

Size-Dependent Proinflammatory Effects of Ultrafine Polystyrene Particles: A Role for Surface Area and Oxidative Stress in the Enhanced Activity of Ultrafines

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Studies into the effects of ultrafine particles in the lung have shown adverse effects considered to be due in part to the particle size. Air pollution particles (PM₁₀) are associated with exacerbations of respiratory disease and deaths from cardiovascular causes in epidemiological studies and the ultrafine fraction of PM₁₀ has been hypothesized to play an important role. The aim of the present study was to investigate proinflammatory responses to various sizes of polystyrene particles as a simple model of particles of varying size including ultrafine. In the animal model, we demonstrated that there was a significantly greater neutrophil influx into the rat lung after instillation of 64-nm polystyrene particles compared with 202- and 535-nm particles and this was mirrored in other parameters of lung inflammation, such as increased protein and lactate dehydrogenase in bronchoalveolar lavage. When surface area instilled was plotted against inflammation, these two variables were directly proportional and the line passed through zero. This suggests that surface area drives inflammation in the short term and that ultrafine particles cause a greater inflammatory response because of the greater surface area they possess. *In vitro*, we measured the changes in intracellular calcium concentration in mono mac 6 cells in view of the potential role of calcium as a signaling molecule. Calcium changes after particle exposure may be important in leading to proinflammatory gene expression such as chemokines. We demonstrated that only ultrafine polystyrene particles induced a significant increase in cytosolic calcium ion concentration. Experiments using dichlorofluorescein diacetate demonstrated greater oxidant activity of the ultrafine particles, which may explain their activity in these assays. There were significant increases in IL-8 gene expression in A549 epithelial cells after treatment with the ultrafine particles but not particles of other sizes. These findings suggest that ultrafine particles composed of low-toxicity material such as polystyrene have proinflammatory activity as a consequence of their large surface area. This supports a role for such particles in the adverse health effects of PM₁₀. © 2001 Academic Press

Key Words: ultrafine; calcium; oxidative stress; polystyrene; inflammation.

The effect of air pollution particles (PM₁₀) on mortality and morbidity are well documented and include exacerbations of airways disease and deaths from cardiorespiratory causes (Dockery and Pope, 1994). PM₁₀ consists of a range of components, many of which have been implicated in adverse health effects. Candidates include ultrafine particles (MacNee and Donaldson, 1999), endotoxin (Ning *et al.*, 2000), and metals (Gilmour *et al.*, 2000). In long-term, high-dose inhalation studies, ultrafine particles of various types have been shown to cause lung inflammation, increased chemokine expression, epithelial cell hyperplasia, pulmonary fibrosis, and lung tumors (Ferin *et al.*, 1992; Oberdoster *et al.*, 1994; Nikula *et al.*, 1995; Dasenbrock *et al.*, 1996; Driscoll *et al.*, 1996). The effects of the long-term studies have been considered to be a consequence of lung overload (Mauderly, 1996). In the short-term, low-exposure studies with ultrafine carbon black (UfCB) produces mild inflammation compared to fine carbon black (CB), oxidant stress, and modulation of the coagulation system in normal rats (Li *et al.*, 1999).

One important question regarding the mechanism of lung injury caused by ultrafine particles is whether ultrafine particles merely provide a large surface area for the release of transition metals. Transition metals, as mentioned previously, provide a potential mechanism for the effect of PM₁₀ and have been found to explain the inflammatory effects of residual oil fly ash (Dreher *et al.*, 1997) and Provo PM₁₀ (Kennedy *et al.*, 1998). With respect to ultrafine particles we have shown that neither soluble extracts of UfCB nor UfCB particles treated with iron chelators are inflammogenic when instilled into the lungs of rats (Brown *et al.*, 2000) and UfCB, while highly inflammogenic, has a very low transition metal content. We hypothesize that, in the absence of a transition metal effect, inflammation produced in the lungs of UfCB-instilled rats is due to either surface area or particle number effects. Increased oxidative activity of the large surface area is a likely factor and, in the

present study, polystyrene particles of different diameters were investigated for inflammogenicity *in vitro* and *in vivo* and oxidant activity. Polystyrene particles are not complicated by transition metals nor do they have the complexity of minerals, being composed of a polymer.

The *in vivo* model used here served to illustrate the differences in inflammatory potential of the different-sized polystyrene particles. The *in vitro* model has been used previously to demonstrate a greater effect of UfCB than CB on the cytosolic calcium ion concentration of macrophages (Stone *et al.*, 2000). Activation of the proinflammatory transcription factor NF- κ B is regulated via a number of second messengers, including Ca^{2+} (Dolmetsch *et al.*, 1998). On stimulation of the cell, Ca^{2+} is released from the endoplasmic reticulum stores, which leads to a calcium ion influx across the plasma membrane via calcium channels (Hoyal *et al.*, 1998). Different pathogenic particles have been reported to cause changes in calcium ion flux within the cell (Faux *et al.*, 1994; Lim *et al.*, 1997) and there may be a role for calcium homeostasis in the proinflammatory effects of ultrafine particles. We have also used here a cell-free assay system based on the oxidation of dichlorofluorescein diacetate (DCFDA) to a fluorescent product dichlorofluorescein (DCF) (Robinson *et al.*, 1988) to test the oxidative activity of the particle surface in the absence of cells.

