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THE ROLE OF FREE RADICALS IN THE TOXIC AND INFLAMMATORY EFFECTS OF FOUR DIFFERENT ULTRAFINE PARTICLE TYPES

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PM_{10} contains an ultrafine component, which is generally derived from combustion processes. This ultrafine fraction may be a factor in the increases in exacerbations of respiratory disease and deaths from cardiorespiratory causes associated with transient increases in levels of PM_{10}. By using four different ultrafine particles (carbon black, cobalt, nickel, and titanium dioxide), we set out to determine the attributes of the ultrafine particle (surface area, chemical composition, particle number, or surface reactivity) that contribute most to its toxicity and proinflammatory effects both in vivo and in vitro. Instillation of 125 μg ultrafine carbon black (UFCB) and ultrafine cobalt (UFCo) particles induced a significant influx of neutrophils at both 4 and 18 h postinstillation. Accompanying the influx of neutrophils was an increase in macrophage inflammatory protein-2 (MIP-2) (at 4 h) and an increase in γ-glutamyl transpeptidase (at 18 h) in bronchoalveolar lavage fluid (BAL). Ultrafine nickel (UFNi) did not induce a significant increase in neutrophil influx until 18 h postinstillation. The increase in neutrophils induced by UFNi at this timepoint was comparable to that induced by UFCo and UFCB. UFTi did not induce a significant increase in neutrophils following instillation into the rat lung. The levels of MIP-2 observed at 4 h and neutrophil influx at 18 h induced by the particle samples were consistent with the pattern of surface free radical generation (as measured by the plasmid scission assay) whereby UFCo, UFCB, and UFNi all cause significant increases in inflammatory markers, as well as inducing a significant depletion of supercoiled plasmid DNA, indicative of hydroxyl radical generation. A role for free radicals and reactive oxygen species (ROS) in mediating ultrafine inflammation is further strengthened by the ability of the antioxidants N-acetylcysteine (NAC) and glutathione monoethyl ester (GSHme) to block the particle induced release of tumour necrosis factor-α (TNF-α) from alveolar macrophages in vitro. The ultrafine particles in PM_{10} may cause adverse effects via oxidative stress, and this could have implications for susceptible individuals. Susceptible individuals, such as those with COPD or asthma, already exhibit preexisting oxidative stress and hence are in a primed state for further oxidative stress induced by occupational or environmental particles.

There is good evidence to suggest that ambient air pollution contains an ultrafine component derived from combustion sources (Dennekamp et al., 2001; Seaton et al., 1995). It has also been hypothesized that ultrafine particles may be responsible for the detrimental health effects induced by PM_{10}

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(Peters et al., 1997). There is now substantial evidence to suggest that ultrafine particles cause greater damage to the lung and to lung cells than larger particles composed of the same material (Ferin et al., 1992; Li et al., 1996; Stone et al., 1998; Oberdorster et al., 1996). Increased translocation of ultrafine particles from the alveoli into the interstitial space may be an important factor in the toxicity of these particles (Ferin et al., 1992). It is hypothesized that the high particle surface area per unit mass impairs macrophage clearance (Tran et al., 2000) and leads to increased interaction between particles and epithelial cells. This in turn may lead to the release of proinflammatory cytokines and chemokines (Jimenez et al., 2000; Lundborg et al., 1999).

Where transition metals are present, Fenton chemistry may be involved in free radical generation (Gilmour et al., 1997) although metals are not necessary for ultrafine-particle-mediated free radical generation (Brown et al., 2000). The generation of oxidants may lead to oxidative stress causing further toxicity, as well as cytokine and chemokine release (Driscoll, 2000).

The study of Zhang et al. (1998a) indicated differences in inflammogenicity caused to the rat lung after intratracheal instillation of three ultrafine particles (UFTi, UFNi, and UFCo) that had similar mass median aerodynamic diameters (MMAD) and surface areas. This study and others indicate that size may not be the only factor in ultrafine-particle-mediated toxicity but surface reactivity may also be a factor (Zhang et al., 1998a, 1998b; Lay et al., 1999). Zhang et al. (1998a) indicated that both UFCo and UFNi were more inflammogenic (as measured by neutrophil influx in the lung) than UFTi, as well as possessing more free radicals. They found, however, that UFNi was markedly more inflammogenic than UFCo, a difference that was not obvious upon measurement of the free radical generation.

