Effects of Submicrometer Particle Compositions on Cytokine Production and Lipid Peroxidation of Human Bronchial Epithelial Cells

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Epidemiologic studies have demonstrated increases in cardiovascular and respiratory morbidity and mortality in association with elevated mass concentration of ambient particulate matter, especially that of fine particles (Dockery and Pope 1996). Because respiratory epithelium and macrophages are the cells that come in direct contact with inhaled particles, in vitro experiments have often used these two cell types to study the toxicity of particles, including studies addressing concerns about the effects of particle size and specific particle components.

Fine particles contain various combustion products, including transition metals and acids, and are better associated with health effects than are coarse particles. However, this has not been consistently reflected in in vitro studies. In fact, in experiments where macrophages or monocytes were used, coarse particles sometimes caused greater cellular responses than fine particles. This could in part be attributed to the sensitivity of macrophages to endotoxin, which was more abundant in coarse particles (Dockery and Pope 1996). Because respira-

To identify the size and components related to toxicity of ambient particles, we used a trichotomous impactor to collect 17 sets of particles in three size ranges—submicrometer (diameters < 1 µm; PM0.01), fine (diameters between 1 and 2.5 µm; PM1.0–2.5), and coarse (diameters between 2.5 and 10 µm; PM2.5–10)—at stations monitoring background, urban, traffic, and industrial air in Taiwan. Elemental contents, carbon contents, soluble ions, and endotoxin content of particles were determined by X-ray fluorescence spectrometry, thermal analysis, ion chromatography, and the Limulus amebocyte lysate assay, respectively. Human bronchial epithelial BEAS-2B cells were exposed to particle extracts at 100 µg/mL for 8 hr, and interleukin-8 (IL-8) concentrations in the medium and lipid peroxidation products were measured. Particle-induced tumor necrosis factor-α (TNF-α) production by mouse macrophage RAW 264.7 cells was also measured. PM0.01 stimulation resulted in significantly higher IL-8 production and lipid peroxidation than PM2.5–10, whereas the responses elicited by PM1.0–2.5 were not significantly higher than blank filters. Untreated and polymyxin B-pretreated PM0.01 also stimulated more TNF-α production by RAW 264.7 cells than PM2.5–10 and PM1.0–2.5. Cytokine production was significantly associated with metal contents of PM0.01. IL-8 correlated with Cr and Mn, and TNF-α correlated with Fe and Cr. Lipid peroxidation in BEAS-2B cells correlated with elemental and organic carbon contents. Our study found that size and composition of ambient particles were both important factors in inducing cytokine production and lipid peroxidation. Key words: cytokine, human bronchial epithelial cell, lipid peroxidation, macrophage, submicrometer particle.

Methods

Particle collection. We collected ambient particles at four ambient air monitoring stations of the Taiwan Air Quality Monitoring Network, which were representative of background, urban, traffic, and industrial air pollution patterns. We used a trichotomous particle sampler (Particle Technology Laboratory, MN, USA) with a flow rate of 40 ft3/min to collect submicrometer (PM0.01), fine (PM1.0–2.5), and coarse (PM2.5–10) ambient particles. We collected a total of 17 sets of ambient air samples between September and December of 2000. Each set included PM0.01 on two 47-mm Teflon filters and one 8 inches × 10 inches quartz filter, PM1.0–2.5 on two 47-mm Teflon filters, and PM2.5–10 on one 47-mm Teflon and one 2.5 inches × 7 inches quartz filter. The sampling duration lasted for 8–37 hr, depending on local particle concentrations.

X-ray fluorescence analysis. Particles on Teflon filters were examined by an energy-dispersive X-ray fluorescence system (model Ex6600AF, Jordan Valley Applied Radiation, Austin, TX, USA) to determine the contents of 26 elements: Na, Mg, Al, Si, P, S, Cl, K, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, as polycyclic hydrocarbons has only begun to accumulate (Bonvallot et al. 2001). To examine the size and component effects of ambient particulates, particle samples in three size ranges, PM0.01, PM1.0–2.5 (diameters between 1 and 2.5 µm), and PM2.5–10, were extensively characterized and correlated with cytokine-inducing and oxidative stress–inducing bioactivities in respiratory epithelial cells. We also examined whether cytokine production in RAW 264.7 cells could be affected by different sizes and components of ambient particulates. Considering the sensitivity of macrophages to bacterial endotoxin, we performed assays for particles with and without polymyxin B pretreatment.