Finally, the expression of the proinflammatory cytokine interleukin-8 (IL-8) has been shown to be induced in airway epithelial cells with exposure to residual oil fly ash (ROFA) particles (Carter *et al.*, 1997). Rapid induction of IL-8 mRNA suggests that the biological effect is an initial acute lung inflammation. We tested the hypothesis that particle size plays a major role in the initiation of lung inflammation by measuring IL-8 mRNA expression in A549 epithelial cells.

MATERIALS AND METHODS

Polystyrene Microsphere Characterization

Polystyrene microspheres were purchased from Polysciences (Park Scientific, Northampton, U.K.) in three sizes: 64, 202, and 535 nm average diameter. Microspheres were suspended at a concentration of 1 mg/ml in saline. In terms of particle number, 1 mg contained 6.95×10^{12} , 2.21×10^{11} , and 1.19×10^{10} particles for 64, 202, and 535 nm, respectively. Five microliters of each suspension were pipetted on to the surface of 200-mesh size carbon-coated electron microscope (EM) grids (Agar Scientific). Grids were placed on filter paper and allowed to dry at room temperature, after which they were examined by transmission electron microscopy (TEM).

Microsphere Instillation

Female Sprague-Dawley rats approximately 4 months old and 300 g body weight were used throughout. Animals were anesthetized with halothane and cannulated using a laryngoscope to expose the trachea, and 125 μ g or 1 mg of each particle suspension contained in 0.5 ml saline was instilled into the lungs. A separate group of animals received 0.5 ml saline. Each experiment consisted of one rat for each treatment and experiments were repeated three times. In a separate experiment, we investigated the effect of instillation of a lysinated product of *N*-acetyl cysteine, nacistelin (NAL), in combination with 64-nm polystyrene particles on polymorphonucleocyte (PMN) recruitment. Particles were suspended at a concentration of 2 mg/ml in 10 mM NAL in saline. Five hundred microliters of this suspension were instilled into three rats. A separate triplicate group of rats received particles suspended in saline. All animals were

conscious within minutes of the instillation procedure and suffered no ill effects. Animals were euthanized after 24 h.

Bronchoalveolar Lavage

Rats were killed by single ip injection of pentobarbitone; the lungs were cannulated and removed and lavaged with 4×8 ml volumes of sterile saline. The first lavage was placed in a separate tube for LDH and protein estimations. Tubes were centrifuged at 900g for 5 min at 4°C and the supernatant was removed; the cell pellet from the first lavage was combined with the cells from the same lavage and resuspended in 1 ml PBS, pH 7.2. Total cells were counted, and cytocentrifuge smears, which were stained with Diffquick, were prepared for differential cell counts (Raymond A. Lamb, London, U.K.). Three hundred cells per slide were counted and the results were expressed as the total number of neutrophils in the lung lavage.

Protein Assay

This assay is based on the Bradford assay that relies on the change in absorbance in Coomassie blue G-250 upon binding of protein (Bradford, 1976). Bio-Rad stock reagent (Bio-Rad) was diluted 1/5 with distilled water and filtered. Two hundred microliters of diluted reagent was pipetted into wells on a 96-well plate, with triplicate groups per test and per sample. Five microliters of BAL sample were then added to the appropriate well, mixed, and incubated at room temperature for 15 min before reading on a plate reader at 450 nm. Standards consisted of BSA solutions ranging from 1 to 0.0625 mg/ml.

LDH Assay

This assay is based on the ability of damaged cells to release LDH (Henderson *et al.*, 1985) and was developed in-house from the Sigma protocol. Fifty microliters of 0.75 mM aqueous sodium pyruvate (Sigma) solution containing NADH (Sigma) at a concentration of 1 mg/ml was pipetted into each well of a 96-well plate and incubated at 37°C for 5 min. A series of standards were prepared to give a range of dilutions representing 0–2000 units/LDH/ml. Fifty microliters of pyruvate/NADH solution gave a concentration of 2000 LDH units/ml. Ten microliters of test sample or appropriate standard was added to each well in triplicate groups and thoroughly mixed. The plate was incubated for exactly 30 min at 37°C. Fifty microliters of 2,4-dinitrophenylhydrazine (20 mg/dl) (Sigma) solution dissolved in 1 M HCl was added to each well and incubated at room temperature for 20 min. To develop the final color, 50 μ l of 4 M NaOH was added to each well and the plate was mixed and allowed to stand for 5 min. The plate was read at 540 nm on an automatic plate reader.

Calcium Measurements

Mono Mac 6 (MM6) cells were grown in continuous culture in RPMI medium (Sigma) containing 1 mM sodium pyruvate, 1 mM oxaloacetate, 1 μ g/ml holotransferrin, nonessential amino acids, L-glutamine, penicillin/streptomycin, and 20% fetal calf serum (all Sigma). Cells were pooled into a single tube and adjusted to 4.5×10^5 cells/ml. Ten milliliters of this cell suspension was added to 75-cm² flasks and incubated at 37°C until required for the assay. Cells were transferred to a universal tube, centrifuged at 900g for 2 min, the medium was removed and cells were resuspended in 1 ml PBS and transferred to an Eppendorf tube. The cells were centrifuged at 145g for 2 min at 4°C, the PBS was removed, and cells were resuspended in serum-free RPMI medium containing 23 mM Hepes buffer. Cells were loaded with 1 μ g/ μ l Fura 2-AM (Sigma) in DMSO, 2 μ l/ml cell suspension and the tube was wrapped in foil and incubated in a shaking water bath for 20 min at 34°C. After incubation, the tube was centrifuged at 145g for 2 min at 4°C and the medium was removed and replaced with 1.5 ml fresh RPMI without serum.

