Effects of aqueous extracts of PM\textsubscript{10} filters from the Utah Valley on human airway epithelial cells

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\textsuperscript{1}Department of Medicine and Environmental Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642; \textsuperscript{2}Human Studies Division, United States Environmental Protection Agency, and \textsuperscript{3}Center for Environmental Medicine and Lung Biology, University of North Carolina, Chapel Hill, North Carolina 27599

Frampton, Mark W., Andrew J. Ghio, James M. Samet, Johnny L. Carson, Jacqueline D. Carter, and Robert B. Devlin. Effects of aqueous extracts of PM\textsubscript{10} filters from the Utah Valley on human airway epithelial cells. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L960–L967, 1999.—We hypothesized that the reduction in hospital respiratory admissions in the Utah Valley during closure of a local steel mill in 1986–1987 was attributable in part to decreased toxicity of ambient air particles. Sampling filters for particulate matter < 10 \textmu m (PM\textsubscript{10}) were obtained from a Utah Valley monitoring station for the year before (year 1), during (year 2), and after (year 3) the steel mill closure. Aqueous extracts of the filters were analyzed for metal content and oxidant production and added to cultures of human respiratory epithelial (BEAS-2B) cells for 2 or 24 h. Year 2 dust contained the lowest concentrations of soluble iron, copper, and zinc and showed the least oxidant generation. Only dust from year 3 caused cytotoxicity (by microscopy and lactate dehydrogenase release) at 500 \textmu g/ml. Year 1 and year 3, but not year 2, dust induced expression of interleukin-6 and -8 in a dose-response fashion. The effects of ambient air particles on human respiratory epithelial cells vary significantly with time and metal concentrations.

INCREASED CONCENTRATIONS of fine particles in the ambient air have been associated with increases in adjusted mortality rates and in respiratory morbidity, with a 50 \textmu g/m\textsuperscript{3} increase in the concentration of particulate matter < 10 \textmu m (PM\textsubscript{10}) associated with a 3–8% increase in the relative risk of death (2, 8, 12). Associations between particle concentrations and health effects have been found consistently in locations with widely varying emission sources, confounding efforts to identify chemical species and mechanisms that could be causative (31). However, an increasing number of studies have implicated transition metals as possible mediators of particle-induced airway injury and inflammation (11, 13, 16, 17, 25, 28). Iron and other ionizable metals with labile or vacant coordination sites may initiate lipid peroxidation through production of oxygen-based free radicals.

The Utah Valley in 1986–1988 provided a unique opportunity for evaluating the health consequences of changes in concentrations and point sources of particulate air pollution. Located in central Utah, the Utah Valley was home to ~188,000 residents in 1990. Only ~5% of the area’s adult population smoked. During winter temperature inversion episodes, local emissions become trapped in a stagnant air mass near the valley floor. Monitoring of the particle mass has been conducted by the Utah State Department of Health in accordance with the Environmental Protection Agency’s reference method at three sites: Utah Valley-Lindon, Provo, and Orem.

A principal point source of particle pollution in the Utah Valley is an integrated steel mill built during World War II and located near the shore of a freshwater lake on the west side of the populated corridor. When in operation, the mill contributed ~50–70% of total Utah Valley PM\textsubscript{10} emissions (23). Because of a labor dispute and subsequent change in ownership, the mill was shut down for a 13-mo period from 1 August 1986 to 1 September 1987. Pope (22) and others reported that winter ambient particle concentrations were reduced by approximately one-half during the closure, with reductions in PM\textsubscript{10} concentrations accompanied by improvement in health indicators among the local population, including decreases in 1) daily mortality (24), 2) age-adjusted death rates for malignant and nonmalignant respiratory diseases (1), 3) respiratory hospital admissions for pneumonia, pleurisy, bronchitis, and asthma (21), 4) bronchitis and asthma admissions for preschool-age children (22), 5) pulmonary function abnormalities (23), and 6) elementary school absences (27).