Free radicals either produced on the surface of particles or produced by inflammatory cells have been shown to alter cellular glutathione (GSH) levels (Stone et al., 1998). Alterations in cellular GSH levels can cause the upregulation of nuclear factor kappa B (NF-κB) (Rahman et al., 2001; Schreck et al., 1992), the release of inflammatory mediators (Li et al., 1996), and the upregulation of adhesion molecule expression (Rahman & MacNee, 2000). All of these factors are involved in the recruitment of inflammatory cells, which may further alter GSH levels, leading, potentially, to inflammation-induced lung pathology.

The factors that are implicated in the toxicity of ultrafine particles are surface area, chemical composition, particle number, and surface reactivity. It was therefore the aim of this study to determine the attributes that contribute most to the toxicity and proinflammatory effects of various ultrafine particles in vivo and in vitro.

METHODS AND MATERIALS

Particles

The following ultrafine particles were used: ultrafine cobalt (Co_3O_4) (UFCo) (gift from Dr. Zhang, Fukui Medical School, Japan), ultrafine ti-

tanium dioxide (TiO_2) (UFTi) (Degussa, Germany), ultrafine nickel (UFNi) (gift from Dr. Zhang, Fukui Medical School, Japan), and ultrafine carbon black (UFCB) (Degussa, Germany).

Particle Preparation

Particles were weighed and suspended in the appropriate medium [saline for in vivo study, DMEM (Dulbecco's modified Eagle's medium) for the in vitro study] to give a final concentration of 1 mg/ml. The suspension was then sonicated for 10–15 min before adjusting to the required concentration.

Instillation and Bronchoalveolar Lavage

Healthy male Wistar rats, weighing 200–300 g, aged 10–12 wk were housed 2 per cage in a regulated animal facility where a 12-h light/dark cycle was maintained. The rats were fed standard laboratory food and received water ad libitum. Rats were intratracheally instilled with 125 μg (in 0.5 ml saline) of ultrafine particles or 0.5 ml saline control. The rats were sacrificed either 4 or 18 h after instillation by an intraperitoneal injection of 2 ml pentobarbitone (Rhone Merieux, Essex, UK). The lungs were exposed and the trachea cannulated before lavaging the bronchoalveolar space with 8 ml of saline at 4°C (Baxter, Newbury, UK). The procedure was carried out 4 times, with the first 8 ml recovered being kept separate from subsequent lavage and used for biochemical analysis. All bronchoalveolar lavage (BAL) samples were centrifuged at 717 × g for 3 min and the pelleted cells were resuspended in 2 ml DMEM (Gibco, Paisley UK) containing 10% fetal calf serum (FCS) (Gibco, Paisley UK). The primary lavage from the rat lung was taken for biochemical analysis and was stored at –80°C until required for analysis of glutamyl transpeptidase (γ-GT), total protein, and MIP-2. Total cell number was assessed using a hemocytometer (Weber Scientific International, Teddington, Middlesex UK). In addition, cytospin slide preparations were made using 40,000 cells centrifuged at 179 × g for 5 min onto glass slides. A differential cell count of the cytospin preparations was carried out following staining with Diff Quick dyes (Raymond Lamb, Eastbourne, UK).

γ-Glutamyl Transpeptidase Determination in BAL

The method for measuring γ-GT was based on a Sigma diagnostic kit (number 545) and adapted for use on a 96-well microtiter plate. The assay produces a pink azo dye, which can be measured at a wavelength of 550 nm and is proportional to the activity and hence quantity of γ-GT enzyme present.

Protein Assay

The bicinchoninic acid (BCA) assay was used to determine total protein content of BAL. Protein standards (0–250 μg/ml) (Sigma, Poole UK) were prepared before 10 μl of either standard or test sample was added to designated wells of a 96-well plate. Detection buffer was prepared by adding 10 ml of bicinchoninic acid solution (Sigma, Poole, UK) to 200 μl of copper(II)

sulfate 4% (w/v) (Sigma, Poole UK); 190 µl of detection buffer was added to each well and incubated at 37°C for 30 min, after which the absorbance was read at 570 nm on a microtiter plate reader (Dynex, UK).

