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CONTRIBUTION OF ENDOTOXIN IN MACROPHAGE CYTOKINE RESPONSE TO AMBIENT PARTICLES IN VITRO

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Ambient particles may cause pulmonary inflammation with ensuing morbidity. Particle-induced production of proinflammatory cytokines in vitro has been used as an indicator of particle toxicity. To identify particle components that were related to particle toxicity, Andersen dichotomous impactors were used to collect ambient fine ($PM_{2.5}$) and coarse ($PM_{2.5\text{-}10}$) particles in central Taiwan with extraction in endotoxin-free water. Mouse monocyte–macrophage cell line RAW 264.7 cells were exposed to particle extracts at 40 μg/ml for 16 h, and tumor necrosis factor-alpha (TNF-α) was measured in the medium by enzyme-linked immunosorbent assay (ELISA). Cell viabilities were all greater than 82%. Coarse particles stimulated higher TNF-α production than fine particles, and this was associated with greater particulate endotoxin content. Polymyxin B inhibited 42% of TNF-α production elicited by coarse particles and 32% of TNF-α production elicited by fine particles. In fine particles, TNF-α production was negatively correlated with Zn content, while no element in coarse particles correlated with TNF-α production. Results suggest that endotoxin and other components may be important factors for TNF-α production by macrophages in vitro.

Recent epidemiological studies identified acute health effects associated with ambient particulate matter exposure. Cardiovascular and respiratory morbidity and mortality were associated with increased mass concentration of particles, especially those of fine particles (Dockery & Pope, 1994, 1996).

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The mechanisms of particle-induced adverse health effects may involve inflammatory processes in the lungs, which may result in lung injury, reduced clearance of pulmonary pathogens, release of inflammatory mediators to systemic circulation, and hypoxemia in compromised hosts (Becker & Soukup, 1998, 1999; Stringer & Kobzik, 1998; Omara et al., 2000).

In addition to particle size, the toxicity of particles may depend on chemical composition. Several transition metals, notably Fe, As, V, Ni, and Zn, were found to cause physiological changes in cells, such as phosphorylation of kinases, which in turn result in enhanced transcription of proinflammatory cytokines (Dreher et al., 1997; Samet et al., 1998; Broeckaert et al. 1999). However, despite clear evidence from studies with residual oil fly ash or metal components added to cell cultures or animal exposure systems (Carter et al., 1997; Kodavanti et al., 1998; Dye et al., 1999), only a few investigations were able to demonstrate a correlation between metal components and the proinflammatory activity of ambient particles. Broeckaert et al. (1999) found that elimination of particles with their metal content was associated with suppression of tumor necrosis factor-α (TNF-α) production in alveolar macrophages. The ability of particle samples collected in Utah Valley to induce cytokine production was correlated with their metal contents (Frampton et al., 1999), and the use of iron chelators identified iron as an important component in NF-κB activation in respiratory epithelial cells (Jimenez et al., 2000). Goldsmith et al. (1998) showed that metal chelation with deferrioxamine significantly inhibited particulate-induced oxidant production and TNF-α content in alveolar macrophages. Although transition metals are presumed to be the bioactive component, in one study particle-induced oxidant generation in leukocytes was related to insoluble Si, Fe, Mn, Ti, and Co content of particles, but not to soluble transition metals (Prahalad et al., 1999). The variability in results indicates that differences in particle composition as well as limitation in our knowledge in particle toxicity are confounding factors. Dose-dependent effect, physical coexistence of multiple components, and interactions of components are all potential problems that need to be considered.

