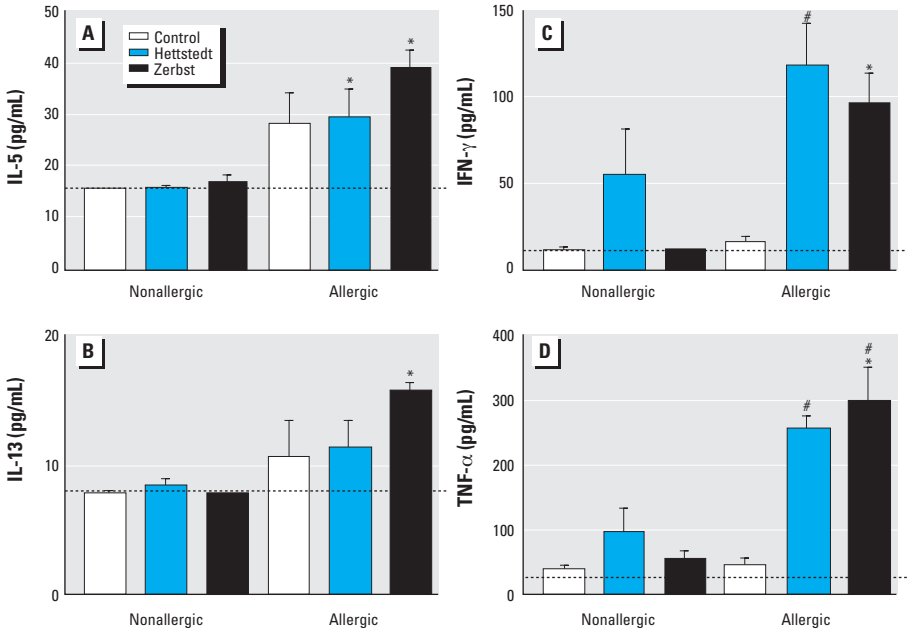

Figure 7. Levels of BAL supernatant cytokines: (*A*) IL-5, (*B*) IL-13, (*C*) IFN-γ, and (*D*) TNF-α recovered 2 days after OVA challenge in nonallergic and allergic mice exposed before challenge phase. The LOD is shown as a dashed line in each panel. Data shown are mean and SEM.
*$p < 0.05$ versus nonallergic mice, same $PM_{2.5}$ exposure. #$p < 0.05$ versus allergic mice exposed to control filter extract.

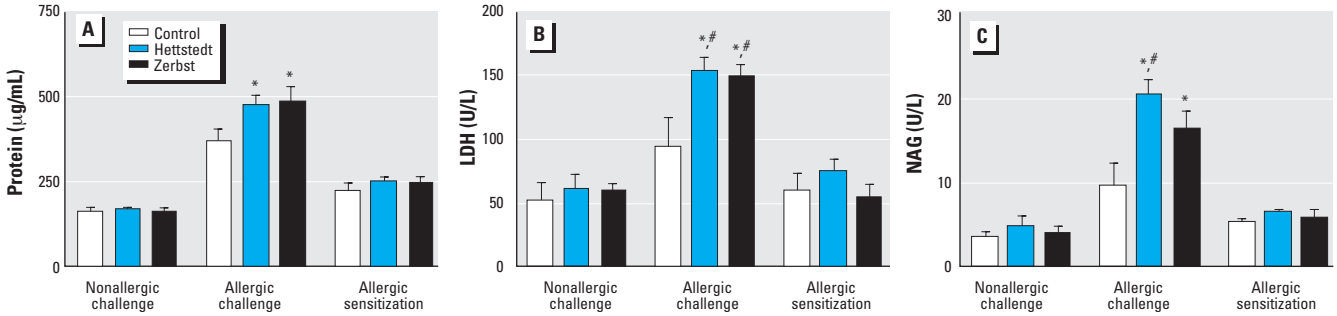

Figure 6. Levels of (*A*) BAL supernatant protein, (*B*) LDH, and (*C*) NAG recovered 2 days after OVA challenge in nonallergic mice exposed at challenge phase, allergic mice exposed at challenge phase, and allergic mice exposed at sensitization phase. Data shown are mean and SEM.
*$p < 0.05$ versus nonallergic mice, same $PM_{2.5}$ exposure. #$p < 0.05$ versus allergic mice exposed to control filter extract.

