Proinflammatory and Cytotoxic Effects of Mexico City Air Pollution Particulate Matter in Vitro Are Dependent on Particle Size and Composition

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Exposure to urban airborne particulate matter (PM) is associated with adverse health effects. We previously reported that the cytotoxic and proinflammatory effects of Mexico City PM10 (≤ 10 µm mean aerodynamic diameter) are determined by transition metals and endotoxins associated with these particles. However, PM2.5 (≤ 2.5 µm mean aerodynamic diameter) could be more important as a human health risk because this smaller PM has the potential to reach the distal lung after inhalation. In this study, we compared the cytotoxic and proinflammatory effects of Mexico City PM10 with those of PM2.5 using the murine monocytic J774A.1 cell line in vitro. PMs were collected from the northern zone or the southeastern zone of Mexico City. Elemental composition and bacterial endotoxins on PMs were measured. Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) production by J774A.1 cells was measured in the presence or absence of recombinant endotoxin-neutralizing protein (rENP). Both northern and southeastern PMs contained endotoxin and a variety of transition metals. Southeastern PM10 contained the highest endotoxin levels, 2-fold higher than that in northern PM10. Northern and southeastern PM2.5 contained the lowest endotoxin levels. Accordingly, southeastern PM2.5 was the most potent in causing secretion of the proinflammatory cytokines TNF-α and IL-6. All PM2.5 and PM10 samples caused cytotoxicity, but northern PMs were the most toxic. Cytokine secretion induced by southeastern PM2.5 was reduced 50–75% by rENP. These results indicate major differences in PM10 and PM2.5. PM2.5 induces cytotoxicity in vitro through an endotoxin-independent mechanism that is likely mediated by transition metals. In contrast, PM10 with relatively high levels of endotoxin induces proinflammatory cytokine release via an endotoxin-dependent mechanism. Key words: apoptosis, cytotoxicity, endotoxin, IL-6, interleukin-6, J774A.1 cells, Mexico City, particle composition, particulate matter, PM10, PM2.5, TNF-α, tumor necrosis factor-α. Environ Health Perspect 111:1289–1293 (2003). doi:10.1289/ehp.5913 available via http://dx.doi.org/[Online 25 April 2003]

Daily exposure to airborne particulate matter (PM) pollution in urban zones is associated with an increase in morbidity and mortality (Krewski et al. 2000). The increase in health risk occurs even when ambient PM concentrations are below established air quality standards. Epidemiologic associations have been done using current air quality standards that only encompass particle size and concentration; these studies did not address the issue of particle composition. Epidemiologic findings from several cities around the world indicate that the greatest health risks correlate with smaller particles, which have the ability to reach the distal regions of the lung after inhalation (Levy et al. 1999). However, the existence of some variation in the biologic effects of PMs from different cities has brought attention to possible roles of the various organic and inorganic components in the PM mixture in mediating adverse health effects (Krewski et al. 2000; Levy et al. 1999; Mar et al. 2000; Pozzi et al. 2003; Schins et al. 2002).

In particular, components such as transition metals, polycyclic aromatic hydrocarbons, and bacterial components (e.g., endotoxin) are known to be associated with PM, either through adherence to the core particle or as an integral component of the particle (Alfaro-Moreno et al. 2002; Bonner et al. 1998; Dreher et al. 1997; Dye et al. 2001; Holian et al. 1998; Monn and Becker 1999; Nel et al. 2001). We previously reported that the cytotoxic and proinflammatory effects of Mexico City PM10 (≤ 10 µm mean aerodynamic diameter) are largely determined by variations in metal and endotoxin content (Alfaro-Moreno et al. 2002; Bonner et al. 1998). The primary objective of this study was to compare PM10 with PM2.5 (≤ 2.5 µm mean aerodynamic diameter) in order to determine if Mexico City PM2.5 also mediates its biologic effects in the same way as PM10. Here we report that PM2.5, unlike PM10, possesses lesser quantities of endotoxin and therefore likely causes cytotoxicity through an endotoxin-independent mechanism that could involve transition metals.