Macrophage Inflammatory Protein-2 and TNF-α ELISA

The rat MIP-2 enzyme-linked immunosorbent assay (ELISA) assay was based on a diagnostic kit (number KRC1022) obtained from Biosource International (Camarillo, CA). The rat TNF-α ELISA assay was based on a kit obtained from Immunokontact (Abingdon, UK) (number 31390164005).

Rat Alveolar Macrophage Preparation

Male Wistar rats (250–350 g) were sacrificed with 2 ml pentabarbitone (Rhone Merieux, Essex, UK) before exposing the lungs which were then lavaged 4 times with 8 ml (total 32 ml) phosphate-buffered solution (PBS) (Ca/Mg free) (Gibco, Paisley, UK). The resulting BAL was centrifuged at 900 × g for 2 min. The supernatant was removed and resuspended in 3 ml RPMI 1640 medium (Gibco, Paisley, UK) containing 1% bovine serum albumin (BSA), 60 units penicillin/streptomycin (Gibco, Paisley, UK), and 200 n*M* glutamine (Gibco, Paisley, UK).

The BAL cells were counted and assessed for viability using 0.5% trypan blue (Sigma, Poole UK) before diluting to yield 2×10^5 cells/ml. To each well of a 96-well plate (Bibby Sterilin, UK), 200 µl of cell suspension in RPMI 1640 medium containing 1% BSA, 60 units penicillin/streptomycin, and 200 n*M* glutamine was added and cultured for 24 h at 37°C, 5% CO_2. After this time the cells were treated with ultrafine particles (50 µg/ml) either in medium alone, medium containing 5 m*M* *N*-acetylcysteine (NAC), or medium containing 5 m*M* glutathione monoethyl ester (GSHme) (Sigma, Poole UK) for 4 h at 37°C, 5% CO_2. The supernatant was collected and stored at –80°C until required for analysis of TNF-α protein content by ELISA.

Plasmid Assay

The ability of particles to break strands of supercoiled ϕX174 RF DNA plasmid (Gibco, Paisley UK) was used to detect particle surface-generated free radicals. In this assay, 20 µl of either UFCo, UFTi, UFCB, or UFNi, all at a concentration of 1 mg/ml in Chelex-treated water (Sigma, Poole UK), was incubated with 1 µl plasmid ϕX174 RF (1 µg/ml) for 6 h at 37°C. Untreated plasmid DNA was used as a control in each experiment. After this time 4 µl orange/blue loading dye (Promega, UK) was added to the treated and control cells. The plasmid samples were then separated by agarose gel (0.8%) (Sigma, Poole, UK) electrophoresis for 16 h at 20 V using TBE electrophoresis buffer (Promega, UK). The resulting agarose gel was soaked for 1 h in a 0.02% solution of ethidium bromide (Sigma, Poole UK). The supercoiled band intensity was measured using software obtained from Genzyme UK. Free radical damage to the plasmid was expressed as depletion of the supercoiled plasmid band intensity relative to the control.

Surface Area Determination

Surface area was determined using the Brunauer, Emmet, and Teller (BET) method of N_2 adsorption and was carried out by Morgan Materials Technology Ltd. (Worcestershire, UK).

Statistical Analysis

Values are expressed as the mean ± standard error. The in vivo instillation experiments were conducted using three rats for each treatment and time point. The data was analyzed by one-way analysis of variance with Tukey's multiple comparison test set to a level of significance (alpha) of .05 for all comparisons (Minitab version 10).

RESULTS

Particle Characteristics

All of the primary particles were held together in aggregates, but the singlet particles are similar in diameter with the UFCB being 6 nm smaller than the other 3 particles (Table 1). With respect to surface area, the three metallic particles (UFCo, UFNi, and UFTi) all have approximately the same surface area. Again in contrast, UFCB has a surface area five to six times greater than the other particles, as measured by the N_2 absorption method.

Cellular Analysis of BAL

Both UFCo and UFCB induced a significant increase in the percentage of neutrophils in BAL 4 h after instillation compared to saline-instilled animals (Figure 1). The neutrophil influx (%) induced by UFCo and UFCB 4 h after instillation (Figure 1 and Table 2) was still evident 18 h later (Figure 1 and Table 3). UFNi induced a slower inflammatory response, not reaching a significant increase in neutrophil influx until 18 h. In addition, the percentages of neutrophils induced by UFCB and UFNi were also significantly greater than for UFTi (15.4 ± 2.3) instilled animals at 18 h ($p < .01$).

