Toxicology and Applied Pharmacology **172,** 119–127 (2001)
doi:10.1006/taap.2001.9128, available online at http://www.idealibrary.com on IDEAL®

Impairment of Alveolar Macrophage Phagocytosis by Ultrafine Particles

L. C. Renwick, K. Donaldson, and A. Clouter[1]

School of Life Sciences, Napier University, 10 Colinton Road, Edinburgh, EH10 5DT, Scotland

Received August 7, 2000; accepted December 14, 2000

Impairment of Alveolar Macrophage Phagocytosis by Ultrafine Particles. Renwick, L. C., Donaldson, K., and Clouter, A. (2001). *Toxicol. Appl. Pharmacol.* **172, 119–127.**

We investigated whether slowed clearance after exposure to ultrafine particles was due to a failure in alveolar macrophage phagocytosis. This was achieved by measuring the ability of a macrophage cell line (J774.2 MΦ) to phagocytose 2-μm indicator latex beads following 8-h exposures to a number of test particles. Particles utilized were fine titanium dioxide (TiO2), ultrafine titanium dioxide (UTiO2), carbon black (CB), or ultrafine carbon black (UCB). Cytotoxicity of particles was measured by means of MTT activity. In a preliminary study, we assessed the effects of conditioned medium from particle-treated macrophages on the phagocytic ability of naive macrophages. Ultrafine and fine particles had no significant cytotoxic effects on J774.2 MΦ. A significant reduction in the ability of macrophages to phagocytose the indicator beads occurred after exposure to 0.39 μg/mm^2 ($p < 0.001$) of UCB and 0.78 μg/mm^2 ($p < 0.001$) of all particle types compared to the control. Furthermore, ultrafine particles were shown to significantly ($p < 0.001$) impair macrophage phagocytosis at a lower dose than their fine counterparts (0.39 and 0.78 μg/mm^2, respectively). At all doses, UCB resulted in a greater number ($p < 0.001$) of nonphagocytic macrophages compared to the other test particles. We tested whether a diffusable mediator being released from particle-exposed cells inhibited the phagocytic activity of adjacent macrophages. The conditioned medium from particle-exposed macrophages had no significant effect on the phagocytic ability of macrophages, suggesting that cell–cell contact is responsible for the pattern of failed phagocytosis (data not shown). We have demonstrated that ultrafine particles impair macrophage phagocytosis to a greater extent than fine particles compared on a mass basis. Therefore, we conclude that slowed clearance of particles, specifically the ultrafines, can in part be attributed to a particle-mediated impairment of macrophage phagocytosis.

Key Words: **phagocytosis; alveolar macrophages; ultrafine particles; fine particles; carbon black; titanium dioxide; free radicals.**

It has been well documented that particles such as titanium dioxide (TiO_2) and carbon black (CB), when inhaled at high concentrations, can cause chronic pulmonary inflammation, pulmonary fibrosis, and the development of lung tumors in exposed rats (Driscoll *et al.*, 1996; Heinrich *et al.*, 1995; Lee *et al.*, 1985; Muhle *et al.*, 1991; Nikula *et al.*, 1995; Warheit *et al.*, 1997). These responses have been linked to excessive lung burdens, which lead to "particle overload" (Morrow, 1988). Central to the particle-overloaded lung is hypothesized impairment of alveolar macrophage-mediated lung clearance, which occurs when the deposition rate of inhaled particles is greater than the alveolar clearance rate. Consequences of impaired clearance include the accumulation and interstitialization of particles (Ferin *et al.*, 1991, 1992; Oberdorster *et al.*, 1992a, 1994), excessive release of inflammatory mediators from activated phagocytic cells (Becker *et al.*, 1996; Borm *et al.*; 1988; Driscoll *et al.* 1990a, Driscoll and Maurer, 1991; Vanhee *et al.*, 1995), epithelial damage, and activation of fibroblasts (Driscoll *et al.*, 1990b, 1995, 1997; Rom 1991). Continuous exposure under these conditions leads to chronic inflammation, fibrosis, and "overload cancer" in rat studies (reviewed in Mauderley, 1996).

The overload phenomenon, originally proposed by Morrow (1988), focused on the volumetric loading of the alveolar macrophage (AM). Morrow suggested that, when the phagocytosed particle burden occupied 6% of the internal volume of the AM, clearance mechanisms were affected, that is, the AM showed impaired ability to migrate and transport their particle burden to the mucocillary escalator. Morrow further calculated that, when the phagocytosed burden occupied 60% of the internal volume of the AM, clearance mechanisms would be completely inhibited. In support of this theory, Dorries and Valberg (1992) demonstrated that the cytoskeleton of AMs was affected when the phagocytic load occupied 7–8% of the AM volume. Furthermore, Lenhert (1990) and Oberdoster *et al.* (1992b) also found that a particle burden occupying 60% of the AM volume caused almost complete inhibition of particle clearance by AM *in vitro*.