Bacterial endotoxin is another particle component that may be of biological significance (Mayeux, 1997). The size of bacteria and their debris are in the range of fine to coarse particles, and endotoxin may also adsorb on the surface of other particles. Alveolar macrophages possess CD14 and Toll-like receptors on their surface, and endotoxin can activate these cells to produce proinflammatory cytokines. Several studies found that the bioactivity of particles was associated with endotoxin in the insoluble fraction of particle suspensions (Ning et al., 2000; Soukup & Becker, 2001). In addition to producing inflammation by itself, endotoxin may act synergistically with other components of particles (Ning et al., 2000), and lipopolysaccharide (LPS) priming amplified TNF-α production by both rat and human alveolar macrophages in response to ambient particles (Imrich et al., 1999). In contrast, diesel exhaust particles suppressed the LPS-induced alveolar macro-

phage production of TNF-α and IL-1 (Yang et al., 1999). Data thus indicate that adsorbed components on particulate matter are a determinant response factor.

The contribution of endotoxin in assays in vitro to particle toxicity varies with the cell type used, the cytokine response measured, the components of adsorbed matter, and the sources of particles. In various studies, inhibition of endotoxin by polymyxin B or endotoxin-neutralizing protein abrogated from 40% to almost all of particle-induced cytokine release in monocytes or macrophages (Dong et al., 1996; Bonner et al., 1998; Monn & Becker, 1999; Ning et al., 2000). These results suggest that particle-associated endotoxin might elicit inflammatory responses in alveolar macrophages, but the bioavailability of endotoxin, in part determined by particle size and adsorbed content, needs to be addressed. In this study coarse and fine particles were collected in central Taiwan for the determination of factors involved in particle-induced TNF-α production by macrophages.

METHOD

Particle Collection

Particles were collected in September 2000 at four air quality monitoring stations in central Taiwan, which is a subtropical and densely populated area, and has a mixture of residential, traffic, and industrial pollution sources. Particles were collected by two Andersen dichotomous particle samplers (Thermo Andersen, Bedford, MA) in 24-h period for 3 consecutive days at each station. Coarse particles in size ranging from 2.5–10 μm and fine particles in size less than 2.5 μm in aerodynamic diameter were collected simultaneously onto 37-mm Teflon filters. A total of 15 sets of filters of coarse ($PM_{2.5–10}$) and fine ($PM_{2.5}$) particles were subjected to analyses.

Particle Preparation

All filters were processed for weighing and particle extraction within 3 wk after collection. The filters were equilibrated in 30 ± 5% relative humidity for more than 24 h before weighing. The filters were then submerged in 3 ml of endotoxin-free water (Sigma, St. Louis, MO) and sonicated in water bath (Bandelin Sonorex) for 30 min. The filters were weighed again after sonication. The difference in weight before and after sonication was used as the weight of particle extracted from the filters (particle extract), which contained particles either dissolved or suspended in water. The particle suspension was stored at –20°C, and sonicated for 1 min prior to use.

In Vitro Assays of Particle Bioactivity

RAW 264.7 cells (American Type Culture Collection) were maintained in Dubecco's modified Eagle's medium (DMEM, Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (FBS). For exposure experiments, RAW 264.7 cells were seeded onto 48-well tissue culture plates

(Costar, Corning, NY) at 5×10^5 cells/ml, 0.36 ml/well, and cultured for 24 h. The medium was changed to DMEM containing 40 μg/ml particulate and supplemented with 1% FBS. The supernantant was collected 16 h later, and TNF-α concentration was measured by enzyme-linked immunosorbent assay (OptEIA mouse TNF-α set, Pharmingen). For inhibition assays, the particle suspension was preincubated with 10 μg/ml polymyxin B or 1 m*M* deferoxamine (Sigma, St. Louis, MO) for 60 min before cell stimulation. Viability of cells was determined by trypan blue exclusion. Viability of cells after 16 h of incubation ranged from 82 to 92%. Each tissue culture plate included two unstimulated wells, and the background level of TNF production (typically <100 pg/ml) was subtracted from other wells of stimulated cells on the same plate.

Endotoxin Measurement

The concentration of endotoxin in each particle suspension was measured by *Limulus* amebocyte lysate (LAL) assay (QCL-1000 kit, BioWittaker, Walkersville, MD). The assay was performed according to the manufacturer's instructions. In brief, a 50-μl aliquot of particle extract or endotoxin standards was mixed with 50 μl of the LAL and incubated at 37°C for 10 min. Then 100 μl of substrate was added and incubated for another 6 min. Twenty-five percent acetic acid was added to stop the reaction, and the plate was read at 405 nm by a microplate reader (Bio-TEK Instruments, Winooski, VT). The amount of endotoxin in particle extracts was calculated by interpolation to the standard curve.