γ-Glutamyl Transpeptidase Analysis

γ-GT is a membrane-bound enzyme found in Type II epithelial and Clara cells and is capable of the breakdown of GSH into its constituent amino acids. The release of γ-GT into BAL can be used as a marker of tox-

TABLE 1. Physical characteristics of the ultrafine particles

Particle	Diameter (nm)	Surface area (m^2/g)
UFCo	20	36.9
UFCB	14	253.9
UFTi	20	49.8
UFNi	20	36.2

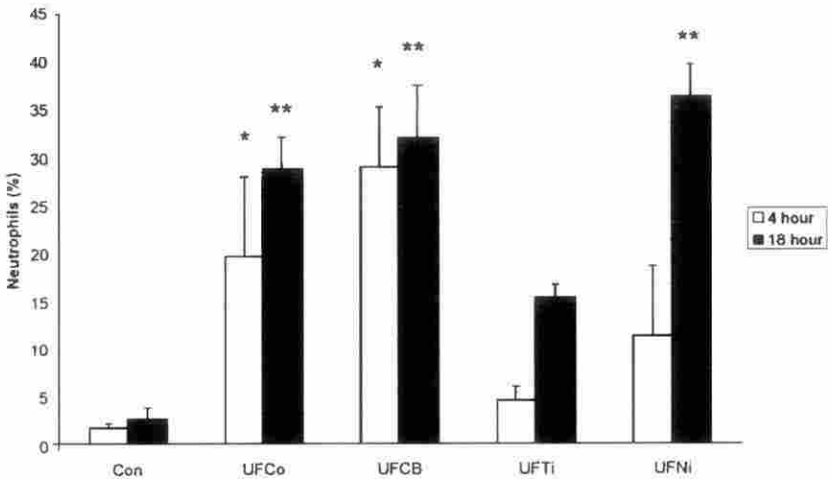

FIGURE 1. Percentage of neutrophils in bronchoalveolar lavage fluid, 4 and 18 h after intracheal instillation of ultrafine particles (125 μg). The results are the mean obtained from 3 rats ± SE. Asterick indicates significant at $p < .05$ and double asterisk at $p < .01$ compared to control levels.

icity of these cells, as damage to the membrane causes release of γ-GT into the airspace, which in turn can be measured in BAL.

After 4 h, there was no significant increase in the γ-GT activity measured in BAL after treatment with any of the ultrafine particles (Figure 2). These results indicate that there was no measurable epithelial cell damage at this timepoint. However, after 18 h, there was a twofold increase in γ-GT activity after treatment with UFCo (191.37 ± 20.74% of control) and UFCB (181.3 ± 17.8% of control) ($p < .05$). UFTi and UFNi induced only modest, but not significant, increases in γ-GT levels, to 140.0 ± 13.0 and 137.3 ± 14.3% of control, respectively.

Measurement of Total Protein in BAL

All of the particles tested caused a modest increase in total BAL protein at 4 and at 18 h postinstillation, with none of the increases being significantly different from the control (data not shown).

TABLE 2. Cellular constituents, expressed as percentages, in BAL 4 h after intratracheal instillation of ultrafine particle (125 μg)

	Total cells ($\times 10^6$)	Macrophages (%)	Neutrophils (%)	Eosinophils (%)	Lymphocytes (%)
Control	2.2 ± 1.1	96.3 ± 1.0	1.9 ± 0.8	0.7 ± 0.4	1.1 ± 0.3
UFCo	5.1 ± 1.9	78.7 ± 6.5[a]	19.9 ± 14.0[a]	0.5 ± 0.1	1.0 ± 0.1
UFCB	4.7 ± 1.3	69.3 ± 6.7[a]	28.9 ± 10.8[a]	0.9 ± 0.1	0.9 ± 0.4
UFTi	4.4 ± 0.7	93.9 ± 1.0	4.6 ± 2.5	0.5 ± 0.3	1.1 ± 0.5
UFNi	3.6 ± 1.1	86.1 ± 6.9	11.3 ± 12.7	0.9 ± 0.4	1.7 ± 0.8

Note. Values are the mean of three rats ± SE.
[a]Significant at $p < .001$.