More recently, it has been demonstrated that ultrafine particles (average diameter $\geq$ 100 nm) have exceptional toxicity and are more likely to induce inflammatory responses and the development of particle-mediated lung diseases than the same mass of fine particles (Ferin *et al.*, 1990, 1991, 1992; Kusaka *et al.*, 1998; Li *et al.*, 1996; reviewed in Donaldson *et al.*,

[1] To whom correspondence should be addressed. E-mail: A.Clouter@napier.ac.uk.

0041-008X/01 $35.00

1998). Oberdorster *et al.* (1994) compared rat AM-mediated clearance of the same mass of ultrafine titanium dioxide ($UTiO_2$, 20 nm) and fine titanium dioxide (TiO_2, 250 nm). They reported that a volumetric loading of 9% with TiO_2 caused a doubling of the retention half-time, whereas a volumetric loading of 2.6% with $UTiO_2$ caused an eightfold prolongation of the clearance half-time of an inhaled radioactive indicator particle. Therefore, impairment of AM clearance was far greater after the loading of $UTiO_2$ than a similar mass of TiO_2. This indicates that the surface area or numbers of particles is most critical to ultrafine particle-mediated lung injury. In agreement with this, Driscoll (1996) reviewed several chronic inhalation studies of various particulates, including toner, TiO_2, CB, diesel soot, and talc. He concluded that overload tumors were best correlated to the surface areas of particulates and not mass or volume. Tran *et al.* (2000) also showed that the onset of inflammation and impairment of clearance was related to lung surface area burden and not lung mass burden for two different nontoxic particles (TiO_2 and Ba_2SO_4) delivered by inhalation; by implication it was also suggested that the high surface area per unit mass of ultrafines might cause this inflammation at a lower mass burden. Several studies have demonstrated that ultrafine particles such as $UTiO_2$ and UCB have a greater ability to generate free radicals and induce oxidative stresses compared to their fine equivalents, and this in turn is thought to be related to surface area (Donaldson *et al.*, 1995, 1996; Gilmour *et al.*, 1996, 1997; Li *et al.*, 1996; Oberdorster, 1996). Donaldson *et al.* (1996) showed that TiO_2 had modest ability to cause hydroxyl radical-mediated DNA strand breakage, but $UTiO_2$ was much more active in this respect. In addition, Stone *et al.* (1998) reported that UCB induced greater oxidative stress, as measured by GSH depletion, in cells than the same dose of CB. Therefore, a unifying hypothesis for the effect of ultrafine particles in causing pathogenic changes via their high surface area per unit mass has evolved with oxidative stress as a likely mechanism.

To date, the majority of research on ultrafine particle-mediated impairment of AM clearance has focused on common features such as decreased macrophage mobility and increased retention and interstitialization of particles (Brown *et al.*, 1992; Ferin *et al.*, 1992; Lehnert *et al.*, 1990; Oberdoster *et al.*, 1992a, 1992b, 1994; Warheit *et al.*, 1997). To our knowledge there are no published studies comparing the direct effect of ultrafine and fine particle exposure on the phagocytic ability of alveolar macrophages. Therefore, we investigated whether slowed clearance following particle exposure could be due to a failure in phagocytosis and whether this effect was greater with ultrafine particles.

TABLE 1
Mean Diameters and Surface Areas of Ultrafine and Fine Particles

Particle type	Mean diameter (nm)	Surface area (m^2/g)
TIO2	250	6.6
UTIO2	29	49.78
CB	14.3	7.9
UCB	260.3	253.9

Note. Surface area was determined by nitrogen adsorption and size was measured using transmission electron microscopy.

TABLE 2
Composition of Particles Used in the Study

Sample	Final concentration/mg particles: Fe (ng/mg)	Cu (ng/mg)	Cr (ng/mg)	Ni (ng/mg)
TIO2 saline	0	35	0	90
TIO2 citrate	0	15	10	0
UTIO2 saline	0	60	0	135
UTIO2 citrate	0	45	0	80
CB saline	0	80	0	85
CB citrate	50	185	0	35
UCB saline	0	55	0	105
UCB citrate	100	30	40	210

Note. See method described by Brown *et al.* (2000).

MATERIALS AND METHODS

Chemicals and solutions. RPMI 1640 medium (without L-glutamine), penicillin–streptomycin (1000 μg/ml), L-glutamine (200 mM), fetal bovine serum (FBS), (10×) Dulbecco's phosphate-buffered saline (PBS) with calcium and magnesium salts, and a Hemacolour rapid staining set were obtained from Gibco BRL (Paisley, UK). 3-(4,5-Dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT); dimethyl sulfoxide (DMSO); latex fluorescent beads (2 μm), and DPX were obtained from Sigma Chemicals Company (Poole, Dorset, UK). Sterile water was purchased from Baxter.

Particles. The particles used in this study were (a) fine carbon black (CB), obtained from H.Haeffner & Co Ltd (Chepstow, UK) as Huber 990; (b) ultrafine carbon black, obtained from Degussa as Printex 90; (c) fine titanium dioxide (TiO_2), provided by Tioxide Ltd; and (d) ultrafine titanium dioxide ($UTiO_2$), obtained from Degussa. The mean diameter and surface area of particulates are shown in Table 1. Surface area was determined by BET analysis, and diameter was measured using transmission electron microscopy. The elemental composition of the particles is shown in Table 2. This was determined as described by Brown and colleagues (2000).