The Fura 2-AM-loaded cells were maintained in suspension in a quartz cuvette using a magnetic stirrer. Fluorescence intensity was measured at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Excitation and emission slit widths were set at 5 nm. During the experiments, the cuvette temperature was kept constant at 37°C. Basal fluorescence was measured for 500 s, after which 100 μ l of the appropriate treatment in

RPMI medium was added to the cuvette and the experiment was allowed to run for a further 1500 s. Treatments consisted of 100 μ l of a 1 mg/ml suspension of each particle type or 100 μ l of medium for the control. After the particle treatment, 7.5 μ l of 20 μ M thapsigargin (Sigma) in DMSO was added to the cuvette and the experiment was continued for a further 500 s. Twenty microliters of 5% Triton X-100 solution was added to the cuvette to lyse the cells to give the maximum fluorescence (R_{max}) and the experiment was continued for 500 s. To give the minimum fluorescence value (R_{min}), 15 μ l of 0.5 M EGTA in 3 M Tris buffer was added to the cuvette. The experiment was terminated after a further 500 s. The ratio of the fluorescence measurements at excitation wavelengths of 340 and 380 nm was converted to calcium concentration values using the method of Gryniewicz *et al.* (1985).

Cell-Free Assay for Free Radical Generation by Particle Surfaces

Complete MM6 medium (1.99 mls) was mixed with 10 μ l 2 mM stock 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma) in *N,N* dimethylformamide. The tube was wrapped in foil and placed in a shaking water bath for 10 min at 37°C. After incubation, the medium was transferred to a quartz cuvette containing a magnetic stirrer, placed in the fluorimeter with heated block, and trace readings commenced. The excitation wavelength was set to 485 nm and emission wavelength was set to 530 nm with slit widths of 10 nm. Data were recorded at 1-s intervals over a 1000-s period. The base readings were continued for 100 s, at which point particle samples (50 μ l of a 2.5% particle suspension) were added. Control traces consisting of 2 ml medium alone, followed by addition of the appropriate particle suspension, were run to account for background fluorescence. The control trace values were subtracted from the corresponding particle traces containing DCFH-DA. A second series of experiments included the antioxidant nacystelin at a concentration of 5 mM.

Cell Culture and RNA Extraction

A549 epithelial cells were grown in continuous culture in Minimal Essential Medium (MEM) (Sigma) containing L-glutamine, penicillin/streptomycin, and 10% fetal calf serum (Sigma). Cells were removed from culture by trypsinization and plated into six-well plates in MEM + 10% FCS at a concentration of 3.5×10^6 cells/ml (2.5 ml/well). The plates were incubated for 24 h at 37°C in a humidified atmosphere of CO₂. After this incubation period, the cells were confluent. The wells were washed twice with PBS (Ca/Mg free) and 1 ml MEM + 10% FCS containing the particle treatments at 1 mg/ml was added to the wells. Controls consisted of medium only or medium containing LPS at a final concentration of 1 μ g/ml. Plates were incubated for the required period, ranging from 2 to 24 h.

The treated A549 monolayers were washed three times with PBS (Ca/Mg free) and 600 μ l Tri reagent (Sigma) was added to each well. The lysed cells were scraped from the surface of the plate using a cell scraper and transferred to Eppendorf tubes. Two hundred microliters of chloroform were added to each Eppendorf, vortexed for 15 s, and allowed to stand at room temperature for 15 min. The resulting mixture was centrifuged at 12,000g for 15 min at 4°C. The colorless upper phase was transferred to fresh Eppendorfs and 450 μ l isopropanol was added and mixed. The samples were allowed to stand for a further 10 min at room temperature. Tubes were centrifuged at 12,000g for 10 min at 4°C, the supernatant was removed, and the RNA pellet was washed in 1 ml 75% ethanol. Samples were vortexed briefly, centrifuged at 7500g for 5 min at 4°C and the RNA pellet was air dried for 10 min. The RNA was then suspended in 50 μ l DEPC-treated water and stored at -70°C until required for quantification and RT-PCR.

IL-8 Protein

An ELISA duoset (R&D Systems, Minneapolis, MN) was used according to the manufacturers instructions. Briefly, wells on a 96-well plate were coated with capture antibody and supernatant was added. The IL-8 concentration was quantified after incubation with detection antibody conjugated to horseradish peroxidase in comparison with a standard curve for IL-8.