X-Ray Fluorescence Analysis

Particles on Teflon filters were examined by an energy-dispersive x-ray fluorescence (XRF) system (model Ex6600AF, Jordan Valley Applied Radiation, Austin, TX) to determine the content of 26 elements, which included Na, Mg, Al, Si, P, S, Cl, K, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Br, Rb, and Pb. Among these, Sc, Co, Ge, As, Se, Br, Cr, and Rb were not included in our statistical analysis due to the low content on filters. The concentration of each element in the particle extract was calculated by multiplying the mass on filter by the extraction ratio, divided by the volume used for sonication of the filter (3 ml). The elemental content used to stimulate cells was calculated by multiplying the concentration by the volume containing 14.4 μg of particle extract (40 μg/ml × 0.36 ml/well).

Statistical Analysis

The difference in particle weight, extraction ratio, endotoxin content, and percentage inhibited by polymyxin B or deferoxamine between coarse and fine particles were compared by Student's *t*-test. Comparison among blanks and fine and coarse particles was conducted by one-way analysis of variance (ANOVA). Pearson's correlation coefficient was used to represent the relation between TNF production and endotoxin or element content. To

assess the contribution of endotoxin in TNF production, linear regression of TNF versus endotoxin content was performed, and *R*-square was calculated. The level of significance for all statistical analyses was set at $p < .05$. All statistical analyses were undertaken using SAS software (release 6.11, SAS Institute, Inc., Cary, NC).

RESULTS

Particle Collection and Extraction

As shown in Table 1, the coarse and fine particles differed significantly in weight, extraction ratio, TNF production capacity, and endotoxin content. The ability of deferoxamine to inhibit TNF production was greater in fine particles, whereas polymyxin B was equally effective in both particulate sizes.

Data in Figure 1 show TNF production elicited by particles. Unstimulated RAW264.7 cells produced TNF spontaneously, typically less than 100 pg/ml. Eleven out of 15 blank samples had very low LPS content (0.0028 ± 0.0005 EU/ml). Extract of these blank filters stimulated some TNF production (range 56–396 pg/ml). Fine particles stimulated higher TNF production than blank filters, but the difference was not statistically significant. TNF production was significantly higher in coarse particles.

Contribution of Bacterial Endotoxin

Extracts of coarse particles had higher LPS content than fine particles. TNF production was significantly associated with the LPS content (Figure 2). By regression analysis, LPS content accounted for 54% of the TNF produced and was markedly higher in coarse particles.

Preincubating particles with polymyxin B significantly attenuated TNF production by 41.9 ± 18.6%, and the reduction was similar with regard to particle size. TNF reduced by polymyxin B correlated significantly with LPS content of particles. Separated by size, the correlation was better for fine particles than coarse particles. After polymyxin B preincubation, coarse par-

TABLE 1. Mass Loadings, Endotoxin Content, and TNF Production of Coarse and Fine Particles in Central Taiwan

	Coarse	Fine
Particle weight (μg)	572 ± 273	379 ± 96[a]
Extraction ratio (%)	83.5 ± 4.8	76.1 ± 4.1[a]
TNF production (pg/ml)	978 ± 488	391 ± 278[a]
Endotoxin content (EU/mg)	2.14 ± 0.88	1.05 ± 0.74[a]
TNF attributable to endotoxin (R^2)	0.24	0.36
TNF reduced by polymyxin B (%)	47.1 ± 17.6	36.7 ± 21.3
TNF reduced by deferoxamine (%)	–4.75 ± 13.9	–24.0 ± 35.3[a]

Note. Data are mean ± SD of 15 samples per group.
[a]Significantly different from coarse ($p < .05$).