Preparation of particles. Particles were suspended in culture medium (RPMI 1640 medium supplemented with 1% penicillin–streptomycin, 1% L-glutamine, and 10% FBS) at concentrations between 125.45 and 15.683 μg/ml, which corresponded to particle densities of 0.78–0.0975 $\mu g/mm^2$, and sonicated (Grant Ultrasonic bath XB6) for 20 min prior to use.

Utilization of J774.2 cell line. J774.2 mouse (BALB/C) tumor monocytic-macrophage cell line was obtained from the European Collection of Animal Cell Cultures (Salisbury, England). J774.2 macrophages are an adherent, phagocytic murine cell line. Of the cells cultured, approximately 55% were phagocytic, in comparison to >90% of primary rat alveolar macrophages. All nonadherent cells were disregarded. Cells were then suspended in fresh me-

dium (RPMI 1640 medium supplemented with 1% penicillin–streptomycin, 1% L-glutamine, and 10% FBS) by scraping the flasks with sterile cell scrapers (Helena BioSciences). The cell suspensions were centrifuged at 900*g* for 10 min at 4°C and the pellets were resuspended in 3 ml of culture medium. Cell counts and viability were determined using a hemocytometer and trypan blue dye exclusion (cell viability > 90%). Cells were then resuspended in culture medium according to the concentration required. Cultures were kept in a humidified incubator at 37°C with 95% air and 5% CO_2. Culture conditions were maintained as above unless otherwise stated.

Assessment of cytotoxicity. J774.2 macrophages were resuspended in culture medium at a concentration of 5×10^6/ml. Cell suspensions (200 μl; 1×10^5 cells) were then dispensed (six control wells and triplicate wells for each particle type) into 96-well plates (Helena BioSciences) and incubated overnight (16 h) to allow for cell adherence. Culture medium was replaced with 200 μl of particle/culture medium suspensions (125.45 μg/ml = 0.78 μg/mm^2) and plates were further incubated for 4, 8, 24, and 48 h. In addition, a dose–response study was carried out over 8 h in which cells were exposed to various particle doses (0.78–0.0975 μg/mm^2). Cytotoxicity of particles was assessed using an MTT assay based on Mosmann (1983), Denizot and Lang (1986), and Carmicheal *et al.* (1987). Briefly, MTT (20 μl, 5 mg/ml of PBS) was added to each well and incubated for 1 h. Supernatants were removed and replaced with 200 μl of DMSO. The 96-well plates were then centrifuged at 900*g* for 15 min at 18°C to remove any particles present in the supernatant. Supernatants (100 μl) were realiquoted onto new 96-well plates and the absorbance was read at 540 nm in a MRX Microplate Reader.

Three different controls were used for each assay and treated as the test samples: control 1, six wells (6) containing macrophages and culture medium; control 2: six wells of culture medium only; and control 3: three wells for each particle type containing particle/culture medium suspension but no cells.

Control cell MTT activity was calculated as

$$\text{control 1} - \text{control 2} = X$$

Test absorbencies were calculated as

$$\text{test} - \text{control 3}.$$

All test results were normalized to *X*. Test results were then expressed as MTT activity and as percentage survival, where any decrease from the control would indicate toxicity. The significance of the results were statistically analyzed by the two-sample assuming equal variances *t* test.

Phagocytic response of J774.2 macrophages to latex beads (2 μm) after exposure to ultrafine and fine particles. The phagocytic ability of J774.2 macrophages after 8-h exposures to varying doses (0.78–0.0975 ug/mm^2) of ultrafine and fine particles was assessed by measuring their ability to phagocytose 2-μm latex beads (Harper *et al.*, 1994) after particle exposure. J774.2 macrophages were resuspended in culture medium at a concentration of 5×10^5 cells/ml. Cell suspensions were then transferred (10 ml; 5×10^6 cells) to sterile 25-cm^2 culture flasks (Helena BioSciences) and incubated overnight (16 h) to allow for cell adherence. Cells were incubated with particle/culture medium suspension (10 ml) for 8 h and then washed two times with PBS to remove excess particles. Culture medium (10 ml) containing latex beads at a bead-to-macrophage ratio of 5:1 was transferred to the culture flasks. Macrophage and bead suspensions were then incubated for 24 h to allow for phagocytosis. Beads not phagocytosed were removed and J774.2 macrophages were resuspended in 5 ml of fresh culture medium. Cell counts and viability was then determined, as described previously (Tables 2 and 3). Cytospins were prepared, air dried, stained using the Hemacolour rapid staining set, and then mounted in DPX. The controls consisted of culture flasks containing the relevant concentration of J774.2 macrophages and volumes of culture medium, minus the particles, and were treated as the test samples.