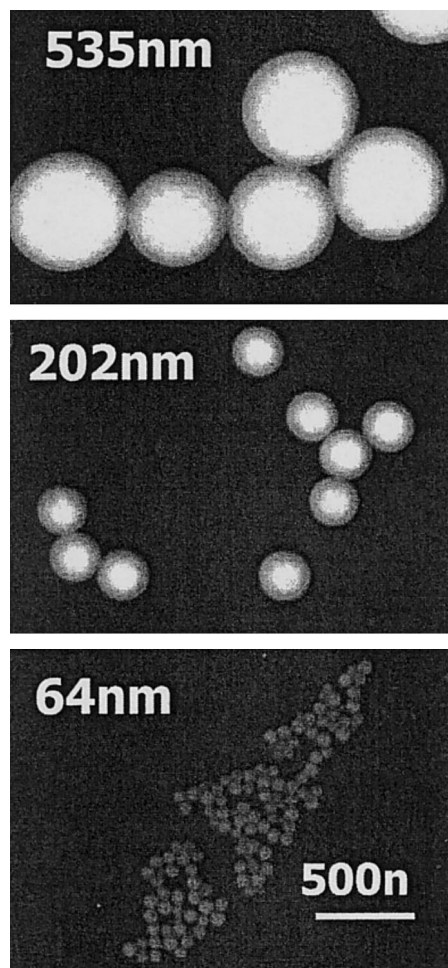


FIG. 1. Transmission electron micrographs showing polystyrene particles of 64, 202, and 535 nm diameter.

Quantification of RNA

Two microliters of RNA suspension were diluted with 98 μ l of DEPC-treated water and measured spectrophotometrically at 260 and 280 nm, and the RNA concentration was calculated from the absorbance at 260 nm. The RNA was diluted to 0.03 μ g/ml with DEPC-treated water and used at this concentration for PCR.

RT-PCR

This procedure was carried out using the Promega Access Kit. Briefly, a master mix of the kit reagents was prepared according to the manufacturer's instructions. Ten microliters of RNA at 0.03 μ g/ml was added to 40 μ l of the master mix containing 10 μ l of the appropriate primer (GAPDH or IL-8). Tubes were placed in a thermal cycler set to run for 25 cycles for the GAPDH treatments or 30 cycles for the IL-8 treatments. The resulting products were separated using a 0.5% agarose gel containing ethidium bromide and viewed under UV light. Bands were quantified by densitometry, and IL-8 was expressed as a percentage of the corresponding GAPDH band. These results were then expressed as a percentage of the untreated control.

Data Analysis

Data from all experiments were analyzed using analysis of variance with the Tukey multiple comparison test.

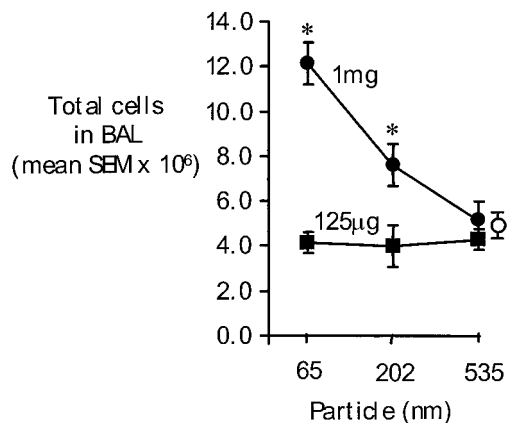


FIG. 2. Mean and SEM total cells in rat lung lavage 24 h after instillation of 1 mg or 125 µg polystyrene particles. Open circle represents the control. *Significant difference from control ($p < 0.05$).

RESULTS

Characterization of Microspheres

TEM images of the microspheres are illustrated in Fig. 1. The microspheres were virtually monodispersed by visual inspection of micrographs and the average diameter closely matched the sizes given by the supplier. No further characterization was carried out.

Bronchoalveolar Lavage after Intratracheal Instillation of Microspheres

At 125 µg of instilled particles there was no effect of any of the treatments on total cell influx into BAL (Fig. 2). However, the 1-mg instillation caused significant increases with the 202- and 64-nm particle sizes compared to the control. In addition, all of the 1-mg particle treatments caused significant increases in BAL PMN compared to the control. In contrast, none of the 125-µg treatments produced differences in BAL PMN number compared to the the control.

Instillation of ultrafine particles in combination with the nacystelin resulted in a significant ($p < 0.05$) decrease in the total number of PMN in the BAL (Table 1).

TABLE 1

Total Cells and Total Neutrophils in BAL from Rats Treated with Polystyrene Particles in Saline or Nacystelin

	Total cells $\times 10^6$	Total neutrophils $\times 10^6$ ^a
Particles (1 mg)	9.0 \pm 0.66	4.29 \pm 0.33
Particles (1 mg) + Nacystelin (10 mM)	6.8 \pm 1.31	1.77 \pm 0.7

Note. Data are the mean total number \pm SEM of cells per treatment with three animals per treatment.

^a Size of particles is 64 nm diameter. Significant difference ($p < 0.05$) between groups.

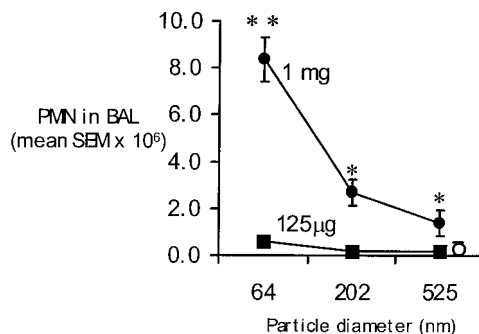


FIG. 3. Mean and SEM total PMN in rat lung lavage 24 h after instillation of 1 mg or 125 µg polystyrene particles. *Significant difference from control ($p < 0.05$). **Significant difference from control ($p < 0.01$).