TABLE 3. Cellular constituents, expressed as percentages, in BAL at 18 h after intratracheal instillation of ultrafine particle (125 μg)

	Total cells ($\times 10^6$)	Macrophages (%)	Neutrophils (%)	Eosinophils (%)	Lymphocytes (%)
Control	5.7 ± 0.8	96.2 ± 1.0	2.8 ± 1.9	0.3 ± 0.01	0.7 ± 0.02
UFCo	4.5 ± 0.2	68.7 ± 7.5[a]	28.8 ± 5.7[a]	1.9 ± 1.0	0.7 ± 0.1
UFCB	4.9 ± 0.7	66.2 ± 5.3[a]	32.1 ± 9.4[a,b]	1.1 ± 0.4	0.7 ± 0.2
UFTi	5.6 ± 0.4	82.1 ± 0.3	15.4 ± 2.3	2.0 ± 1.4	0.6 ± 0.2
UFNi	5.2 ± 0.5	62.5 ± 3.2[a]	36.2 ± 5.9[a,b]	0.6 ± 0.2	0.6 ± 0.1

Note. Values are the mean of three rats ± SE.
[a]Significant at $p < .01$ compared to control values.
[b]Significant at $p < .01$ compared to UFTi-instilled animals.

Measurement of MIP-2 in BAL

Four hours after instillation of UFCo and UFCB there was a significant ($p < .001$) increase in the MIP-2 content of BAL, increasing to 668.6 ± 75.3 pg/ml and 655.0 ± 82.5 pg/ml, respectively, compared to control values of 209.8 ± 20.4 pg/ml (Figure 3). BAL content of MIP-2 after treatment with UFNi (474.4 ± 150.3 pg/ml) was not significantly altered from the control. There was no significant increase in MIP-2 after the instillation of UFTi at this time (333.4 ± 58.4 pg/ml). Eighteen hours after instillation with UFCo, UFCB, and UFNi, the concentration of MIP-2 in BAL had returned to control levels.

Effect of Antioxidants on the Production of TNF-α After Treatment with Ultrafine Nickel Particles

To investigate whether oxidative stress played a role, primary rat alveolar macrophages were assessed for TNF-α production after treat-

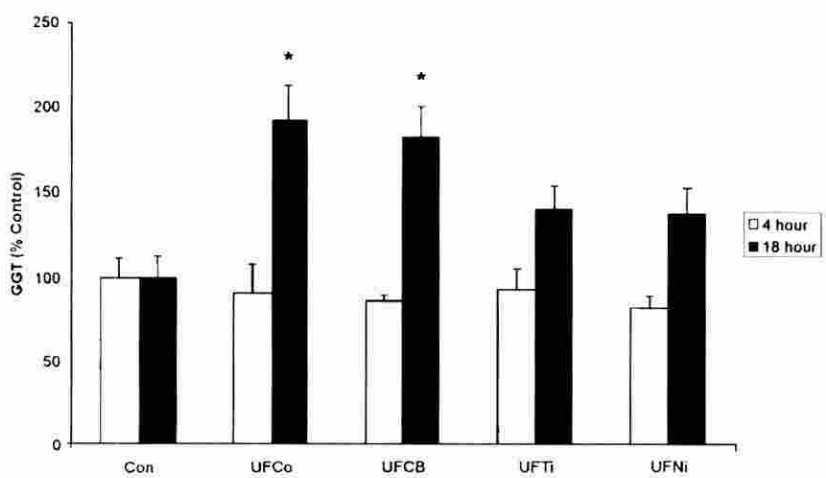

FIGURE 2. γ-GT activity in BAL 4 or 18 h after instillation of 125 μg of ultrafine particles. Values are expressed as a percentage of the control values at 4 h (62.57 ± 7.76 U/ml) and 18 h (62.32 ± 10.12 U/ml). Values are the mean of three rats ± SE. Asterick indicates significant at $p < .05$.