TABLE 3
Percentage of J774.2 Macrophages That Phagocytosed the Only Indicator Latex Beads

	J774.2 macrophages that phagocytosed only the indicator latex beads (%)			
	Particle dose (μg/mm^2)			
Test particle	0.0975	0.195	0.39	0.78
TIO2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
UTIO2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
CB	0 ± 0	0 ± 0	0 ± 0	0 ± 0
UCB	5.2 ± 1.0	1.2 ± 0.3	1.0 ± 0.2	0 ± 0

Note. Macrophages did not phagocytose the test particles but could phagocytose ≥2 indicator latex beads. Results are the mean ± SEM of three separate experiments.

From each slide (four slides/treatment) 100 cells were counted and placed into one of four categories:

1. Cells that could still phagocytose ≥2 indicator latex beads after the uptake of particles (Fig. 1A). This parameter measured the total phagocytic ability of the macrophage population after the uptake of test particles.
2. Cells that phagocytosed the test particles but were then unable to further phagocytose the indicator latex beads (Fig. 1B). This parameter assessed the ability of phagocytosed particles to inhibit the phagocytic activity of the cell.
3. Cells that did not phagocytose either the test particles or the indicator latex beads (Fig. 1C). This parameter measured the total proportion of cells that were nonphagocytic.
4. Cells that did not phagocytose the test particles but phagocytosed ≥2 indicator beads (Fig. 1D). This final parameter represents the population of macrophages that were phagocytic but failed to phagocytose the test particles. We did not anticipate this group to be large.

Results were expressed as a percentage of the total number of J774.2 macrophages. Tukeys one-way analysis of variance with multiple comparisons was used to evaluate significant differences between the effects of the treatments.

Additional Experiments: Effect of conditioned media on the phagocytic ability of J774.2 macrophages. J774.2 macrophages were incubated with particle/culture medium suspensions (0.39 μg/mm^2) for 8 h. The medium was aspirated and centrifuged to remove excess particles. The conditioned medium was then transferred to freshly cultured cells and left for 8 h. Medium was replaced with latex bead/culture medium suspensions and treated as described previously. Cells that contained ≥2 latex beads were counted as phagocytic.

RESULTS

Cytotoxicity

Preliminary studies were carried out to establish a dose and time of particulate exposure that would not be cytotoxic to cells. This was assessed by means of MTT activity. Significant ($p < 0.05$) cytotoxicity occurred after a 48-h exposure to 0.78 μg/mm^2 UCB, however, this was minimal compared to the control. (data not shown). Therefore, 8 h exposures to ultrafine and fine particles at doses < 0.78 μg/mm^2 was assessed.