When all of the PMN data in Fig. 3 were plotted against the surface area of particles instilled in the 125-µg or 1-mg dose, a straight line relationship passing through zero was evident (Fig. 4).

The lavage fluid from animals instilled with 1 mg particles was retained to estimate the protein content and LDH activity. There was a significantly greater increase in BAL protein in treatments with the 64- and 535-nm particles ($p < 0.05$) but not with the 202-nm particles (Fig. 5). The LDH activity in BAL only showed a significant increase over control after treatment of the rats with 64-nm particles ($p < 0.05$) (Fig. 6) The magnitude of the LDH effect suggested that cell death in the ultrafine particle-treated rat lungs was about three times the control level.

Intracellular Ca^{2+} Concentration in Particle-Treated MM6 Cells

Figure 7 shows the effect of particles on the intracellular Ca^{2+} concentration in MM6 cells. The 64-nm particles caused

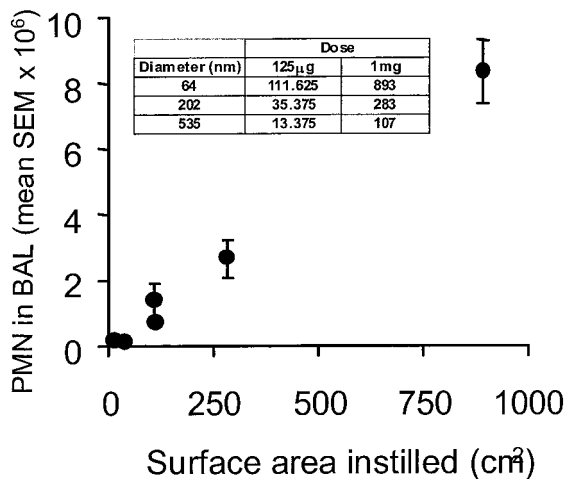


FIG. 4. Mean PMN response to 64-, 202-, and 535-nm particles at 125 µg and 1 mg, replotted as the instilled surface area dose. Inset table shows the actual values for the surface area instilled at the different doses of the different-sized particles.

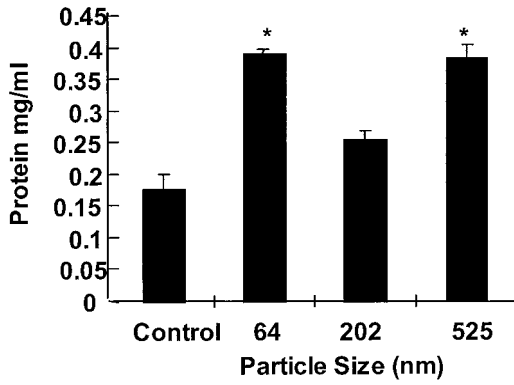


FIG. 5. Mean and SEM of total protein (mg/ml) in lung lavage of rats 24 h after instillation of 1 mg different-sized polystyrene particles. *Significant difference from control ($p < 0.05$).

an increase in the intracellular Ca^{2+} concentration from approximately 50 nM in control cells to approximately 300 nM, while the other sizes had no effect. This effect of 64-nm particles was more evident after thapsigargin stimulation when the intracellular Ca^{2+} concentration increased to more than 600 nM, which was significantly different from the control thapsigargin-induced Ca^{2+} concentration ($p < 0.05$). The larger particles produced effects that were not significantly different from the control.

IL-8 Gene Expression and Protein in Particle-Treated A549 Cells

Treatment of control A549 cells with the LPS caused an increase in IL-8 mRNA that just failed to attain statistical significance, but TNF- α (1 ng/ml) treatment produced a highly significant 2.4-fold increase in IL-8 mRNA (Fig. 8). Treatment of A549 cells with microspheres for 2 h resulted in modestly but significantly increased IL-8 mRNA with the 64-nm particles. There was no significant effect of the larger particles compared to control. At 4 h there was no difference between

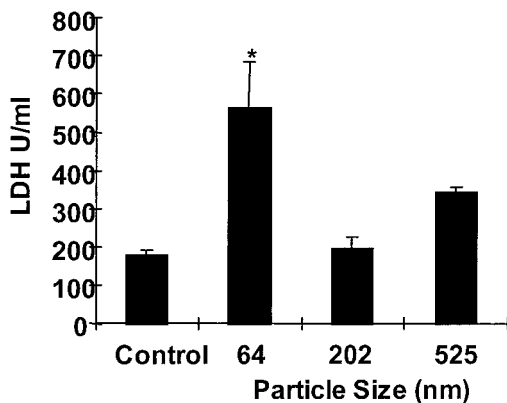


FIG. 6. Mean and SEM of LDH (units/ml) in lung lavage from rat lungs instilled with 1 mg polystyrene particles 24 h previously. *Significant difference ($p < 0.05$) compared to control.