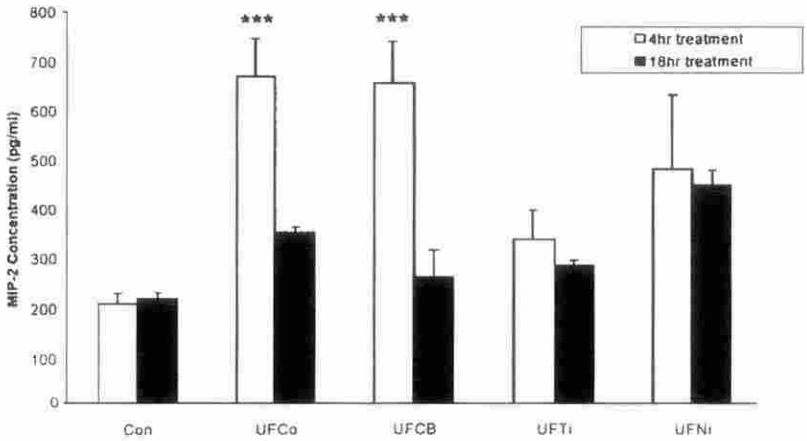

FIGURE 3. MIP-2 content of BAL, 4 and 18 h after instillation of 125 μg of ultrafine particles. Values are the mean of three rats ± SE Triple asterick indicates significant at $p < .001$ compared to saline instilled animals.

ment with UFNi and interventions with antioxidants were carried out.

The effect of UFNi with and without antioxidant intervention on TNF-α secretion from rat alveolar macrophages is shown in Table 4. Cells treated with UFNi produced a significant increase ($p < .001$) in TNF-α protein levels up to 43.4 ± 7.4 pg/ml compared to control levels (11.9 ± 5.3 pg/ml). The addition of NAC and GSHme was able to significantly ($p < .001$) prevent TNF-α production compared to UFNi-treated cells with no antioxidant intervention. The coincubation with NAC and UFNi reduced TNF-α production by 45% compared to UFNi-treated cells alone. The coincubation with GSHme and UFNi with the rat alveolar macrophages also induced a significantly ($p < .001$) lower TNF-α production compared to alveolar macrophages incubated with UFNi alone.

TABLE 4. TNF-α cytokine production by rat alveolar macrophages in response to treatment with ultrafine nickel (50 μg/ml) for 4 h and when also treated with the antioxidants NAC or GSHe (5 mM)

	Control	UFNi
Untreated	11.87 ± 5.30	43.39 ± 7.36[a]
Treated with NAC	9.48 ± 4.10	19.81 ± 3.06[b]
Treated with GSHe	7.41 ± 1.89	17.26 ± 3.68[b]

Note. The results were obtained from the combined means of three experiments in duplicate ± SE.

[a]Significant at $p < .001$ compared to control levels (medium alone).

[b]Significant at $p < .001$ compared to UFNi untreated cells.

Measurement of Particle Free Radical Activity

The presence of surface free radicals was detected using the plasmid assay (Figure 4). After electrophoresis, the intensities of the DNA bands formed, which corresponded to the supercoiled structure, and was quantified by densitometry.

UFCo (49.0 ± 13.5% of control), UFCB (44.9 ± 24.8% of control), and UFNi (54.7 ± 25.2% of control) produced significant ($p < .05$) free radical damage as shown by the depletion of supercoiled plasmid DNA compared to the untreated control (100 ± 17.8). UFTi (82.8 ± 25.2% of control) produced no significant reduction in supercoiled plasmid DNA band intensity.

DISCUSSION

The ability of ultrafine particles to induce a greater detrimental effect in vivo compared to fine particles of the same material has been shown after inhalation and instillation of various materials (Brown et al., 2000, 2001; Ferin et al., 1992; Li et al., 1996; Zhang et al., 1998a, 1998b). This study demonstrates that four different ultrafine particles, composed of different materials, exhibit different degrees of inflammation and lung injury following instillation on an equal mass basis. The degree of lung injury appears to be related not to their size or surface area but to their ability to generate surface free radicals and to cause subsequent oxidant damage. Previous studies in which UFNi particles were instilled at high mass into the rat lung reported maximal neutrophil influx of 24 h after instillation, causing a persistent and sustained inflammation for up to 30 days later (Zhang et al., 1998b). These previous results are consistent with the study presented here