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Keywords: cytokine, human bronchial epithelial cell, lipid peroxidation, macrophage, submicrometer particle.

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Ga, Ge, As, Br, Rb, and Pb. The means of three different spots scanned on each Teflon filter were used to calculate elemental contents of each sample.

Particle preparation. All Teflon filters were equilibrated in 50 ± 5% relative humidity for more than 48 hr and weighed before and after air sampling to obtain particle mass. After X-ray fluorescence spectrometry (XRF) analysis, the 47-mm Teflon filters were submerged in 2.5 mL endotoxin-free water (Sigma, St. Louis, MO, USA) and sonicated in water bath (Bandelin Sonorex, Mörfelden-Walldorf, Germany) for 30 min to extract particles. After sonication, the filters were weighed again to determine the weight of water-extractable particles in the samples. Accordingly, the particle suspension contained particles either dissolved or suspended in water. For samples with lower PM1.0 mass, two filters were extracted sequentially in the same 2.5 mL water to achieve adequate particle concentrations. The particle extraction fractions were 53 ± 15% for PM1.0, 65 ± 16% for PM1.0–2.5, and 64 ± 10% for PM2.5–10. The particle suspension was stored at –20°C for more than 48 hr and weighed before air sampling to obtain particle mass.

The particle suspension was stored at –20°C and after air sampling to obtain particle mass. Accordingly, the particle suspension contained particles either dissolved or suspended in water. For samples with lower PM1.0 mass, two filters were extracted sequentially in the same 2.5 mL water to achieve adequate particle concentrations. The particle extraction fractions were 53 ± 15% for PM1.0, 65 ± 16% for PM1.0–2.5, and 64 ± 10% for PM2.5–10. The particle suspension was stored at –20°C and sonicated for 1 min before cell stimulation. In total, there were 17 samples for in vitro assay using human bronchial epithelial BEAS-2B cells. For samples with sufficient amount, which were 9, 14, and 13 for PM1.0, PM1.0–2.5, and PM2.5–10, respectively, we also performed in vitro assay using RAW 264.7 cells.

In vitro assays of particle bioactivity. Human bronchial epithelial BEAS-2B cells (CRL-9609, American Type Culture Collection) were maintained in serum-free LHC-9 basal medium supplemented with growth modifiers (BEGM SingleQuots, Clonetics, San Diego, CA, USA) on 100-mm culture dishes coated with a protein mixture (0.01 mg/mL fibronectin, 0.03 mg/mL vitrogen 100, and 0.01 mg/mL bovine serum albumin in 0.5 mL LHC-9 medium). For exposure experiments, BEAS-2B cells at 5.0 × 10^5 cells/mL were seeded onto 48-well (PM1.0) or 24-well (PM1.0–2.5 and PM2.5–10) tissue culture plates (Costar, Corning, NY, USA) in duplicates and cultured for 24 hr. The medium was then changed to F-12 medium containing 100 ng/mL of particles and supplemented with 1% fetal bovine serum (FBS). The supernatant was collected 8 hr later and kept at –20°C, and IL-8 concentration was measured by enzyme-linked immunosorbent assay (ELISA; Endogen, Woburn, MA, USA). Each plate included two to four wells of unstimulated cells, and the mean value of background production of cytokines was subtracted from other wells of the same plate. The cells were frozen at –70°C, and the content of malonaldehyde and 4-hydroxyalkenals was measured by an LPO-586 assay kit (Oxis Research, Portland, OR, USA).

RAW 264.7 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM; Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% FBS. For exposure experiments, RAW 264.7 cells were seeded onto 48-well tissue culture plates at 5 × 10^5 cells/mL, 0.36 mL/well, and cultured for 24 hr. The medium was changed to DMEM containing 100 µg/mL particle and supplemented with 1% FBS. The supernatant was collected 16 hr later, and tumor necrosis factor-α (TNF-α) concentration was measured by ELISA (OptEIA mouse TNF-α set; Pharmingen, San Diego, CA, USA). For inhibition assays, the particle suspension was preincubated with 10 µg/mL polymyxin B (Sigma) for 60 min before cell stimulation. Viability of cells was examined by trypan blue exclusion.