Materials and Methods

Reagents. We used the Limulus assay (BioWhittaker, Walkersville, MD, USA) to detect endotoxin; the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method (Roche, Mannheim, Germany) to detect apoptosis; and enzyme-linked immunosorbent assay (ELISA) to detect tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (both kits from R&D Systems, Minneapolis, MN, USA). We obtained the II XTT cell proliferation-cytotoxicity kit from Roche Diagnostics Corporation (Indianapolis, IN, USA). We purchased TNF-α from Upstate Biotechnology (Lake Placid, NY, USA) and bovine fetal serum from Harlan (Indianapolis, IN, USA). Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 was obtained from Sigma (St. Louis, MO, USA). Recombinant endotoxin-neutralizing protein (rENP) neutralizes the bioactivity of LPS as measured by the Limulus amebocyte lysate assay when used in a 1:1 ratio (weight) of rENP/LPS (Cape Cod Associates, Cape Cod, MA, USA).

PM sampling. Particles were collected in the northern zone (Gustavo A. Madero) and southeastern zone (Iztapalapa) of Mexico City. The northern zone is characterized by industrial activity, and much of the PM in the southeastern zone is derived from windborne dust from the dried lake basin of Texcoco. Both northern and southeastern zones have heavy motor vehicle traffic, which...
also contributes to PM composition. PMs were collected using high-volume particle collectors (Sierra Andersen, Smyrna, GA, USA). We used fiberglass filters (type A/E glass; Gelman Sciences, Ann Arbor, MI, USA) for 24-hr sampling (1.13 m²/min). Samples were collected during 7 days of the dry-warm season (March–May) of 2000. Filters were stabilized at 22°C ± 3°C, 50% ± 5% humidity during 24 hr. Filters were weighed before and after air sampling, using an analytical balance (A200-S; Sartorius, Goettingen, Germany). After weighing, filters were dry sonicated for 45 min to recover particles by smooth sweeping with a brush in an endotoxin-free flask. PM₁₀ and PM₂.₅ were pooled by zone and stored in endotoxin-free glass vials in a drier at 4°C until their use. Sterile suspensions of PM (1 mg/mL) were prepared immediately before cell exposure. Although contamination of the PM samples with fibers from the filters does occur, the amount of fibers found at the concentrations used for cell experimentation does not cause detectable biologic effects (Martinez-Romero 2001).

**Elemental analysis of PM samples by proton-induced X-ray emission.** Samples of airborne particles were ground using an agate mortar and then introduced in bags made of 3.5-µm-thick Mylar. These samples were analyzed using proton-induced X-ray emission (PIXE) for elements heavier than argon (atomic number Z = 19), using the external beam setup developed by Ruvalcaba et al. (2001). A 2.95 MeV proton beam was employed as primary radiation (Pelletron Accelerator; National Electrosportics Corporation, Middleton, WI, USA), and the emitted X-rays were collected with a Canberra LEGE detector (Canberra Industries, Meriden, CT, USA). Standard electronics were used to process the signals from the detector, and spectra were collected using an Oxford PCA3 multichannel analyzer (Oxford Industries-Tennecle, Oak Ridge, TN, USA). The spectra were deconvoluted using the QXAS computer code (IAEA 1995). No exact quantitative results can be given for elemental concentrations in this experiment because of sample characteristics; therefore, ratios of each element’s X-ray peak areas to those of the Kα line from atmospheric argon (which serves as an equivalent beam total charge integration) were used as an indication of the elemental contents in every sample. Soil-related and non-soil-related elements were defined according to a previous report (Miranda et al. 2000).