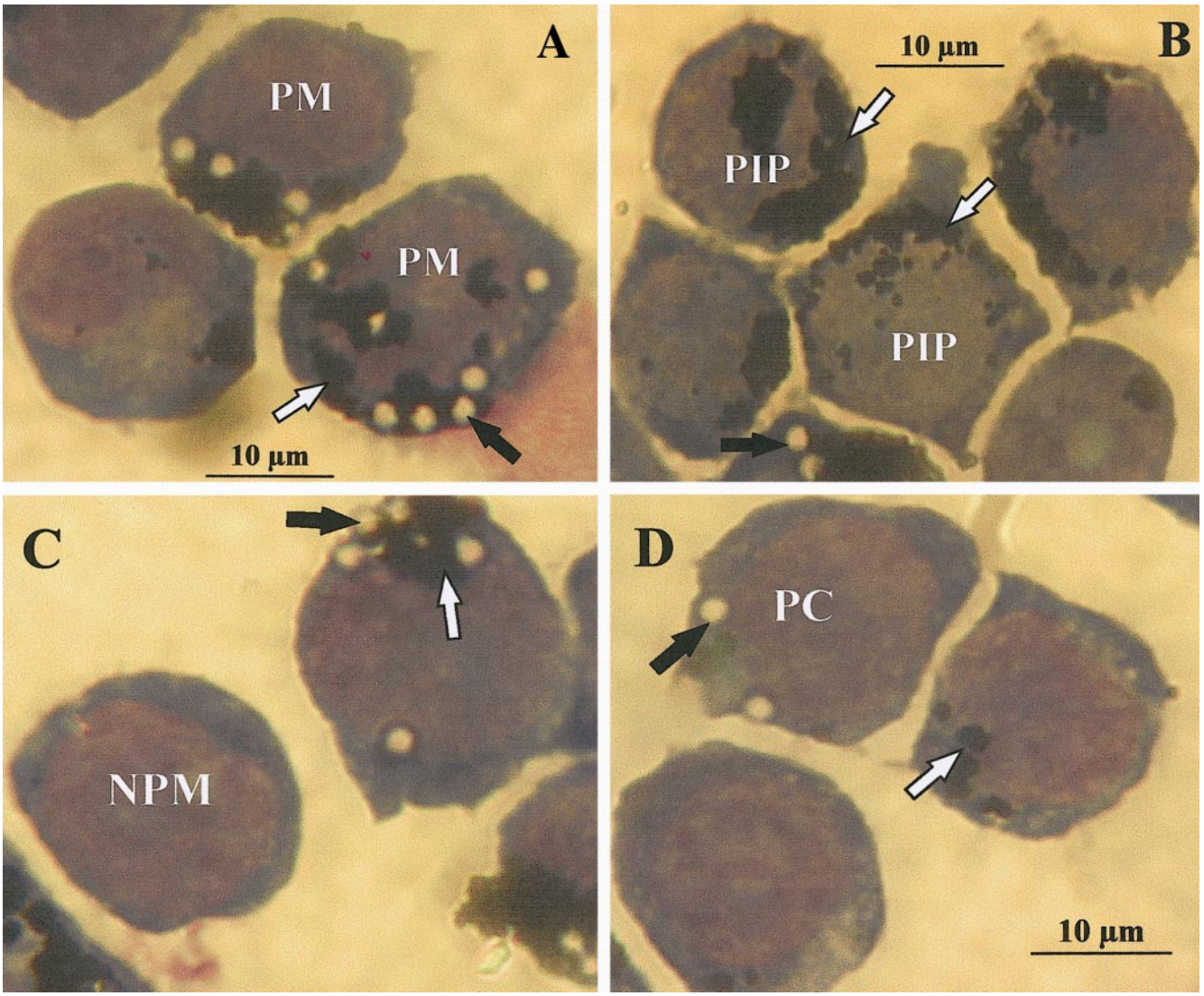

FIG. 1. Photomicrographs of J774.2 macrophages (original magnification 1000×). White arrow, CB particles; black arrow, indicator latex beads. (A) Cells that could still phagocytose ≥2 indicator beads after the uptake of particles (PM, phagocytic macrophages). (B) Cells that were unable to phagocytose indicator beads following the uptake of particles (PIP, particle-inhibited phagocytosis). (C) Cells that did not phagocytose either the particles or indicator beads (NPM, nonphagocytic macrophages). (D) Cells that phagocytosed only the indicator beads (PC).

Particle dose (≤0.78 $\mu g/mm^2$) had no significant cytotoxic effects on J774.2 macrophages (data not shown). Thus, macrophage phagocytosis assays utilized particle doses ≤ 0.78 $\mu g/mm^2$ and an exposure time of 8 h.

Phagocytic Response after Particle Exposure

The phagocytic response of J774.2 macrophages to 2-μm latex beads after 8-h exposures to ultrafine or fine particles (0.0975–0.78 $\mu g/mm^2$) was assessed.

Total proportion of cells that could phagocytose >2 indicator beads following the uptake of test particles. This parameter measured the total phagocytic ability of the macrophage population after the uptake of test particles. A significant reduction in the phagocytosis of indicator latex beads occurred after exposure to 0.39 $\mu g/mm^2$ of UCB ($p < 0.01$) and 0.78 $\mu g/mm^2$ of all other particles types ($p < 0.001$) compared to the control (Figs. 2a and 2b). In addition, macrophage phagocytosis was significantly ($p < 0.01$) impaired following exposure to UCB at a lower dose (0.39 $\mu g/mm^2$) than with the fine equivalents (CB) (Fig. 2b). A decrease ($p < 0.01$) in latex bead phagocytosis was also observed for all particles as the dose was increased from 0.0975 to 0.78 $\mu g/mm^2$ (Figs. 2a and 2b). The phagocytic activity of macrophages after exposure to 0.78 $\mu g/mm^2$ of

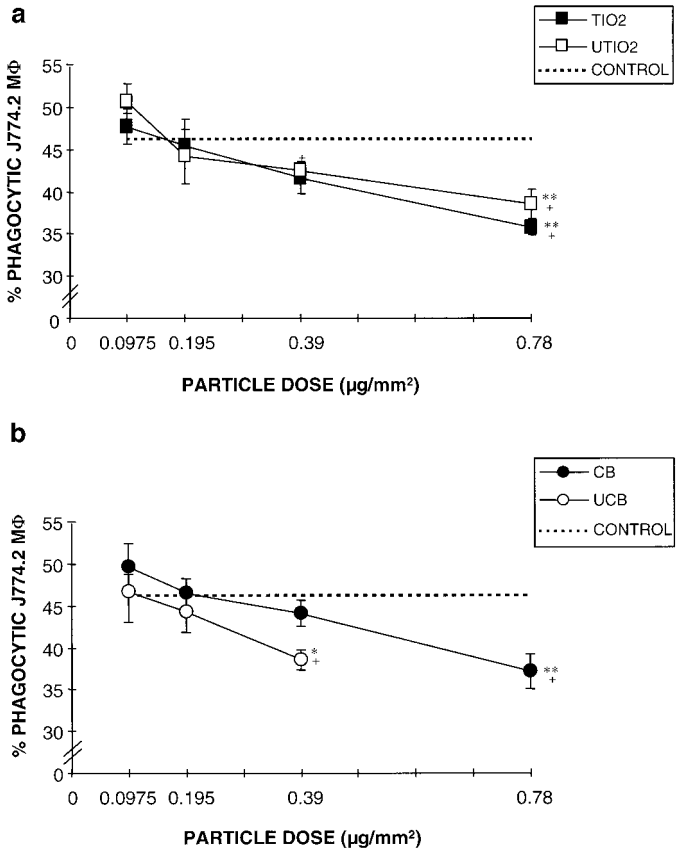

FIG. 2. Percentage of cells capable of phagocytosing ≥2 indicator latex beads after the uptake of test particles (i.e., phagocytic macrophages) (a) titanium dioxide and ultrafine titanium dioxide; (b) carbon black and ultrafine carbon black. Results are the means ± SEM of three separate experiments. *Significantly different from control value, $p < 0.01$. **Significantly different from control value, $p < 0.001$). +Significant differences between the lowest particle dose (0.0975 μg/mm^2) and doses > 0.0975 μg/mm^2, $p < 0.01$. ++ Significant differences between the lowest particle dose (0.0975 μg/mm^2) and doses > 0.0975 μg/mm^2, $p < 0.001$).