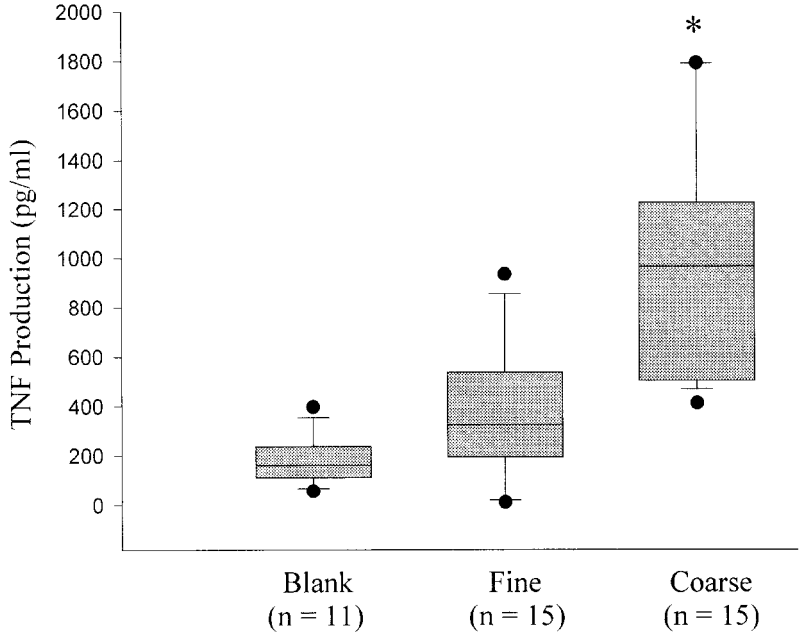

FIGURE 1. Comparisons of TNF-α production in RAW 264.7 cells between coarse and fine particles. Data are box plots displaying the 10th, 25th, 50th, 75th, and 90th percentiles of TNF production in each group. RAW 264.7 cells were incubated with 40 μg/ml particulate matter for 16 h. Asterisk represents significant difference compared with blank ($p < .05$).

ticles still stimulated higher TNF production than fine particles (Table 1). It is noteworthy that the inhibiting effect of polymyxin B (10 μg/ml) preincubation was more effective for fine than coarse particles, as the remaining TNF-inducing capability still correlated with LPS content in coarse but not fine particles. Preincubating particles with deferoxamine was unable to reduce TNF production by RAW cells.

Relationship Between Elemental Composition and TNF Production

As shown in Table 2, the most abundant elements in coarse particles were sodium, sulfur, aluminum, and silica. Among transition metals, iron, copper, and zinc were the most abundant. Fine particles contained fewer amounts of elements that could be identified. The major elements in fine particles were sulfur, silica, and sodium. The content of transition metals was generally lower in $PM_{2.5}$ than in $PM_{2.5-10}$. The amounts of elements contained in 40 μg of extracts (the amount used in cell stimulation experiments) were calculated. Correlation analysis was used to examine if any of the elements was associated with TNF production. For coarse particles, none of the elements was significantly associated with TNF production. For fine particles, only one element, Zn, was significantly associated with the TNF elicited by particle extracts (Figure 3). Since TNF production may be associ-

ated with a major endotoxin component, the correlation between elements and TNF production was examined with polymyxin B-pretreated particle extracts. Results were not markedly different from untreated particle extracts. Incidentally, Zn was also negatively associated with endotoxin content of fine particles. Endotoxin did not seem to confound the negative relation between Zn and TNF, as Zn was also associated with the TNF production elicited by polymyxin B-pretreated particles. Zn was independently associated with TNF production in regression analysis adjusted for endotoxin content.