Endotoxin measurement. The concentration of endotoxin in each particle suspension was measured by Limulus amebocyte lysate (LAL) assay (QCL-1000 kit, BioWittaker, Walkersville, MD, USA). The assay was per- formed according to the manufacturer’s instructions. Briefly, a 50-µL aliquot of particle extracts or endotoxin standards was mixed with 50 µL LAL and incubated at 37°C for 10 min. Then, 100 µL of substrate was added and the mixture incubated for another 6 min; the reaction was stopped, and the plate was read at 405 nm by a microplate reader. The amount of endotoxin in particle extracts was calculated by comparison with the standard curve and transformed to mass units by a conversion factor of 0.1 ng for one endotoxin unit.

Carbon analysis. A strip of quartz filters representing 1–5% of collected particle mass of PM1.0 and PM2.5–10 at each location was used in carbon analysis by a nondispersive infrared radiation method (total carbon analyzer; Shimadzu, Tokyo, Japan). To measure elemental carbon (EC), the filters were baked for 350°C for 30 min and then heated at 950°C for 3 min. To measure total carbon (TC), the filters were heated at 950°C for 3 min without prior baking. The amount of organic carbon (OC) was the difference between TC and EC. The total carbon analyzer was calibrated with four glucose standards ranging from 0.047 to 0.510 mg.

Soluble anions analysis. One hundred microliters of particle suspension were diluted to 500 µL and filtered by a 0.22-µm Millipore filter. Soluble nitrates and sulfates in the filtered extracts were determined by ion chromatography (DX–120 ion chromatograph; Dionex, Sunnyvale, CA, USA).

Statistical analysis. To compare IL-8, TNF-α and lipid peroxidation induced by blank, PM1.0, PM1.0–2.5, and PM2.5–10 samples, one-way analysis of variance (ANOVA) with Scheffe mean comparison test was used. Pearson’s correlation coefficient (R) was used to evaluate the relations among IL-8, TNF-α, lipid peroxidation, and particle components. Linear regression models were used to assess...
the contribution of individual particle compositions to IL-8 production and lipid peroxidation. The level of significance for all statistical analyses was chosen as \( p < 0.05 \). All statistical analyses were made using SPSS software (version 9.0; SPSS Inc., Chicago, IL, USA).

### Results

In preliminary tests, we found that ambient particles at 50, 70, and 100 \( \mu \text{g/mL} \) dose dependently stimulated BEAS-2B cells to produce IL-8. The viability of BEAS-2B cells after the incubation with particle extracts at 100 \( \mu \text{g/mL} \) for 8 hr averaged 89 ± 6%, which was not significantly different from that of untreated cells. Particles of the three size ranges stimulated BEAS-2B cells with marked difference in bioactivity (Figure 1). PM\(_{1.0}\) stimulated the highest production of IL-8, which was significantly higher than for blank filters (ANOVA, \( p < 0.05 \)). PM\(_{1.0}\) from different filters had a wide range of effect, with the majority stimulating 2- to 3-fold IL-8 productions compared with blank controls. PM\(_{1.0-2.5}\) did not stimulate BEAS-2B cells to produce IL-8. PM\(_{2.5-10}\) elicited less than double the IL-8 production of controls and was not statistically significant.

Figure 2 shows lipid peroxidation products induced by particles of the three size ranges. There was considerable overlapping in the range of lipid peroxidation products among the four groups. Only PM\(_{1.0}\) resulted in significantly higher lipid peroxidation than that for blank controls (ANOVA, \( p < 0.05 \)). All particle samples taken together, IL-8 production and lipid peroxidation were significantly correlated (\( R = 0.50, p < 0.01 \)), reflecting the greater potency of submicrometer particles regarding the two biologic endpoints. Separated by size, there was little correlation between IL-8 and lipid peroxidation among PM\(_{1.0}\) samples; in contrast, the correlation was significant among PM\(_{2.5-10}\) samples (correlation coefficient = 0.69).

Results of particle-induced cytokine response in macrophage RAW 264.7 cells are shown in Figure 3. PM\(_{1.0}\) induced higher TNF-\( \alpha \) production than particles of the other two size ranges. There was no significant difference in TNF-\( \alpha \) production between PM\(_{1.0-2.5}\) and PM\(_{2.5-10}\). Polymyxin B pre-treatment significantly reduced TNF-\( \alpha \) production, indicating the major role of endotoxin response in macrophages to ambient particles. In polymyxin B–treated particles, PM\(_{1.0}\) stimulated significantly higher TNF-\( \alpha \) production than PM\(_{1.0-2.5}\), which was in turn higher than that for PM\(_{2.5-10}\) (ANOVA with Scheffe test, \( p < 0.05 \)).