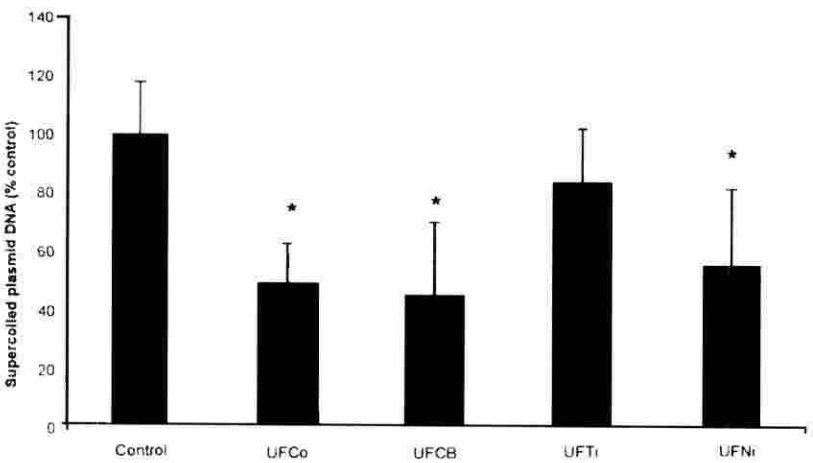

FIGURE 4. Depletion of supercoiled DNA by ultrafine particles. The results are shown as mean obtained from teo experiments in triplicate ± SE. Asterick indicates significant at $p < .05$ compared to control levels.

and suggest that a slower influx of neutrophils is induced by UFNi, which may result in a more prolonged inflammation. This is supported by previous studies demonstrating that neutrophil influx induced by UFCo at 24 h is not as great as is observed with UFNi (Zhang et al., 1998a).

Previous instillation studies investigating UFCB have indicated that there is an induction of neutrophil influx into the rat lung at 6 h postinstillation followed by a maximal increase at 24 h (Li et al., 1999). Again these results are consistent with the results observed in this study.

The hydroxyl radical can be generated in living systems by reactions such as the Fenton reaction which occur on contact between the reduced forms of transition metal ions and H_2O_2 (Halliwell & Gutteridge, 1989). Certain conditions may also allow the generation of hydroxyl radicals after the reaction between superoxide anion and NO (Department of Health, 1993). The surface of ultrafine particles provides the initial interaction between the particle and a biological system, and it has been hypothesized that the toxicity of these particles is based on the presence of surface associated free radicals or free radical generating systems (Donaldson et al., 1998; Donaldson & McNee, 1996). Therefore, any free radicals associated with the ultrafines present in this study may explain their toxicological, oxidative, or proinflammatory effects.

Reactive oxygen species (ROS) have been implicated in the mediation of cell damage and inflammation induced by ultrafine particles (MacNee et al., 1991; Stone et al., 1998; Li et al., 1996, 1999; Zhang et al., 1998a). Inflammation observed in this study at 18 h was consistent with the degree of surface free radical generation, as measured by the plasmid assay, whereby UFCo, UFCB, and UFNi all cause a significant increase in neutrophil influx, as well as inducing a significant depletion of supercoiled plasmid DNA, indicative of hydroxyl radical generation (Gilmour et al., 1997). The increase in neutrophils measured in BAL 18 h postinstillation is also consistent with MIP-2 changes observed at 4 h. This would indicate that an early alteration in this proinflammatory chemokine subsequently leads to an infiltration of neutrophils at a later time of 18 h. A role for free radicals and ROS in ultrafine inflammation is further strengthened by the ability of antioxidants (NAC and GSHme) to block the release of the cytokine TNF-α from alveolar macrophages in vitro after treatment with UFNi. The antioxidants NAC and GSHme were used as they are both thiol antioxidants capable of entering the cell and replenishing depleted antioxidant stores (MacNee et al., 1991; Grattagliano et al., 1995).