**Endotoxin.** PMs were suspended (1 mg/mL) in endotoxin buffer (0.05 M potassium phosphate and 0.1% triethylamine, pH 7.5) and sonicated for 60 min at 20°C (Bath Sonicator 5200; Branson Ultrasonics, Danbury, CT, USA). After 1 min of centrifugation at 16,600 × g supernatants were diluted and endotoxin was measured by a Limulus amebocyte lysate assay kit according to manufacturer’s specifications (BioWhittaker). Blank filters do not contain detectable endotoxin, and storage does not add endotoxin to the PM samples (Bonner et al. 1998).

**Cytotoxicity.** Cytotoxicity was measured using the crystal violet method (Kuang et al. 1989). To assess cytotoxicity, proliferating cultures of J774A.1 cells were exposed to particle concentrations of 20, 40, and 80 µg/cm² of PM samples. Cells were seeded in 96-well plates at a density of 15,000 cells/cm² to determine cell viability after 72 hr of exposure. Cytotoxicity was determined measuring the residual cell number by crystal violet staining with an ELISA plate reader at 595 nm (Multiscan MS 352; LabSystems, Helsinki, Finland). Percentage viability was calculated comparing the absorbance of exposed cultures with the absorbance of nonexposed cultures.

**Apoptosis assays.** Apoptosis was measured in J774A.1 cells using the TUNEL assay (Gorczyca et al. 1993) using a commercial kit. Cells were plated on two-well culture slides (BD Falcon, Bedford, MA, USA) at a density of 15,000 cells/cm² in 1% fetal bovine serum–Dulbecco’s modified Eagle medium (FBS-DMEM) and antibiotics. After 8 hr, the medium was changed for complete medium (10% FBS-DMEM + antibiotics), and particles from both zones were added at 80 µg/cm². Cells were exposed to particles for 24 hr and then fixed with 4% formaldehyde. After fixation, the TUNEL assay was performed following the manufacturer instructions (Roche Diagnostics Corporation). Cells were analyzed under fluorescence microscopy. Fluorescent cells were considered apoptotic and were expressed as a percentage of the total number of cells counted in 10 randomly selected fields.

**Cytokine assays.** J774A.1 cells cultured were grown to confluency in 24-well plates, maintained in serum-free DMEM for 24 hr and then exposed to increasing concentrations of PM₁₀ (20, 40, and 80 µg/cm²) in 1 mL serum-free DMEM. After 24 hr, cell supernatants were collected, centrifuged at 14,000 × g for 15 min, and frozen at −70°C. Nonexposed cells were used as negative controls, and cells exposed to 10 µg/mL LPS from *E. coli* 055:B5 (Sigma) as positive controls. We measured TNF-α and IL-6 in the supernatants using undiluted samples and an ELISA kit following manufacturer recommendations (R&D Systems). Experiments were run in parallel using 2 µg/mL rENP. Results are expressed in picograms per milliliter.

**Conditioned medium cytotoxicity on L929 cells.** To assess biologic activity for the TNF-α produced by J774A.1 cells stimulated by particles, the L929 cytotoxicity assay was used (Branch et al. 1991). L929 cells were plated on 96-well plates at a density of 30,000 cells/well. After 24 hr, medium was changed and 50 µL fresh medium and 50 µL of the medium collected from J774A.1 cells exposed to particles was added in the presence of 1.2 µg/mL actinomycin D. After 18 hr, we added 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and carried out the procedure following manufacturer instructions (Roche Diagnostics Corporation). All supernatants