DISCUSSION

It was shown that coarse particles stimulated higher TNF production than fine particles in murine macrophages. This is consistent with the results of Soukup and Becker (2001) with human alveolar macrophages, in which endotoxin in the insoluble component of coarse particles was identified as a major bioactive moiety. The endotoxin concentrations in Taiwan particulate matter varied from 0.24 to 4.14 EU/mg in particles, with a mean value of 1.59 EU/mg. This range was similar to particle samples collected in Boston (mean of 2.3 EU/mg) (Ning et al., 2000). Polymyxin B binds LPS sto-

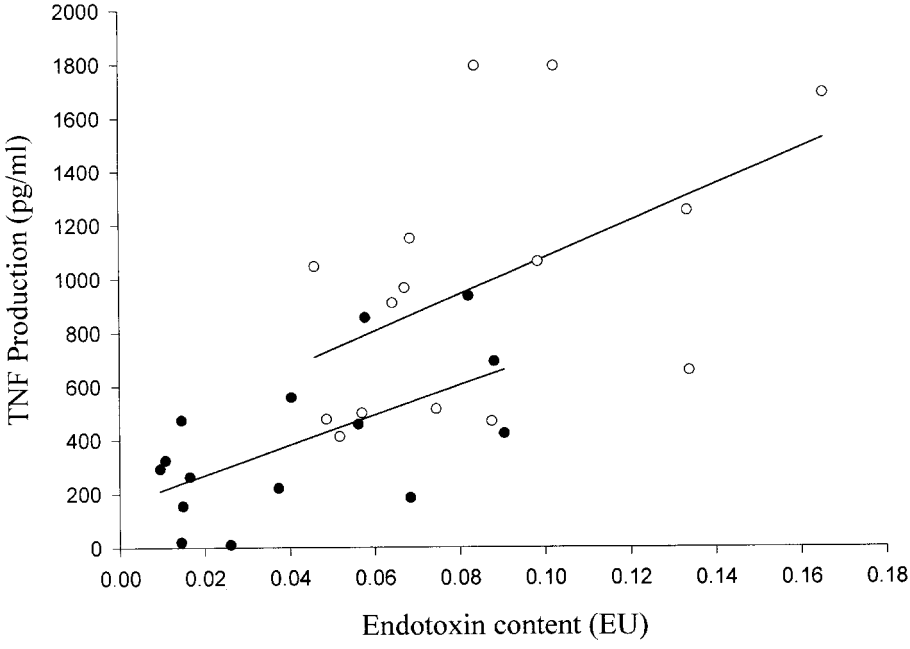

FIGURE 2. Relationship between endotoxin and TNF-α production in RAW 264.7 cells exposed to coarse (○) and fine (●) particles. RAW 264.7 cells were incubated for 16 h with particle extracts. TNF was measured in the medium, and endotoxin concentration in the extracts was determined by LAL assay.

TABLE 2. Mass of 14 Major Elements in Coarse and Fine Particles, and Correlation with TNF Production

	Coarse particles			Fine particles		
	Content (ng/well)	R	R_{PB}	Content (ng/well)	R	R_{PB}
Al	4.84 (ND–15.2)	–0.39	–0.32	0.83 (0.15–1.81)	0.16	0.23
Cu	0.25 (ND–3.46)	0.06	–0.08	0.04 (ND–0.41)	–0.13	–0.03
Fe	0.96 (0.17–3.34)	–0.06	–0.11	0.45 (0.00–0.86)	–0.46	–0.10
Ga	0.34 (0.03–6.13)	–0.03	0.32	0.40 (0.01–1.86)	0.00	0.09
K	0.54 (0.05–0.98)	–0.13	–0.20	0.70 (0.18–1.51)	–0.09	–0.07
Mn	0.07 (ND–0.46)	–0.02	–0.17	0.04 (ND–0.09)	–0.24	–0.09
Na	10.0 (1.45–42.5)	0.26	0.29	2.37 (0.86–4.01)	0.10	–0.03
Ni	0.03 (ND–0.26)	0.05	–0.06	0.04 (ND–0.44)	–0.40	–0.30
P	0.51 (ND–2.71)	0.03	0.08	0.09 (0.03–0.21)	–0.44	–0.56
Pb	0.04 (ND–0.14)	–0.11	–0.23	0.09 (ND–0.32)	–0.22	0.28
S	7.85 (0.86–17.8)	–0.35	–0.30	5.46 (0.97–10.7)	–0.45	–0.35
Si	3.19 (0.91–9.11)	–0.17	–0.08	2.59 (0.72–9.50)	0.49	0.56
V	0.02 (ND–0.10)	–0.19	0.03	0.02 (0.00–0.04)	0.04	0.20
Zn	0.12 (ND–0.37)	–0.14	–0.30	0.17 (0.03–0.26)	–0.82[a]	–0.68[a]