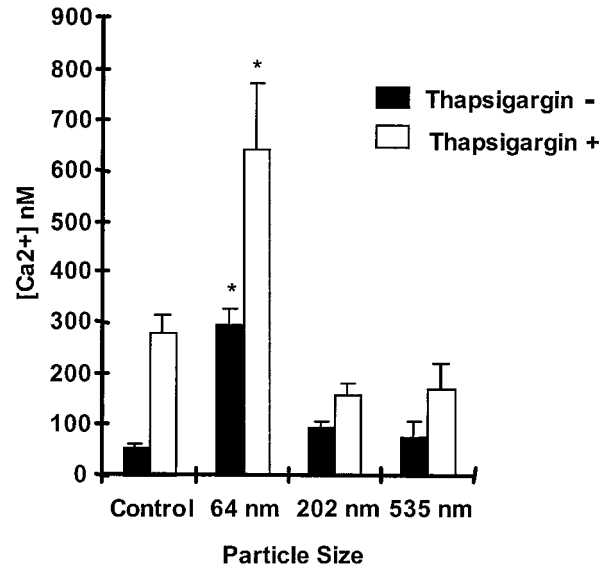


FIG. 7. Mean and SEM Ca^{2+} concentration in MM6 cells following treatment with particles in the presence or absence of thapsigargin. *Significant difference from respective control ($p < 0.05$). Results from three separate experiments.

any particle treatments and the control (data not shown). IL-8 protein was not detectable at 2 h in supernatants from A549 cells exposed to 64-nm particles, although by 4 h the levels were comparable to the control levels (Fig. 9). Both 202- and 535-nm particles stimulated an increase in IL-8 protein by A549 cells at both of the time points tested.

Free Radical Activity of Microspheres

Figure 10 illustrates the fluorescence intensity of DCF, a measure of reactive oxygen species, after particle treatment in

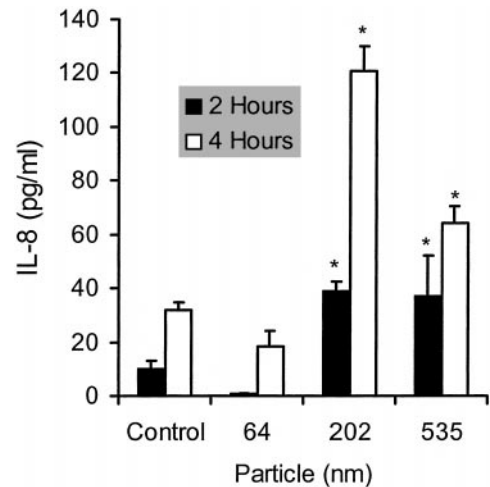


FIG. 8. Mean and SEM of IL-8 expression in A549 cells treated for 2 h with polystyrene particles. IL-8 is expressed as a ratio of GAPDH and then expressed as a percentage of the control. *Significant difference from control ($p < 0.05$).

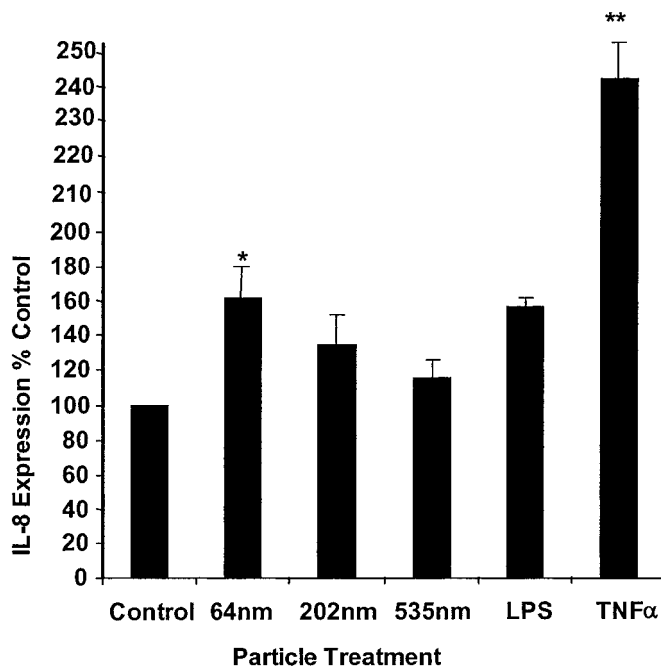


FIG. 9. Mean and SEM IL-8 protein release from A549 cells exposure to polystyrene particles of various sizes. *Significant difference from control ($p < 0.05$). **Significant difference from control ($p < 0.01$). Data from duplicate wells in three separate experiments.

the absence of cells. The ultrafine particles produced a dramatic increase in fluorescence compared with 202- and 535-nm particles ($p < 0.005$), which were not significantly different from the control.

DISCUSSION

Increases in particulate air pollution have been linked with increased morbidity and mortality (Bates, 1992; Schwartz, 1994). For example, exacerbations of airways disease are associated with increased levels of PM₁₀ (Dockery *et al.*, 1989; Pope *et al.*, 1991). In addition to the size range of the particles, factors such as the chemical composition and the availability of adsorbed carbon compounds and transition metals on the particle surface may be important in causing lung inflammation (MacNee and Donaldson, 1999). We have demonstrated that the *in vitro* proinflammatory effects of PM₁₀ on epithelial cells were diminished by chelating the transition metals (Jimenez *et al.*, 2000) and similar results have been produced in many other studies, e.g., using ROFA (Carter *et al.*, 1997).

