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.

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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 (MSP-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/c; 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 exposed to 100 µg of Hettstedt or Zerbst PM$_{2.5}$, or an equivalent volume of control filter extract 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/c J 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-exposed groups) 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

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[(\text{Post-value} - \text{Pre-value}) / \text{Pre-value}] \times 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 following 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 (Hytkema 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.](image-url)
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 34, 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.
while Zerbst PM2.5 and control filter extract increased PenH 120% and 44%, respectively (Figure 2B). Of these three groups, only the response to Hettstedt PM2.5 was significant compared to responses in nonallergic mice. In allergic mice administered PM2.5 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 PM2.5 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 PM2.5 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 PM2.5 or control filter extract and nonallergic mice exposed to Hettstedt PM2.5 (Figure 3C). The Mch responsiveness in allergic Hettstedt PM2.5-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 PM2.5 and in comparison with allergic mice exposed to Zerbst PM2.5 (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 PM2.5 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 PM2.5 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 PM2.5 significantly increased BAL neutrophil numbers compared with control filter extract (Figure 5B). Eosinophil numbers in allergic mice exposed to Hettstedt PM2.5 before challenge were significantly increased 3-fold compared with allergic mice exposed both to control filter extract and to Zerbst PM2.5 and compared with nonallergic mice exposed to Hettstedt PM2.5 (Figure 5C). There were no significant differences in lymphocyte numbers among the exposure groups (Figure 5D). Exposure to PM2.5 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 PM2.5 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 PM2.5 administered before challenge in allergic mice caused similar significant increases in protein, LDH, and

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**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 PM2.5 at challenge phase of response to OVA (2 hr before OVA challenge). (B) Responses in allergic mice exposed to control filter extract or 100 µg Zerbst or Hettstedt PM2.5 at challenge phase of response to OVA (2 hr before OVA challenge). (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.

*Statistically significant (Figure 5A). Hettstedt and Zerbst PM2.5 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 PM2.5 significantly increased BAL neutrophil numbers compared with control filter extract (Figure 5B). Eosinophil numbers in allergic mice exposed to Hettstedt PM2.5 before challenge were significantly increased 3-fold compared with allergic mice exposed both to control filter extract and to Zerbst PM2.5 and compared with nonallergic mice exposed to Hettstedt PM2.5 (Figure 5C). There were no significant differences in lymphocyte numbers among the exposure groups (Figure 5D). Exposure to PM2.5 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).

**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 PM2.5 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 PM2.5 or control filter extract.
metals caused significant increases in a number of parameters of allergic lung disease in mice relative to PM2.5 with lower levels of metals. The increase in lung inflammation and airway responsiveness in allergic mice exposed to PM2.5 from Hettstedt relative to Zerbst provides coherence with previous epidemiologic findings in children (Heinrich et al. 1999) and points to PM2.5 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 PM10 (PM < 10 µm aerodynamic diameter) pollution of the Utah Valley, where hospital admissions for bronchiolitis and asthma correlated strongly with PM10 levels associated with the operation of a local steel mill (Pope 1989). Extracts of PM10 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 PM2.5 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 PM2.5 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 PM2.5 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 PM2.5 than in Zerbst PM2.5 (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 PM2.5 than in Zerbst PM2.5. 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

Discussion

The results of this study show that ambient PM2.5 with high levels of toxic and transition metals caused significant increases in a number of parameters of allergic lung disease in mice relative to PM2.5 with lower levels of metals. The increase in lung inflammation and airway responsiveness in allergic mice exposed to PM2.5 from Hettstedt relative to Zerbst provides coherence with previous epidemiologic findings in children (Heinrich et al. 1999) and points to PM2.5 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 PM10 (PM < 10 µm aerodynamic diameter) pollution of the Utah Valley, where hospital admissions for bronchiolitis and asthma correlated strongly with PM10 levels associated with the operation of a local steel mill (Pope 1989). Extracts of PM10 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 PM2.5 composition is a critical factor in the enhancement of allergic respiratory disease.

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The relative differences between Hettstedt and Zerbst in levels of transition metals and other elements shown here for PM2.5 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 PM2.5 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 PM2.5 than in Zerbst PM2.5 (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 PM2.5 than in Zerbst PM2.5. 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
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 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).

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 PM10 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 PM10, 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 bronchoconstrictor 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 PM2.5 2 hr before OVA challenge had the most significant changes in respiratory tract responsiveness and pulmonary inflammation measured 2 days after challenge, whereas Zerbst PM2.5 had no significant physiologic responses and fewer inflammatory effects. The effects observed in Hettstedt PM2.5-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 PM2.5 such as ROFA (Lambert et al. 1999; 2000) and diesel exhaust particles (Diaz-Sanchez et al. 1999; Kobayashi 2000), neither Hettstedt nor Zerbst PM2.5 administered before sensitization enhanced allergic responses, with the exception that Hettstedt PM2.5 significantly increased serum protein (Figure 7).
responsiveness. Exposure of allergic mice to 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 airways 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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