The change in particle source during the Utah Valley steel mill closure and the availability of particle-monitoring filters from before, during, and after the closure provided an opportunity to determine whether the observed reductions in respiratory illness were explained by changes in ambient particle composition in addition to reductions in particle concentration. The degree to which health effects are influenced by particle composition is important in determining whether regulatory efforts should focus on particle concentration alone or on particle composition and source. We hypothesized that ambient particles collected in the Utah Valley during the closure of the steel mill would contain lower concentrations of ionizable metals, have a reduced capacity to induce production of reactive oxygen species, and have a reduced potency for induction of epithelial injury and inflammatory cytokine production.
Materials and Methods

Preparation of particle extracts. Particle collection filters used for monitoring of PM₁₀ at the Lindon monitoring station in the Utah Valley were obtained from the Air Monitoring Center, Utah Division of Air Quality (Salt Lake City, UT). A total of 36 filters were obtained: 12 before closure of the steel mill (January–March 1986, year 1), 12 during closure of the steel mill (January–March 1987, year 2) and 12 after reopening of the steel mill (January–March 1988, year 3). These months were chosen to correspond with the timing of published epidemiological studies (1, 21–24, 27). The filters were stored at room temperature before extraction.

Because intact particles could not be physically separated from the filters, we obtained aqueous extracts of the particles. Each filter was agitated in a 50-ml conical polypropylene tube containing 40 ml of deionized water for 96 h. Filters were removed, and the aqueous extract was centrifuged at 2,500 g for 30 min to pellet insoluble matter. The supernatant fluid from all 12 filters was removed, lyophilized, pooled, and stored at room temperature before extraction.

Measurement of ionizable concentrations of metals. Aliquots of the water-soluble dust extract were agitated in 1.0 N HCl (1.0 mg/ml) for 1 h at room temperature and centrifuged for 1 h at 2,500 g. The supernatant was removed for analysis. Concentrations of zinc, nickel, iron, vanadium, copper, and lead were measured in the supernatants by inductively coupled plasma emission spectroscopy (model P40, Perkin-Elmer, Norwalk, CT). Metals were individually analyzed in duplicate. These metals were chosen for analysis because recent animal and in vitro studies have implicated first-row transition metals as possible mediators of particle toxicity (10, 25). The metal content of the supernatant fraction of the aqueous extract was found to be identical to that of the whole aqueous extract, indicating that all the metals tested were water soluble.

Measurement of in vitro oxidative stress. Oxidant generation by the three different aqueous extracts of the particles was measured with thiorbarbituric acid (TBA)-reactive products of deoxyribose as an end point. The reaction mixture containing 1.0 mM deoxyribose, 1.0 mM H₂O₂, 1.0 mM ascorbate, and 500 µg of dust extract was incubated at 37°C for 60 min with agitation and then centrifuged at 1,200 g for 10 min. One milliliter of 1.0% (wt/vol) TBA and 2.8% (wt/vol) TCA was added to 1.0 ml of supernatant, heated at 100°C for 10 min, and cooled in ice, and the chromophore concentration was determined by its absorbance at 532 nm.

To determine whether the oxidation of deoxyribose associated with exposure to the fraction of the air pollution particles could be inhibited by metal chelators and hydroxyl radical scavengers, deeroxamine, DMSO, and dimethylthiourea were added to the reaction mixtures (final concentration 1.0 mM). Chromophore concentration in the presence or absence of the chelators and scavengers was determined in triplicate by absorbance at 532 nm.

Exposure of cells to particle extracts. BEAS-2B cells (S6 subclone, passage 59) were obtained courtesy of Drs. Curtis Harris and John Lechner from the National Institutes of Health. This is an immortalized line of normal human bronchial epithelium derived by transfection of primary cells with simian virus-40 early-region genes, which undergoes squamous differentiation in response to serum. Cells were grown in KGM (Clonetics, San Diego, CA), which is essentially MCDB 153 medium supplemented with 5 ng/ml human epidermal growth factor, 5 ng/ml insulin, 0.5 ng/ml hydrocortisone, 0.15 mM calcium, bovine pituitary extract, 0.1 mM ethanolamine, and 0.1 mM phosphoethanolamine. For these experiments, cells (passages 88–95) were grown to confluence in 12-well plates (Costar, Cambridge, MA) and then cultured for 24 h in KGM supplemented with additional calcium (1.2 mM) to induce epithelial differentiation (20). Cells were then fed 1 ml/well of fresh KGM; 30 min later, 100 µl of medium were removed and replaced with 100 µl of particle extract at final concentrations of 0, 125, 250, and 500 µg/ml. After 2 or 24 h, the supernatant was removed for cytokine protein and lactate dehydrogenase (LDH) analysis, and the cells were washed twice with PBS (Life Technologies, Grand Island, NY) in preparation for RNA extraction.