In all of the experiments conducted, treatment of UFTi treatment had much less effect than any of the other ultrafine particles, with no significant free radical activity in the plasmid assay, no significant neutrophil influx, and no significant increases in MIP-2. Consequently, this study shows that the surface reactivity of ultrafines of similar size is a factor in dictating the ability to cause inflammation. These experiments are different from those that have previously demonstrated proinflammatory effects of UFTi (Ferin et

al., 1992). However, the Ferin et al. (1992) study was conducted under overload conditions and the particles were delivered by high inhalation exposure. The UFTi sample used in the Ferin study was also different from that used here, and differences between the surface areas of these UFTi samples could be present (Nolan et al., 1987). Recently, Tran and colleagues indicated that the surface area threshold for overload inflammation in nontoxic particles is around 200–300 cm^2 (Tran et al., 2000). These data were based on an inhalation study of two non-ultrafine particles (TiO_2 and barium sulfate) but may explain the inflammation observed after the instillation of UFCB (125 μg was 317.4 cm^2) but not UFTi, UFCo, or UFNi.

The generation of oxidative stress in the airspaces by particle surfaces may initiate the early stages of pulmonary inflammation. The induction and release of proinflammatory cytokines by UFCB are well documented both in vitro (Beck-Speier et al., 2001) and in vivo (Li et al., 1999) and support the data presented here. Both nickel (Rice et al., 2001) and cobalt (Bucher et al., 1999) salts are well documented to cause inflammation, and the large surface area of metallic ultrafine metal used here has already been suggested to have extra inflammogenicity (Serita et al., 1999; Zhang et al., 1998a).

Recently ultrafine carbon black particles have been shown to stimulate the entry of extracellular Ca^{2+} (via plasma membrane channels) into rat alveolar macrophages and a macrophage cell line (Stone et al., 2000; Brown et al., 2000). An increase in intracellular Ca^{2+} levels may be responsible for the activation of a number of transcription factors, which could lead to the expression of proinflammatory genes (Pahl & Baeuerle, 1997). The effect on Ca^{2+} influx was inhibited by mannitol (a hydroxyl radical scavenger), thus reinforcing the data presented here, such that surface free radicals cause inflammation via a pathway involving oxidative stress.

In conclusion, differences between the particles in vivo can be distinguished on the basis of their inflammogenicity in the rat lung (both at 4 and 18 h). There are also clear differences between the particles with respect to their ability to induce MIP-2 production (at 4 h) and γ-GT release (at 18 h). All of these measurements indicate that at the time points investigated, both UFCo and UFCB and to a lesser extent UFNi induce greater effects on the lung when compared to UFTi. The slightly anomalous activities of UFNi, that is, active in the plasmid assay to the same extent as the UFCB and UFCo, but less so than γ-GT and MIP-2, and later onset inflammation, confirm that there are other modifying factors involved in the pulmonary inflammatory response arising from these ultrafine particles. It is also likely that some effects of UFNi would have been significant if more rats had been instilled, but we are ethically constrained by university regulations to use low numbers of animals. The inflammation observed is likely mediated by the surface free radical activity, indicated by the fact that UFCo, UFCB, and UFNi are all capable of producing hydroxyl radicals (as shown in the plasmid scission assay) and all had more activity than UFTi, which had little or no free radical activity. These data also indicate that the UFNi, and pos-

sibly the other inflammogenic ultrafines induced production of TNF-α by alveolar macrophages, a result ameliorated by antioxidants.

From this study we propose that surface area, chemical composition, and surface reactivity appear to play a role in the toxicity of ultrafines. PM_{10} is known to contain an ultrafine fraction, which is likely to be carbon based. If, as with UFCB, carbon-based PM_{10} particles have a large surface area, then this could contribute to the free radical activity associated with PM_{10} (Donaldson et al., 1997; Li et al., 1996). However, adding to the complexity of PM_{10} are metals (Costa & Dreher, 1997), gram negative bacterial products (Soukup & Becker, 2001), and the potential for interaction among all of these components.

Several studies indicate that PM_{10} causes adverse effects via oxidative stress (Jimenez et al., 2000; Carter et al., 1997), and the ultrafine component may contribute to this stress. Susceptible individuals, such as those with chronic obstructive pulmonary disease (COPD) or asthma, already exhibit preexisting oxidative stress and hence are in a primed state for further oxidative stress induced by ultrafine particles.

This study provides important information regarding the effect the role of free radicals in mediating inflammation after exposure to instilled ultrafines. Further studies are warranted to investigate the effect that such particles will have after inhalation exposure, as well as to investigate the health effects after exposure to ambient ultrafine particles.

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