Table 1 shows the amount of some particle components that may be related to bioactivity, including major transition metals, EC and OC, anions, and endotoxin, contained in 100 \( \mu \text{g} \) of particle extract. Among transition metals, Mn and Fe were more abundant in coarse particles, whereas the contents of Cu and Zn were highest in PM\(_{1.0-2.5}\). The contents of Ni, V, and Cr were not significantly different among the three size ranges. EC and OC contents were both significantly higher in PM\(_{1.0}\) than in PM\(_{2.5-10}\) because carbon accounted for a higher percentage of mass in PM\(_{1.0}\) (38–63%) than in PM\(_{2.5-10}\) (9–20%). Nitrate content was higher in PM\(_{1.0-2.5}\) and PM\(_{2.5-10}\) than in PM\(_{1.0}\), whereas sulfate content increased with decreasing particle size. Bacterial endotoxin lipopolysaccharide (LPS) was most abundant in coarse particles.

Because PM\(_{1.0}\) induced the most significant cytokine production and lipid peroxidation, we limited the following discussion to this size fraction. Correlation analysis was used to examine whether any of the particle contents were associated with the biologic end points (Table 2). Among transition metals, only Mn was correlated with IL-8 production, with marginal significance. The contribution of metals to IL-8 production was further examined by linear regression analysis. The combined amount of Mn and Cr was significantly associated with IL-8 (\( R^2 = 0.28, p = 0.051 \); Figure 4). Combining other metal components did not improve the regression model. TNF-\( \alpha \) production induced by untreated particles in macrophages was not significantly correlated with any of the particle components; however, as shown in Table 2, TNF-\( \alpha \) production elicited by polymyxin B–pretreated particles was significantly associated with Fe and Cr. Carbon content and LPS were not significantly associated with IL-8 production in BEAS-2B cells. For anions, nitrate content was highly correlated with IL-8 production (\( R = 0.84, p < 0.01 \)). However, the correlation became low with borderline statistical significance (\( R = 0.31, p = 0.13 \)) after one extreme

| Component | IL-8 | Lipid peroxidation | TNF-\( \alpha \) by RAW 264.7 cells |
|-----------|------|-------------------|-----------------------------|
| PM\(_{1.0}\) |      |                   |                             |
| PM\(_{1.0-2.5}\) |      |                   |                             |
| PM\(_{2.5-10}\) |      |                   |                             |

**Table 1.** Comparison of the major components (ng/100 \( \mu \text{g} \) of particle) of three particle sizes (mean ± SD).
data point with high nitrate and IL-8 values was removed.

We then examined the relationship between particle components and PM$_{1.0}$-induced lipid peroxidation in BEAS-2B cells. No transition metal was positively associated with lipid peroxidation; V was negatively correlated with marginal significance. EC and OC were each significantly correlated with particle-induced lipid peroxidation. EC and OC contents were significantly correlated with each other ($R = 0.74$, $p < 0.01$). Total carbon content (the sum of EC and OC) had a slightly better correlation with lipid peroxidation ($R = 0.56$, $p = 0.012$; Figure 5). There was little correlation between lipid peroxidation and either nitrate, sulfate, or LPS.

Discussion

This study tried to better define the size and component effects of ambient particles on respiratory epithelial cells. Particles collected by a trichotomous sampler were extensively characterized to identify particle components responsible for bioactivity. We demonstrated that the ability of ambient particles to elicit inflammatory cytokine production and to cause lipid peroxidation was dependent on size. The effect of components was less defined. The results suggested that cytokine production and lipid peroxidation were associated with different sets of particle components.

The particle-induced cytokine production by epithelial cells was size dependent, being greatest for submicrometer particles and least for PM$_{1.0-2.5}$. For TNF-$\alpha$ production in macrophages, the major factor was bacterial endotoxin, which was responsible for approximately 77% of TNF-$\alpha$ responses. Previous studies have demonstrates that coarse particles elicit higher inflammatory response in monocytes/macrophages than fine particles, with significant contribution of bacterial endotoxin that is more abundant in the coarse fraction (Monn and Becker 1999; Soukup and Becker 2001; Huang et al. 2002). Endotoxin content was greater in our coarse particles, but we observed that submicrometer particles elicit the highest TNF-$\alpha$ response, which remained true after the endotoxin was neutralized by polymyxin B. Therefore, the BEAS-2B and RAW 264.7 data are consistent and support the finding that submicrometer particles cause greater cytokine release than do particles of the other two size ranges. Consistent with our results, a recent study using different sizes of coal fly ash showed that submicrometer particles resulted in more IL-8 production than did fine or coarse fractions in lung epithelium A549 cells (Smith et al. 2000).