Note. Data presented are mean and range of 15 samples per group. R is the Pearson correlation coefficient of elemental content and TNF production elicited by particle extract; R_{PB} is the correlation coefficient of elemental content and TNF production elicited by polymyxin B-pretreated particle extract.

[a]Significant association ($p < .05$).

ichiometrically, but the exact mode of association between polymyxin B and LPS is not clear (Tsubery et al., 2000). Polymyxin B at 10 μg/ml has been used to neutralize endotoxin associated with particles and was able to abrogate macrophage MIP-2 production induced by 50 ng/ml LPS (Ning et al., 2000), which is much higher than the endotoxin content of ambient particles. Polymyxin B was reported to inhibit about 41% of MIP-2 and 82% of TNF-α production by ambient particles (Ning et al., 2000) and completely inhibited TNF production by alveolar macrophages (Dong et al., 1996).

In this study two methods were used to estimate the contribution of endotoxin to particle-induced TNF production: (1) the percentage of TNF production that could be inhibited by polymyxin B, and (2) linear regression analysis of TNF production versus endotoxin content in particle preparations. For fine particles, both methods yielded the same estimation, with about 36% of TNF production attributable to endotoxin. For coarse particles, although 47% bioactivity was inhibited by polymyxin B, the contribution of endotoxin as assessed by linear regression was only 24%, suggesting the presence of other active components that may travel together with the LPS content.

Imrich et al. (2000) suggested that endotoxin is mostly associated with the insoluble fraction of particles, and that endotoxin embedded in particles may not be inhibited by polymyxin B. Our study showed that polymyxin B was not able to neutralize all the LPS in coarse particles, as TNF production

stimulated by polymyxin B-treated particle extracts were still significantly associated with the original endotoxin content of coarse particles. Thus, the percentage of cytokine production inhibited by polymyxin B may underestimate the contribution of endotoxin or other components. In contrast, the lower estimation of the contribution of endotoxin by linear regression (24%) suggested that not all endotoxin, as detected by LAL assay, had the same magnitude of bioactivity. The discrepancy in estimation (47% vs. 24%) indicated that polymyxin B may have overestimated the contribution of endotoxin. The geometry of the particle and the actual location of endotoxin on the particle may affect the bioavailability of endotoxin. This consideration is especially relevant for coarse particles. An alternative explanation for the discrepancy is that other particle components may also interfere with the LAL assay, suggesting that polymyxin B alone is ineffective in complete inhibition of TNF production.

Cellular recognition of endotoxin is facilitated by LPS-binding protein (LBP), CD14, and Toll-like receptors on macrophages (Mayeux, 1997). Under normal conditions, because of the relative scarcity of LBP, the endotoxin associated with inhaled particles may be sequestered in the lipid layer of surfactant or bound to surfactant-associated proteins, which will render the endotoxin functionless (Martin, 2000). However, a recent study demon-