Quantification of mRNAs by RT-PCR. The relative concentrations of mRNAs coding for interleukin (IL)-6 and IL-8 in cultured cells were estimated by sequential reverse transcription and cDNA amplification (RT-PCR) by a modification of the method of Becker et al. (3). We assumed that mRNA sequences are proportionally represented in the first-strand cDNAs, so that the relative amount of a cDNA sequence represents the relative amount of the corresponding mRNA sequence in the total RNAs. Because we made no adjustment for reverse transcription efficiencies, these estimates reflect relative rather than absolute steady-state mRNA levels.

Cells were lysed with 4 M guanidine thiocyanate (Boehringer Mannheim, Indianapolis, IN), 50 mM sodium citrate, 0.5% Sarkosyl, and 0.01 M diethioctetol. After the cells were dislodged from wells with scrapers (Costar), lysates were sheared with four passes through a 22-gauge syringe. RNA was pelleted by ultracentrifugation through 5.7 M cesium chloride (Boehringer Mannheim) and 0.1 M EDTA. One hundred nanograms of total RNA were reverse transcribed (Moloney murine leukemia virus reverse transcriptase; Life Technologies). The resultant cDNA was amplified for 24, 32–34, and 34–36 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IL-8, and IL-6, respectively, in separate reactions with gene-specific primers. Oligonucleotide primer sequences were synthesized using a DNA synthesizer (model 391, Applied Biosystems, Foster City, CA) based on sequences published in the GenBank DNA database. The following sense and antisense sequences were employed: 5'-CCATGGAAGGCTGGG-3' and 5'-CAAAGTGTCTCGTAC-3' for GAPDH, 5'-AACCCTCCTGACCACTGATC-3' and 5'-TCCCTCTCAATCACTCTCA-3' for IL-8, and 5'-CTTTCCTCAGGCGCTTC-3' and 5'-GGCAAGTCTCCTATGGACC-3' for IL-6.

Amplification products were separated on a 2% denaturing agarose gel, stained with ethidium bromide, and photographed under ultraviolet illumination. The resulting negative (type 55 film, Polaroid, Cambridge, MA) was quantitated using a densitometer (Bioimage, Ann Arbor, MI). For each concentration of dust, the integrated optical densities of the IL-8 and IL-6 DNA bands were divided by that of the GAPDH DNA band (a housekeeping gene) to correct for variation in the amount of amplifiable cDNA in each sample and expressed as the percentage of control wells (0 µg/ml).

The concentrations of IL-8 and IL-6 protein in the culture supernatant fluids were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN).

Cytotoxicity. Cytotoxicity of the dust extract was assessed by light-microscopic morphology at 2 and 24 h of exposure and
by release of LDH into the culture medium. The concentration of LDH was measured using a kit purchased from Sigma Chemical and modified as described previously (7). The results were expressed as percentage of control wells. In some experiments, cells in control wells were lysed with 1% Triton X-100 to provide total cellular LDH.

Scanning electron microscopy. For scanning electron-microscopic examination, BEAS-2B cells were grown on 12-mm round glass coverslips and treated with particle extracts as previously described. At specific intervals, the coverslips with adherent cells were immersed in primary fixative consisting of 2% glutaraldehyde-2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The cells were subsequently postfixed in buffered 1% osmium tetroxide, dehydrated through a graded ethanol series, and subjected to critical point drying. The coverslips were mounted on conventional scanning electron-microscopic specimen mounts with silver paste and sputter coated with 60:40 gold-palladium. All specimens were examined in an ETEC Autoscan at an accelerating voltage of 20 kV.

Statistics. Values are means ± SE unless otherwise indicated. Results were analyzed using a two-way ANOVA examining the effects of the year in which the dust was collected and concentration of the dust. The analysis also included a test of interaction between year and concentration. A significant interaction indicated that the effects of concentration differed among the three years; individual effects were examined only if there was no significant interaction. Significance was assumed at $P < 0.05$.

RESULTS

Particle characterization. Table 1 provides the mass of ambient particles collected in each of the three years and their metal content. Iron, copper, and zinc were the most abundant metals, but the concentrations and proportions varied significantly by year of collection. For example, zinc was most abundant in year 1, but copper showed the highest concentration in year 3. All three metals showed the lowest concentration in year 2, during the steel mill closure. Vanadium, which has been shown to stimulate epithelial cell cytokine production associated with aqueous extracts of residual oil fly ash (ROFA) particles (28), was virtually absent.