### Table 1. Mean particle mass on the filters used for experimentation, March–May 2000.

| Particles | Zone          | PM₁₀ (µg/m²) | PM₂.₅ (µg/m²) | PM₁₀/PM₂.₅ |
|-----------|---------------|--------------|---------------|------------|
|           | Northern      | 4.0 ± 4      | 54 ± 6        | 0.04 ± 0.08 |
|           | Southeastern  | 82 ± 9       | 114 ± 6       | 0.04 ± 0.08 |

| *Values represent the mean ± standard error from seven 24-hr samples. |

### Table 2. Ratios of X-ray peak areas to argon Kα X-rays.

| Element       | PM₁₀                 | PM₂.₅                | PM₁₀/PM₂.₅                |
|---------------|----------------------|----------------------|---------------------------|
|               | 2.459 (0.131)°        | 1.037 (0.051)°       | 2.574 (0.164)°            |
|               | 7.114 (0.264)°        | 1.242 (0.122)°       | 5.033 (0.030)°            |
|               | 2.674 (0.141)°        | 0.465 (0.029)°       | 2.667 (0.138)°            |
|               | 0.000 (0.000)         | 0.217 (0.023)°       | 0.293 (0.022)°            |
|               | 0.016 (0.000)         | 0.217 (0.023)°       | 0.293 (0.022)°            |
|               | 2.473 (0.131)°        | 0.011 (0.004)°       | 0.084 (0.053)°            |
|               | 0.027 (0.000)         | 0.609 (0.039)°       | 0.303 (0.003)°            |
|               | 2.469 (0.125)°        | 2.667 (0.138)°       | 8.094 (0.063)°            |
|               | 0.000 (0.000)         | 0.027 (0.000)°       | 0.000 (0.000)°            |
|               | 3.516 (0.100)°        | 0.116 (0.004)°       | 0.116 (0.000)°            |
|               | 0.032 (0.007)°        | 0.076 (0.010)°       | 0.076 (0.003)°            |
|               | 0.047 (0.008)°        | 0.047 (0.008)°       | 0.047 (0.008)°            |

| *Elements with larger areas are included in each sample. |

### Table 3. Endotoxin concentrations on the particulate matter.

| Particles | Zone          | PM₁₀ (EU/mg) | PM₂.₅ (EU/mg) |
|-----------|---------------|--------------|--------------|
|           | Northern      | 21.69         | 12.22         |
|           | Southeastern  | 30.09         | 59.34         |

| EU, endotoxin units. |
from the J774A.1 cells exposed to PM$_{2.5}$ and PM$_{10}$ from northern and southeastern Mexico City were tested. Results are expressed as the percentage of viable cells in relation to supernatants from unexposed J774A.1 cells.

**Statistical analysis.** All experiments were repeated at least three times, and results are expressed as mean ± standard deviation. Statistical significance was evaluated by analysis of variance for a factorial design (Armitage and Berry 1987). Factors tested were area, particle size, and particle concentration. Also, when appropriate, the role of rENP was tested as an additional independent factor. Models were developed to test all significant interactions among these factors. All analyses were performed with Intercooled Stata for Windows, Version 6.0 (Stata Corporation, College Station, TX, USA). Alpha level was set at $p = 0.05$.

**Results**

**Particle levels.** Gravimetric results from PM$_{2.5}$ and PM$_{10}$ samples collected in northern and southeastern Mexico City during the dry-warm season of the year 2000, as well as PM$_{2.5}$:PM$_{10}$ ratios, are presented in Table 1. Particle levels were approximately 40% higher in the southeastern zone compared with the northern zone, although the PM$_{2.5}$:PM$_{10}$ ratios were similar in both zones.

**Elemental analysis of PM samples.** A total of 13 elements were found. Ratios of the X-ray peak areas for every element to those of atmospheric argon are shown in Table 2. The elements are more abundant in PM$_{2.5}$ than in PM$_{10}$, as expected, given that the first fraction includes the second one. However, differences are observed for both zones. Contents of elements associated with soil (potassium, calcium, titanium, manganese, iron, strontium, and zirconium) predominate in all samples. However, so-called anthropogenic elements (copper, zinc, nickel, and lead) presented larger ratios in the northern zone than in the southeastern zone.