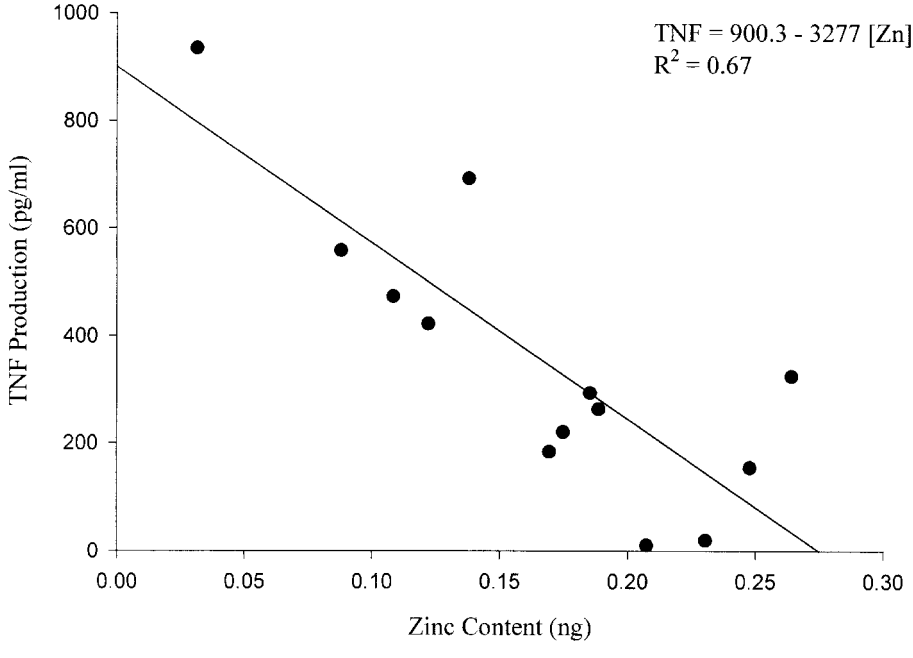

FIGURE 3. Relationship between zinc contents and TNF production in RAW 264.7 cells exposed to fine particles.

strated an inverse relation between house dust endotoxin exposure and the frequency of interferon-γ-producing T cells, where inhaled endotoxin, although at low concentrations, produced a significant physiological effect (Gereda et al., 2000). The proinflammatory effects of endotoxin associated with ambient particles in humans remain to be evaluated.

The relation between metal components and cytokine responses in vitro were difficult to demonstrate. Prahalad and colleagues (1999) observed significant association between radical generation in polymorphonuclear leukocytes and insoluble content of particles. Similarly, metallic content in particles was attributed to contribute to inflammatory processes in alveolar macrophages (Broeckaert et al., 1997, 1999; Dreher et al., 1997). In contrast, Imrich et al. (2000) found no correlation between macrophage production of MIP-2 and TNF with any of a number of elements quantified within insoluble particle samples. The discrepancy may be due to differences in the cell used, variation in components attached to particles, and the bioactivity measured. In addition, XRF analysis does not indicate the electric charge and chemical form of the elements, which may be vital determining factors in bioavailability, such as solubility.

In this study a negative association was observed between zinc and TNF production. Zinc and several other transition metals at relatively high concentrations (500 μ*M*) were shown to produce kinase activation and IL-8 production in human airway epithelial cells (Samet et al., 1998). In contrast, preincubating human monocytes with zinc (20 μ*M*) reduced cytokine response when these cells were stimulated with LPS later (Koropatnick & Zalups, 1997). Zinc may also bind protein thiols and inhibit NF-κB binding to DNA (Shumilla et al., 1998). Metal concentration is a factor to be considered in interpreting the results; another concern is the matrix effect of particle extracts. With the complex metal composition and the presence of other bioactive components in particle extracts, the effect of individual metal elements may be modified and may become dependent on the concentration as well as the matrix. Furthermore, the negative association between zinc and cytokine response could also be specific to the cell type used and the cytokine measured.

The lack of inhibition by deferoxamine in this experiment is reasonable, considering the absence of correlation between TNF production and most metals. It is also consistent with previous reports showing that antioxidants did not inhibit urban particle-induced TNF production by rat macrophages (Dong et al., 1996) and that deferoxamine inhibited cytotoxicity but not cytokine production by outdoor particles (Monn & Becker, 1999).

In summary, endotoxin was found to contribute differently to the in vitro bioactivity of coarse versus fine particles. For fine particles, endotoxin contributed to ~36% of TNF production, while for coarse particles, the contribution was difficult to assess, since not all endotoxin could be inhibited by polymyxin B. Bioavailability and matrix effect may be important factors in linking particle component with bioactivity.

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