Figure 1 shows the oxidant generation capacity of the particle extracts as determined by TBA-reactive products of deoxyribose. Oxidant capacity paralleled the metal content of the extracts and was increased in years 1 and 3 relative to year 2. Addition of the hydroxyl radical scavengers dimethylthiourea or DMSO or the metal chelator deferoxamine significantly inhibited the oxidant-generating capacity of the particle extracts.

**Cytokine protein and mRNA expression.** Year 1 and year 3, but not year 2, particle extracts induced release of IL-8 and IL-6 protein (Fig. 4) into the cell culture medium in a dose-response fashion. The increases were detectable after 24 h, but not after 2 h, of exposure. The year 3 extract showed the largest effect. At the mRNA level (Fig. 5), the pattern of response for IL-8 was similar to the protein expression, with year 2 extract showing no increase and year 1 and year 3 extracts inducing IL-8 mRNA expression in a dose-response fashion after 2 h of exposure. At 24 h after exposure, only year 3 extracts showed a dose-response pattern of IL-8 mRNA expression. For IL-6, a similar pattern was seen at the lower concentrations, but ANOVA showed no statistically significant differences. IL-6 mRNA appeared to decrease with year 3 extract exposure at 500 µg/m³, possibly reflecting cytotoxicity or mRNA instability.

**Electron microscopy.** Scanning electron microscopy was performed on cells after exposure to determine whether differences in insoluble particle size, shape, or uptake may have contributed to the differences in cytotoxicity and cytokine expression. As shown in Fig. 6, visible cell-associated particles ranged in size from <1 to ~10 µm and often appeared to be covered with a
membrane or protein coat. There was no evident difference in particle size, shape, or cellular uptake among the three extracts. With exposure to year 1 and year 3, but not year 2, extracts, rare cells were seen to be undergoing apparent apoptosis, with rounding of the cell and "blebbing" of the cell membrane (Fig. 6, C and E).

**DISCUSSION**

We have demonstrated that aqueous extracts of ambient air particles, collected in three separate years from a single site in the Utah Valley, varied with regard to oxidant capacity, metal content, cytotoxicity for respiratory epithelial cells, and induction of proinflammatory cytokine expression. Oxidant capacity and metal content were substantially lower in the extract of particles collected during closure of the local steel mill, and this extract demonstrated no cytotoxicity and minimal induction of cytokines at the concentrations used. Year 3 extract was the most effective inducer of IL-8 expression and caused cell detachment and LDH release.
release at the highest concentration studied. In these studies, we compared the effects of the extracts using equivalent mass concentrations; the differences would be more pronounced if adjustments were made for the ambient particle mass concentrations.

Hospital admissions for bronchitis and asthma in the Utah Valley, as reported by Pope (22), were substantially fewer during the steel mill closure than before and after closure; this pattern of variation was not seen in the neighboring Cache Valley, which was not affected by the steel mill operation. It is interesting to note that for preschool-age children, the group most affected in the Pope study, peak hospital admissions were higher after the steel mill closure (40 in February of year 3) than before (25 in January of year 1), even though peak PM<sub>10</sub> levels were highest in year 1 (122 µg/m<sup>3</sup> in January of year 1 vs. 102 µg/m<sup>3</sup> in January of year 3). These data support the conclusion that respiratory health effects are influenced by changes in particle source and composition as well as in exposure concentration. This has obvious implications for future regulatory efforts; identification of components of the PM<sub>10</sub> mix that mediate toxicity would allow control efforts to target sources that contribute the most toxic particles.

There is evidence implicating transition metals as mediators of inflammation and cytotoxicity via oxidative mechanisms. Ambient air particles from a variety of locales contain ionizable metals (13) and produce reactive oxygen species in aqueous solution (14), similar to the findings in the present study. Gilmour and colleagues (14) showed that PM<sub>10</sub> collected from Edinburgh induced DNA damage in vitro that was iron dependent. Kennedy and colleagues (17) demonstrated that aqueous extracts of total suspended particulate filters (not PM<sub>10</sub>) from the Utah Valley, collected in 1982, induced inflammation when instilled into the airways of rats and increased expression of IL-8 and intercellular adhesion molecule-1 in cultured BEAS-2B cells.