OVA-IgE levels measured 2 days after challenge. The two different allergic sensitization protocols used in this study (via the airways before sensitization or intraperitoneally before challenge) achieved roughly equivalent levels of sensitization, as indicated by serum OVA-specific IgE levels in allergic mice exposed to the same $PM_{2.5}$ sample (Figure 4). Therefore, timing of $PM_{2.5}$ exposure and its chemical composition, not sensitization protocol, were the important factors causing differences in allergic responses among the groups of mice. The data suggest that although Hettstedt $PM_{2.5}$ can increase the degree of allergic sensitization, exposure of previously sensitized mice near the time of allergen challenge is more important for increasing airway responsiveness and pulmonary inflammation.

Proallergic cytokines (IL-4, IL-5, and IL-13) may contribute to respiratory symptoms by directly promoting IgE synthesis, eosinophil recruitment, and airway hyperresponsiveness. Exposure of allergic mice to either Hettstedt or Zerbst $PM_{2.5}$ before OVA challenge caused greater increases in BAL levels of proinflammatory cytokines (TNF-α, IFN-γ) than proallergic cytokines. The results imply that TNF-α and IFN-γ also contribute to respiratory symptoms induced by exposure to these $PM_{2.5}$ samples by stimulating inflammation in previously sensitized subjects. Recent studies have shown that TNF-α has adjuvant activity in sensitizing rats to house dust mite antigen and correlates with increased numbers of eosinophils and airway hyperresponsiveness (Lambert et al. 2001). These results suggest that proinflammatory cytokines such as TNF-α may contribute to increased allergic inflammation and hyperresponsiveness.

In contrast to TNF-α, IFN-γ is a Th1 cytokine that inhibits Th2 cell differentiation and subsequent IgE synthesis, allergic inflammation, and airway hyperresponsiveness (Chung 2001; Yoshida et al. 2002). Therefore, it is somewhat surprising that IFN-γ levels were increased in BAL fluid of mice exposed to both Hettstedt and Zerbst $PM_{2.5}$, while airway hyperresponsiveness, OVA-specific IgE, and BAL eosinophils were increased in the Hettstedt $PM_{2.5}$-exposed group. However, recent studies indicate that IFN-γ induces the production of the chemokine CXCL10, which promotes allergic inflammation and airway hyperresponsiveness in mice (Medoff et al. 2002). Therefore, IFN-γ has dual roles, and both TNF-α and IFN-γ may contribute to allergic inflammation and airway hyperresponsiveness observed in mice exposed to Hettstedt $PM_{2.5}$. Other mechanisms must be responsible for the differences in allergic inflammation and airway hyperresponsiveness observed in this study because allergic mice exposed to Zerbst $PM_{2.5}$ had equivalent levels of these cytokines but did not have significantly increased responses.

In summary, we found that Hettstedt $PM_{2.5}$ administered before OVA antigen challenge in sensitized mice significantly increased lung inflammation and airway hyperresponsiveness to Mch aerosol in comparison to mice exposed to Zerbst $PM_{2.5}$. Administration of Hettstedt $PM_{2.5}$ before OVA sensitization increased IgE levels but did not cause increases in allergic airways responses after OVA challenge. This study provides coherence with previous epidemiologic findings and shows that metal composition of $PM_{2.5}$ influences allergic airway responses in previously sensitized subjects. Further analysis of $PM_{2.5}$ samples from additional cities with different levels of allergy and asthma will help clarify the relationship between the composition of $PM_{2.5}$ and the prevalence and severity of allergic airways disease.

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