Ultrafine particles comprise the dominant number of particles in PM₁₀ and we have hypothesized that they play an important role in mediating the adverse effects of PM₁₀ (MacNee and Donaldson, 1999). The role of reactive oxygen species in promoting part of the cellular effects observed with ultrafine particles has been demonstrated by Stone *et al.* (2000) using UfCB. UfCB particles have greater free radical activity than a similar mass of fine CB, demonstrated using a plasmid scission

assay (Stone *et al.*, 1998), and this is not mediated by transition metals (Brown *et al.*, 2000). The purpose of the present study was to investigate ultrafines further using particles made of an inert material—polystyrene—having different diameters down to the ultrafine size range. Such particles are without complications such as mineral habit or contaminating metals and present an opportunity to determine the role of particle size. The study revealed that particles made of a low-toxicity material, such as polystyrene, do have inflammogenic potential when they are in the ultrafine form. The exposure dose used here was large compared to ultrafines in ambient air, but the adverse effects of ambient air particles are seen only in susceptible populations, whereas the model used here was not susceptible. The present study was aimed at demonstrating differences in potency between different-sized particles rather than necessarily studying response to realistic doses, which would require models of susceptibility.

At an instilled dose of 125 μg polystyrene beads, there was no significant influx of PMN. At the 1 mg dose, the ultrafine polystyrene produced increased PMN influx, but the other-sized particles also caused some inflammation. This suggested that there was not a clear cutoff at any particle size in terms of ability to cause inflammation and suggested that another variable, such as surface area, might be driving the inflammation. Only the 64-nm particles caused significant cell death as measured by LDH in the lavage. Increased protein levels in lavage were found with 64- and 535-nm particles but not with 202-nm particles; we cannot explain this finding but the increases in protein that were seen were modest, being only twice the control level.

In order to address the question of whether the ultrafine polystyrene particles represented an extreme case of a large surface area, we replotted the neutrophil response against instilled dose expressed as surface area. This clearly showed a straight line passing through zero. This is an important finding, demonstrating that the short-term PMN inflammatory response in the rat responds to the metric of surface area. Importantly, it

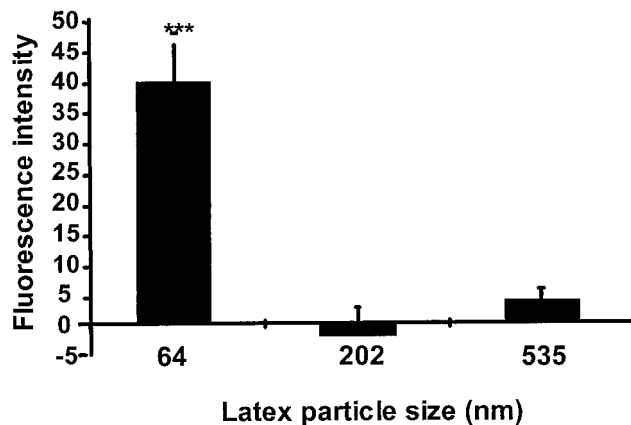


FIG. 10. Mean and SEM fluorescence intensity of DCF after treatment with 50 μl of each particle suspension. Data from of three separate experiments ***Significant difference from control ($p < 0.001$).

suggests that the effects of ultrafine particles occur because they deliver a large surface area dose to the lungs.

Particles below 100 nm have been shown to behave differently from larger respirable particles for a number of materials (MacNee and Donaldson, 1999). The retention of such particles in the lung may be a key event leading to their increased inflammogenicity. Rats exposed to equal masses of titanium dioxide in two aerosol size ranges retained more ultrafine particles in the lung interstitium and developed an increased inflammatory response (Ferin *et al.*, 1990, 1992). A similar mechanism may operate with the ultrafine polystyrene particles tested here, but, at the short time points used, clearance was unlikely to have played a role.

We also attempted to find *in vitro* correlates of the *in vivo* effects of the ultrafine particles. Macrophages were studied because of their important role in particle inflammation and our previous unique finding that the macrophage Ca^{2+} response is especially sensitive to ultrafine particles (Stone *et al.*, 2000). In lung epithelial cells, UfCB but not CB particles interfere with metabolic functions assessed by tetrazolium salt reduction via an oxidative stress mechanism (Stone *et al.*, 1998), and changes in metabolic function may be linked to alterations in calcium homeostasis within the cell (Hoyal *et al.*, 1998). These effects have been investigated by Stone *et al.* (2000) using macrophage-like cells. UfCB was found to affect membrane calcium channels, resulting in influx of extracellular Ca^{2+} following thapsigargin-mediated release of intracellular Ca^{2+} stores (Stone *et al.*, 2000). We used MM6 cells instead of primary rat alveolar macrophages here for humane reasons. However, in another published study, we have demonstrated the same Ca^{2+} effects in primary rat alveolar macrophages with ultrafine particles as we saw here in the MM6 cell line with ultrafine polystyrene particles (Stone *et al.*, 2000).