**Endotoxin content on PM.** Endotoxin levels in the PM$_{2.5}$ and PM$_{10}$ samples are presented in Table 3. All samples had detectable levels of endotoxin. PM$_{10}$ from the southeastern zone had the highest content (59.34 endotoxin units (EU)/mg).

**Cytotoxicity.** PMs caused a concentration-dependent cytotoxic effect that was apparent after 72 hr in proliferating J774A.1 cells from monocytic origin (up to 68%) (Figure 1). Particles from the northern zone showed a statistically significant larger effect compared with particles from the southeastern zone ($p < 0.0001$). The cytotoxic effects induced by PM$_{2.5}$ and PM$_{10}$ from the northern zone were not significantly different. PM$_{2.5}$ from the southeastern zone was significantly more toxic than PM$_{10}$ from the southeastern zone ($p < 0.0012$). Particles do not induce cytotoxic effects under experimental conditions when cells are confluent (Bonner et al. 1998; Alfaro-Moreno et al. 2002).

**Apoptosis.** PMs induced apoptosis in J774A.1 monocytic cells (Figure 2). PMs from the northern zone induced more apoptosis (~30%) than did particles from the southeastern zone (~12%) ($p < 0.0001$). There were no differences in apoptosis between PM$_{10}$ and PM$_{2.5}$.

**Presence of TNF-α and IL-6 in supernatants of J774A.1 cells.** Particles induced TNF-α and IL-6 secretion in J774A.1 monolayer cell lines in a concentration-dependent manner, and maximal secretion was attained with 80 µg/cm$^2$. PM$_{10}$ from the southeastern zone caused a significantly greater production of TNF-α compared with all other particles from the northern and southeastern zones ($p < 0.004$) (Figure 3A). rENP induced a statistically significant ($p < 0.0001$) reduction on TNF-α levels for all doses and PM fractions tested (Figure 3B). Biologic activity of the TNF-α present in those supernatants was tested on susceptible L929 cells (Figure 4). Conditioned medium from J774A.1 monocytic cells exposed to PM$_{10}$ and PM$_{2.5}$ from the northern and southeastern zones induced cytotoxic effects on L929 cells that correlate with the levels of TNF-α present in the supernatants ($p < 0.0001$) (Figure 4A). The supernatants obtained from cells that were exposed to particles in the presence of rENP, in which an inhibition of TNF-α secretion was observed, induced a parallel decrement in cytotoxicity ($p < 0.0001$) (Figure 4B).

PM$_{10}$ from the southeastern zone caused the greatest production of IL-6 ($p < 0.007$) (Figure 5A). In this case, rENP noticeably blocked the secretion of IL-6 stimulated by PM$_{10}$ from the southeastern zone ($p < 0.0001$) (Figure 5B). Negative control unexposed cells secreted 89.32 ± 9.65 pg/mL of TNF-α, whereas cells stimulated with LPS (10 µg/mL) reached levels of 9921.93 ± 40.73 pg/mL. In the case of IL-6, unexposed cells (negative control) secreted 16.18 ± 2.41 pg/mL, whereas cells stimulated with 10 µg/mL LPS secreted 2,356 ± 168.87 pg/mL. Because positive-control experiments in the presence of rENP were done in excess LPS (2 µg/mL rENP vs. 10 µg/mL LPS), the inhibition of TNF-α and IL-6 secretion was not greater than 15%. Levels of cytokines produced by unexposed cells in the presence of rENP were not affected. No cytotoxic effects were observed under these culture conditions, when cells are confluent.
A comparative summary of the cellular effects induced by PM from both zones of Mexico City is presented in a semiquantitative fashion in Table 4.