In another study (25), ambient air particles from 10 different sources induced influx of polymorphonuclear leukocytes and increases in protein content of lung fluids when instilled into the airways of animals. The potency of these particles for inducing inflammation was proportional to the content of soluble ionizable metals. ROFA particles also induce airway injury when instilled intratracheally into rats, and their effects are mimicked by a mixture of soluble metals in proportion to their content in the dust (10). ROFA particles induced inflammatory cytokine expression in normal human bronchial epithelial cells (4); the mechanism may involve inhibition of protein tyrosine phosphatase activity (28), with accumulation of phosphoproteins and subsequent phosphorylation of the nuclear regulatory factor nuclear factor-κB (NF-κB), as well as activation of other proinflammatory pathways. Reactive oxygen species have also been linked directly with activation of NF-κB (19, 29) and with increased expression of IL-8 (18). Finally, reactive oxygen species and iron appear to increase uptake of particles by airway epithelial cells (5, 16).

We therefore speculate that ambient air particles in the Utah Valley induce health effects in part by delivering transition metals to the airway epithelium in soluble form, such as humates, sulfates, citrates, or other molecular forms. Reactive oxygen species and lipid peroxidation products are generated via Fenton chemistry, promoting cytokine gene expression and cytotoxicity in airway epithelial cells. The mechanisms for increased expression of IL-8 and other proinflammatory cytokine genes may involve activation of NF-κB by reactive oxygen species (17), accumulation of intracellular...
lar phosphates (28), or other pathways. The subsequent influx of inflammatory cells enhances and prolongs epithelial injury in susceptible individuals. If this hypothesis is correct, regulatory strategies that reduce transition metal content of ambient particles should be more effective in ameliorating health effects than reduction of particle concentration alone.

Our studies do not indicate which metal(s) is most active in inducing these effects. Deferoxamine reduced the oxidant-generating capacity of the extracts, but this compound chelates iron and copper as well as other metals (30). In two previous studies of IL-8 released by respiratory epithelial cells, one in response to exposure to an oil fly ash (4) and one using an aqueous filter extract (17), IL-8 concentrations in the culture supernatants were diminished after inclusion of deferoxamine in the incubations. Both of these particle extracts had significant copper concentrations. Thus previous experiments with deferoxamine have not clarified which metals are most active.

Fig. 6. Scanning electron microscopy of BEAS cells exposed to particle extracts. A: control well; B and C: year 1; D: year 2; E and F: year 3. Solid arrowheads, particles; open arrowheads, apoptotic cells.

Caution must be exercised in extrapolating the findings of this study to the effects of ambient particles on human airway epithelium in vivo. The human airway represents a complex interaction of cell types, and responses seen in transformed cells in culture may differ from the responses of normal differentiated human epithelial cells (15). However, a preliminary report (6) indicates that instillation of similar extracts into the airways of rats resulted in divergent effects on airway injury and inflammation, with year 1 and year 3 extracts inducing greater release of protein and LDH and larger increases in inflammatory markers than year 2 extracts.

We cannot exclude the possibility that the different effects of the particle extracts observed in this study were caused by some physical or chemical difference other than metal content or oxidant potential. For example, endotoxin has been found to be present in ambient air particles (9) and can contaminate stored samples. However, endotoxin was undetectable in the
particle extracts used in these studies. Furthermore, BEAS-2B cells are known to be unresponsive to endotoxin in the absence of human serum because lipopolysaccharide-binding protein and soluble CD14 are required for activation of epithelial cells by endotoxin (26). Finally, because we studied a soluble extract, and not the original particles themselves, the influence of particle size, shape, and insoluble components in modulating the health effects observed in the Utah Valley is unknown.

We conclude that changes in the source of ambient air particles influence their content of transition metals and their potential for epithelial cell cytotoxicity and induction of IL-8 and IL-6 in vitro. Parallel between these in vitro effects and observed concurrent changes in health effects in the Utah Valley suggest that ambient particle chemistry, specifically transition metal content, is an important determinant of the respiratory health effects of PM10 exposure.

The authors acknowledge the technical assistance of Lisa Dailey and Jacqueline Quay.

These studies were supported in part by National Heart, Lung, and Blood Institute Grant RO1-HL-51701 and National Institute of Environmental Health Sciences Grant RO1-ES-02679. The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratories (US Environmental Protection Agency) and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendations for use.

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Received 18 November 1998; accepted in final form 14 July 1999.

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