The present study revealed that ultrafine polystyrene particles also stimulated the entry of extracellular calcium on treatment with thapsigargin. These rapid effects allow Ca^{2+} to act as an effective intracellular signaling molecule. Transcription factor activation may be mediated in part by changes in calcium ion homeostasis, leading to the expression of proinflammatory genes (Dolmetsch *et al.*, 1998). Ultrafine polystyrene thus joins asbestos (Faux *et al.*, 1994) and quartz (Tarnock *et al.*, 1997) in a list of pathogenic particles that modulate intracellular calcium (Faux *et al.*, 1994; Lim *et al.*, 1997) and these Ca^{2+} changes are very likely related to the oxidative stress that the particles generate (Hoyal *et al.*, 1998; Stone *et al.*, 2000).

It is difficult to quantify the significance of the Ca^{2+} changes for the IL-8 response because the methodological constraints prevented us from assessing the Ca^{2+} response in epithelial cells. However, we intend to develop an assay to measure Ca^{2+} flux in epithelial cells that will allow us to relate any Ca^{2+} changes to the chemokine response.

We have previously demonstrated the UfCB has its increased inflammatory effects via mechanisms other than transition metal (Brown *et al.*, 2000). We therefore examined the role of soluble factors in the Ca^{2+} effects of polystyrene in

macrophages. Soluble extracts of the ultrafine (64 nm) particles had no effect on calcium ion homeostasis (data not shown), once again demonstrating that transition metals were not responsible for this effect of ultrafine polystyrene.

Transcription factor activation and expression of proinflammatory genes are key events in the initiation of inflammation, and IL-8 is a potent neutrophil chemoattractant, which plays a significant role (Standiford *et al.*, 1990). We therefore used A549 epithelial cells to investigate the time-related expression of IL-8 after treatment with particles, using LPS and TNF- α as positive controls; TNF- α did cause significantly increased IL-8 release while LPS caused only modest, insignificant release compared to particle treatments. We demonstrated that only the ultrafine polystyrene particles produced significantly more IL-8 gene expression than controls and that this was most pronounced after a 2-h exposure. At 4 h there was still more IL-8 expression compared to untreated cells, but this was reduced in comparison to the 2-h time point. These findings are in keeping with previous work in which IL-8 expression was increased at 2 h in A549 cells exposed to ultrafine titanium dioxide (unpublished data) and provides an explanation for the increased inflammation seen with the ultrafine polystyrene particles.

IL-8 protein production by A549 cells exposed to the polystyrene particles was also assessed in this study. The A549 cells showed increased IL-8 release with the 202- and 535-nm particles but no effect with the 64-nm particles. We have encountered this phenomenon previously with ultrafine carbon black, where IL-8 release from cells *in vitro* is actually inhibited below the control levels with ultrafine treatment. We believe this to be an artifact of *in vitro* exposure to the high surface area of ultrafine, possibly involving adsorption to the particle surface or inhibition of secretion; this phenomenon requires further investigation. With respect to the 202- and 535-nm particles, the increased protein production was greater than can be explained by the mRNA levels, suggesting that the particles influence posttranscriptional regulation of the IL-8 gene. Particle-induced chemokine release from epithelial cells serves to recruit inflammatory cells and this may facilitate clearance of particles. In this *in vitro* system, the larger particles were able to produce an IL-8 signal that was not produced by the ultrafine particles either due to protein absorption or posttranslational regulation. Failure of this IL-8 signal *in vivo* would hinder recruitment and the fact that we saw PMN recruitment with the ultrafines *in vivo* suggests that this is indeed an *in vitro* artifact. However, there is substantial redundancy in the proinflammatory response and so other mediators could be responsible and the effects of ultrafines on IL-8 production warrant further study.

In control *in vitro* experiments we noted that, in line with a previous report on ROFA particles (Imrich *et al.*, 1999), the 64-nm particle could directly oxidize the oxidant-activated fluorophore DCFH-DA in a cell-free system. We confirmed that the 64-nm particles were active in generating oxidative activity compared with the larger-sized particles, which were not. We have previously demonstrated (unpublished data) that

ultrafine carbon and TiO₂ particles and also PM₁₀, instilled into the lung in combination with the antioxidant thiol NAL, showed a neutrophil response that was reduced to half that seen in the absence of NAL. We hypothesize that the ultrafine particles deposit on the epithelium, where their large surface area causes oxidative stress by an unknown mechanism, which then stimulates IL-8 gene expression. IL-8 secretion leads to neutrophil recruitment and the usual inflammatory consequences. The amelioration of the proinflammatory effect of ultrafine polystyrene by NAL supports this contention. The relative lack of inflammatory activity of 202- and 535-nm polystyrene can be directly linked to their lesser surface area and subsequent lack of oxidative activity as measured by DCF fluorescence. The data presented here provide a clear hypothesis for the acute proinflammatory effects of small polystyrene particles based on their large surface area and ability to cause oxidative stress.

This study using polystyrene as a model particulate provides a likely sequence for the increased inflammatory effects that we have reported for ultrafines (MacNee and Donaldson, 1999). Ultrafine particulates are ubiquitous in ambient urban air, although at levels that would result in much lower exposures than were used in the present study. However, the same surface area mechanisms could be important in mediating adverse effects in susceptible populations whose lungs are in a hyperresponsive state, and further research is required to elucidate this potential role. Other components of urban air, such as endotoxin (Ning *et al.*, 2000) and transition metals, (Jimenez *et al.*, 2000) could synergize with ultrafine particles in proinflammatory effects and this also warrants further investigation.

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