Discussion
The majority of research articles published on air pollution PM relate to PM$_{10}$. However, increasing emphasis and stringent regulations have been placed on PM$_{2.5}$. The primary objective of this study was to determine if Mexico City PM$_{2.5}$ possessed similar biologic effects compared with PM$_{10}$. Our results indicate that PM from Mexico City induced differential biologic effects related to zone of sampling and the size of the particles. Southeastern PM$_{10}$ strongly induced the secretion of TNF-α and IL-6 (Figures 3 and 5), and this was due at least in part to endotoxin, because the rENP reduced cytokine release in response to these particles. Southeastern PM$_{10}$ also contained the highest levels of endotoxin (Table 3). PM$_{10}$ from the northern region and PM$_{2.5}$ from both the northern and southeastern regions of Mexico City had potent cytotoxic effects. Therefore, our data indicate important differences in the biologic effects induced by PM$_{2.5}$ compared with PM$_{10}$.

An evaluation of nearly equal PM$_{2.5}$/PM$_{10}$ ratios from particles from the northern and southeastern zones indicates that the differences in cytotoxicity and proinflammatory cytokine production observed among the PMs were not merely due to particle size. A higher proportion of non-soil-related metals (copper, zinc, nickel, and lead) in particles from the northern zone could account for the differences in cytotoxic effects on proliferating cells, as has been proposed by others using residual oil fly ash particles released from the industrial burning of fuel oil (Dreher et al. 1997) or “real-world” ambient particles collected from urban areas (Dye et al. 2001; Vincent et al. 2001). Endotoxin content on PM$_{10}$ from the southeastern region could explain the proinflammatory potential of these particles because the cytokine stimulatory effects of these particles on confluent cell cultures were blocked by rENP. Moreover, the endotoxin level extracted from PM$_{10}$ from the southeastern region (59.34 EU/mg) was 2-fold higher than the endotoxin level from PM$_{10}$ from the northern region (Table 3). Relatively low endotoxin levels were obtained from PM$_{2.5}$ from the northern (21.69 EU/mg) and the southeastern regions (12.22 EU/mg), and PM$_{2.5}$ caused less proinflammatory cytokine secretion (Figures 3 and 5). Interestingly, composition and the cellular effects induced by PM$_{2.5}$ from the two regions were homogeneous. This suggests that differences on the components present in the coarse fraction

### Table 4. Semiquantitative comparative appreciation of cellular effects induced by PM$_{2.5}$ and PM$_{10}$ from Mexico City.

| Proinflammatory | Cytotoxicity | Apoptosis | TNF-α | IL-6 |
|-----------------|-------------|----------|------|------|
| Northern PM$_{10}$ | +++ | +++ | + | + |
| Northern PM$_{2.5}$ | +++ | +++ | + | + |
| Southeastern PM$_{10}$ | ++ | + | +++ | +++ |
| Southeastern PM$_{2.5}$ | ++ | + | + | + |

*The number of +s indicates the magnitude of the observed effects. See figures for the statistical significance of each observation.*
have also reported that endotoxin mediates proinflammatory cytokine production by either indoor or outdoor air pollution particulates (Mann and Becker 1999). Moreover, we previously reported that Mexico City PM$_{10}$ up-regulated the platelet-derived growth factor α-receptor subtype on lung myofibroblast via an endotoxin-dependent mechanism (Bonner et al. 1998). Thus, our findings clearly indicate that endotoxin is the major component that mediates the proinflammatory effects of PM$_{10}$ from Mexico City.

In conclusion, our results support the hypothesis that particle composition in different PM size fractions may account for the differences in the inflammatory and toxic responses induced by air pollution particles from two different zones of Mexico City. In general, northern zone PMs have higher levels of transition metals. Southeastern zone PM$_{10}$ also contained high levels of endotoxin and was a potent inducer of proinflammatory cytokines via an endotoxin-dependent mechanism. In contrast, PM$_{10}$ from the northern zone and PM$_{2.5}$ from both zones behaved homogeneously and caused cytotoxicity but were not strong inducers of proinflammatory cytokine release. We propose that the use of biologic response parameters, such as the summary presented in Table 4, could prove useful when assessing the biologic impact of complex pollutant particulate mixtures.

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