UCB was not assessed due to the interference of particles in the visualization of latex beads.

Total proportion of cells that phagocytosed the test particles but were then unable to further phagocytose the indicator beads. This parameter measured the population of macrophages whose phagocytic ability was completely inhibited after the uptake of test particles. Increasing particle dose resulted in a greater ($p < 0.01$) inhibition of macrophage phagocytosis, as shown by the increase in particle-laden macrophages (Fig. 3). This increase in particle-laden macrophages correlated with the decrease in phagocytic cells shown in Figs. 2a and 2b. Thus, the observed effect occurred at a lower dose with UCB than with its fine equivalents.

Total proportion of cells that did not phagocytose either the test particles or the indicator latex beads. This parameter assessed the total proportion of cells that were nonphagocytic.

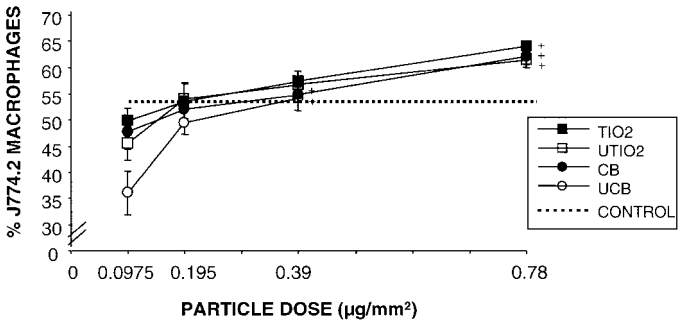

FIG. 3. Percentage of cells that were unable to phagocytose the indicator latex beads following the uptake of test particles (i.e., particle-inhibited phagocytosis). Results are the means ± SEM of three separate experiments. +Significant differences between the lowest particle dose (0.0975 μg/mm^2) and doses > 0.0975 μg/mm^2, $p < 0.01$. + Significant differences between the lowest particle dose (0.0975 μg/mm^2) and doses > 0.0975 μg/mm^2, $p < 0.001$).

At the lowest dose, exposure to UCB caused a significant ($p < 0.001$) increase (sixfold) in the number of nonphagocytic cells compared to the other test particles (Fig. 4). In fact, for all particles, the number of nonphagocytic macrophages was greatest following exposure to the lowest dose (0.0975 μg/mm^2).

Total proportion of cells that did not phagocytose the test particles but phagocytosed indicator beads. This parameter measured the population of macrophages that were phagocytic but failed to phagocytose the test particles. As anticipated, a very small percentage (1–5%) of cells phagocytosed only the latex beads and this occurred following exposure to all doses of UCB (except 0.78 μg/mm^2) (Table 3). This effect did not arise with the other test particles.

Additional Experiments: Effect of Conditioned Medium on the Phagocytic Ability of J774.2 Macrophages

At all doses, UCB resulted in a greater number of nonphagocytic macrophages compared to the other test particles.

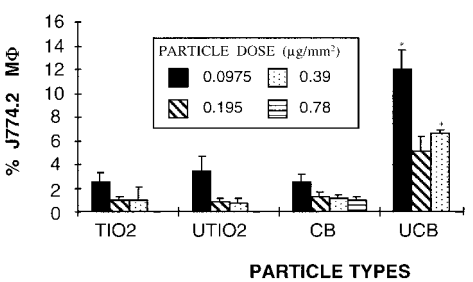

FIG. 4. Percentage of macrophages that did not phagocytose either the test particles or the indicator latex beads (i.e., nonphagocytic cells). Results are the means ± SEM of three separate experiments. *At the lowest dose, UCB resulted in a greater percentage of nonphagocytic cells compared to the other test particles, * $p < 0.001$.

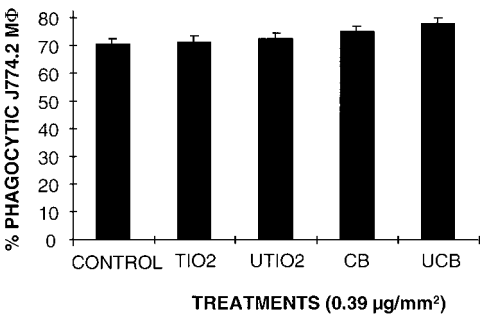

FIG. 5. Effects of conditioned medium taken from particle-exposed macrophages on the phagocytic ability of J774.2 cells. Results are from one experiment ± SD.