We find it unlikely that size effect could be explained by particle components because the amounts of cytokine-inducing components such as Fe, Mn, Cr, and nitrate were smallest in submicrometer particles. This observation suggested that the effect of particle components could be delineated only when restricted to a particular size range. An unexpected result in our study was the lack of bioactivity of PM$_{1.0-2.5}$ in epithelial cells, although these particles were able to induce TNF-$\alpha$ production by macrophages. Incidentally, PM$_{1.0-2.5}$ had the highest content of Cu, which could inhibit cellular generation of superoxide and hydrogen peroxide (Schulter et al. 1995). Such size effects could not be explained by the difference in the ability of particulate matter to adsorb or destroy the IL-8 after it has been secreted. We did find an approximate 40% reduction in IL-8 concentrations in cell-free media after incubating IL-8 with particles for 8 hr. However, the magnitude of reduction was not significantly different among particles of the three size ranges.

The bioactivities of ambient particles have been attributed to the metal components in some studies. For example, the cytokine-inducing properties of particle samples collected in Utah Valley were correlated with their metal contents (Frampton et al. 1999); others have demonstrated a reduction in oxidant and cytokine production when particles are pretreated with metal chelators (Goldsmith et al. 1998; Jimenez et al. 2000). However, studies attempting to identify specific contributions of specific metal components to bioactivity of ambient particles have not generated consistent results. Imrich et al. (2000) found no correlation between macrophage production of cytokines with any of a panel of elements within insoluble particle samples, whereas Prahalad et al. (1999) observed significant association between radical generation in polymorphonuclear leukocytes and insoluble content of particles but not soluble metals. Using submicrometer ambient particles, we showed that Mn and Cr were associated with IL-8 production in epithelial cells, whereas Fe and Cr were associated with TNF-$\alpha$ production in macrophages. Previous in vivo studies showed that chromium could cause pulmonary inflammation in rats and induced pulmonary macrophages to express proinflammatory cytokines and reactive oxygen intermediates (Cohen et al. 1998). However, previous studies have failed to show a good correlation between Cr content in ambient particles and in vitro end points (Prahalad et al. 1999; Imrich et al. 2000). Our findings indicate that the role of metals in cytokine induction could better be illustrated by restricting analyses to submicrometer particles. It is also noteworthy that, although not statistically significant, some metal components such as Cu were negatively associated with cytokine responses. One major limit of the study is the inability to discern soluble and insoluble forms of metals by XRF analysis. Ghio et al. (1999) reported that oxidant generation and cytokine release stimulated by particles in vitro might correlate better to the concentrations of ionizable rather than total metals. Nonetheless, our results support the finding that transition metals in ambient particles play an important role in cytokine induction.

Our findings of EC and OC positively associating with lipid peroxidation induction separately suggest that some organic components might have biologic activity with oxidative property. For example, benzo[al]pyrene in culture caused free radical–induced cell membrane damage, and the effect was enhanced by iron oxide (Garcon et al. 2000). Also, native DEP and organic extracts of DEP were more potent than the stripped DEP in activating nuclear factor-κB and protein kinases (Bonvallot et al. 2001). Another mechanism may be that carbon content serves as an indicator for smaller particle size. Ultrafine particles have higher animal and in vitro toxicity than fine particles, and the effect is independent of the soluble metal content (Brown et al. 2000). This is the first report to correlate the toxicity
of ambient particles to carbon content. Further studies of organic compounds, the relation between carbon content and particle size distribution, and the interaction between carbon and metal contents are likely to be rewarding.

Submicrometer particles were more potent in causing cytokine secretion and lipid peroxidation than were larger particles, but we found no association between the two aspects of particle bioactivity. Cytokine production by residual oil fly ash–stimulated macrophages was inhabitable by antioxidant, suggesting that oxidant production and cytokine production were related (Goldsmith et al. 1998). However, oxidant elicited by various stimuli did not always parallel the cytokine response (Becker et al. 1996; Imrich et al. 1999). Furthermore, cytokine production could be more closely associated with the immediate oxidant production, an indicator of cell activation. The level of lipid peroxidation with 8 hr of particle incubation, as in this study, is more likely a marker of membrane damage. The data suggest that different characteristics of the particles are responsible for each of the two bioactivity measures.

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