This effect could have been a result of cell line proliferation. However, observation of the total cell numbers and cell viabilities following all treatments showed that there was no significant difference between all particle types over the dose range (data not shown). Thus, we tested the possibility of a diffusable mediator being released from particle-exposed macrophages that inhibited the phagocytic activity of adjacent cells. The conditioned medium from particle-exposed macrophages had no significant effect on the phagocytic ability of J774.2 macrophages compared to the control (Fig. 5). Furthermore, there was no difference between the effect of UCB-conditioned medium and the conditioned medium from all other test particles.

DISCUSSION

We have demonstrated that the phagocytic ability of J774.2 macrophages was significantly impaired following an increasing dose of all particle types. This was to be anticipated since the phagocytic ability of individual cells is finite. Of greater interest was the potential for ultrafine particles to impair phagocytosis more than fine particles.

In terms of the overall ability of the macrophage population to phagocytose the indicator latex beads after the uptake of test particles, UCB showed greater potency than other particles (UTiO2, TiO_2, and CB). Ultrafine carbon black significantly impaired macrophage phagocytosis at 0.39 $\mu g/mm^2$, whereas the other particles had this effect only at 0.78 $\mu g/mm^2$.

The proportion of cells that did not phagocytose either the test particles or the latex beads (i.e., nonphagocytic cells) was most striking with UCB. In fact, at all doses, UCB caused a greater number of macrophages to be nonphagocytic compared to the other test particles. For all other particles the number of nonphagocytic cells was more marked at the lowest dose.

This may have been partly artifactual, that is, at such low doses it is difficult to see ultrafine particles inside the cells. Therefore, it is possible that some cells were falsely counted as "nonphagocytic" when, in fact, they contained particles, but these were at low levels and therefore not visible at the magnifications used. It is also a possibility that macrophages "find" particles, that is, they actively move toward them, and the mechanism of failed clearance may be related in some way to a failure by the macrophages to locate the particles.

The paradoxical category of cells that failed to phagocytose the test particles but did phagocytose the indicator beads was, as anticipated, a low 1–5% of the total macrophage population.

The results show that, as the dose of particles increased, there was a change in the macrophage population from cells that could still phagocytose the indicator beads after the uptake of particles to cells that could not. This happened with all particles but was most significant following exposure to the UCB particles. Furthermore, with UCB, the particle-laden macrophages were finally replaced with a smaller, but significant, population of nonphagocytic cells, i.e., cells that phagocytosed neither the test particles nor latex beads. Thus, the major effect of UCB was seen as an increase in nonphagocytic cells. There are two plausible mechanisms for this effect: particle-exposed macrophages released a diffusible mediator that inhibited the phagocytic activity of adjacent cells or particle-exposed macrophages transmitted a message by direct contact to adjacent cells, which in turn inhibited the phagocytic activity of contacted cells.

In our additional experiment we tested the first mechanism, i.e., whether a soluble component was released from the particle-treated macrophages. Conditioned medium was collected from cells exposed to all of the test particles and then the ability of this conditioned medium to affect the phagocytosis of naive macrophages was determined. There was no effect on macrophage phagocytosis following exposure to the UCB-conditioned medium and the conditioned media from macrophages treated with the other test particles. Therefore, we conclude that cell–cell contact is responsible for the observed effect and that contact between macrophages and UCB causes cells to transmit an inhibitory signal that prevents the phagocytic activity of cells with which they make contact. There are several precedents for such an effect. Macrophages have been shown to inhibit bone marrow cell proliferation and cause cytostasis of A549 epithelial cells by a contact-dependent process (Sauty *et al.*, 1994). Also, lipid molecules have been reported to be involved in contact-mediated inhibition of lymphocyte growth (Kato *et al.*, 1998; Stallcup *et al.*, 1986). Further studies on the hypothesis of contact-mediated inhibition of phagocytosis is warranted.

Previous studies, for example, Warheit *et al.* (1997) and Lee *et al.* (1985) have also reported impairment of rat AM phagocytosis and prolonged particle clearance after exposure to increasing doses (5, 50, and 250 mg/m^3) of TiO_2, although this was under overload conditions *in vivo*.

Contrary to these findings and those of Morrow (1988), we show here that, in the case of the ultrafines ($UTiO_2$ and UCB), the volume of particles phagocytosed was not solely responsi-

ble for the impairment of macrophage phagocytosis, but this impairment may be attributable to differences in size, number, and surface area of particles. Our findings of impaired phagocytosis in ultrafine-exposed cells support the findings of Oberdorster *et al.* (1994), who demonstrated that a volumetric loading of AM with $UTiO_2$ results in an eightfold increase in the particle retention half-time. In comparison, a loading with larger-sized TiO_2 only doubled the retention half-time. In our hands, $UTiO_2$ did not have as much inhibitory activity as UCB. This could be explained by the greater surface area of the UCB (253.9 m^2/g) compared to UTiO2 (49.78 m^2/g), which could result in greater free radical generation.

A comparison of the size and surface area of the test particles shows that $UTiO_2$ and UCB have much smaller diameters and far greater surface areas than their fine equivalents. Thus, at all doses there is a greater number of ultrafine particles with corresponding larger surface areas. Due to the larger size and smaller surface area of fine particles, a much greater mass of phagocytosed particles (0.78 $\mu g/mm^2$) was required to impair macrophage phagocytosis of the indicator beads compared to their ultrafine equivalents (0.39 $\mu g/mm^2$). In addition, as UCB has a smaller diameter and larger surface area than $UTiO_2$, we hypothesized that exposure to UCB would inhibit macrophage phagocytosis to a greater extent than $UTiO_2$. Although statistically there was no significant difference between the inhibitory effects of $UTiO_2$ and UCB, the UCB did appear to have a marginally greater effect than $UTiO_2$. Exposure to the highest dose of UCB (0.78 $\mu g/mm^2$) resulted in cells so packed with particles that it was impossible to determine if they were still phagocytic for the indicator beads. This did not occur with $UTiO_2$. Furthermore, a greater percentage of nonphagocytic macrophages was observed following exposure to UCB than $UTiO_2$.

Ultrafine particles have been shown to generate free radicals and induce oxidative stress (Donaldson *et al.*, 1996; Gilmour *et al.*, 1996, 1997; Stone *et al.*, 1998). Furthermore, this study showed that, at an equal mass, the ultrafine particles ($UTiO_2$ and UCB) produced free radicals to a much greater extent than their fine counterparts. This difference in free radical activity was attributed to the larger surface areas of the ultrafine particles. In addition to direct particle surface-derived oxidative stress, the act of particle phagocytosis has been shown to increase and prolong the release of reactive oxygen species by phagocytosing AM, and this effect was greater after exposure to ultrafines (Becker *et al.*, 1996; Nadeau *et al.*, 1996; Rahman *et al.*, 1997). Therefore, the inhibitory effects of the UCB may be due to its ability to induce oxidative stress in phagocytosing macrophages.

In summary, we have demonstrated that increasing the dose of particles to which macrophages are exposed significantly reduces macrophage phagocytic ability and that the effect varies in a particle-specific manner. Thus, UCB impaired phagocytosis to a greater extent than UTiO2 or the fine particles (CB and TiO_2) compared on a mass basis. This could be due to a number of factors, including differences in size, number, surface area, and surface-associated free-radical generation systems. The pattern of phagocytosis inhibition suggested that macrophages that first encounter UCB rapidly transmit a signal by cell–cell contact that prevents particle uptake by adjacent macrophages. We conclude that slowed clearance of particles, specifically the ultrafine with the very high surface area UCB, can in part be attributed to a particle-mediated impairment of macrophage phagocytosis.

Ultrafine particles are increasingly being used in manufacturing and there is increasing likelihood of exposure. The adverse health effects of PM10 in susceptible individuals (reviewed by Pope *et al.*, 1995) have been linked to the ultrafine content (MacNee and Donaldson, 1999), although the daily exposure to ultrafine particles is very low. The data shown here suggest that, in ultrafine particle-exposed lungs, there could be direct inhibition of macrophage phagocytosis with subsequent buildup of particles with obvious consequences for increased retention of particles.

ACKNOWLEDGMENTS

We acknowledge funding from The British Occupational Health Research Foundation for Louise Renwick and the Transco British Lung Foundation Fellow in Air Pollution and Respiratory Health for Ken Donaldson.

REFERENCES

Becker, S., Soukup, J. M., Gilmour, I., and Devlin, R. B. (1996). Stimulation of human and rat alveolar macrophages by urban air particulates: Effects on oxidant radical generation and cytokine production. *Toxicol. Appl. Pharmacol.* **141,** 637–648.

Borm, P. J. A., Pamen, N., Engelen, J. J. M., and Buurman, W. A. (1988). Spontaneous and stimulated release of tumour necrosis factor-α (TNF) from blood monocytes of miners with coal workers' pneumoconiosis. *Am. Rev. Respir. Dis.* **138,** 1589–1594.

Brown, G. M., Brown, D. M., and Donaldson, K. (1992). Persistent inflammation and impaired chemotaxis of alveolar macrophages on cessation of dust exposure. *Environ. Health Perspect.* **97,** 91–94.

Brown, D. M., Stone, V., MacNee, W., and Donaldson, K. (2000). Increased inflammation and intracellular calcium caused by ultrafine carbon black is independent of transition metals or other soluble components. *Occup. Environ. Med.* **57,** 685–691.

Carmicheal, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., and Mitchell, J. B. (1987). Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res.* **47,** 936–942.

Castranova, D., Dalal, N. S., and Vallyathan, V. (1996). Role of surface free radicals in the pathogenicity of silica. In S*ilica and Silica-Induced Diseases*. (D. Castranova, V. Vallyathan, and W. E. Wallace, Eds.), pp. 91–105. CRC Press, Boca Raton.

Denizot, F., and Lang, R. (1986). Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **89,** 271–277.

Donaldson, K., Beswick, P. H., and Gilmour, P. S. (1996). Free radical activity associated with the surface of particles: A unifying factor in determining biological activity? *Toxicol. Lett.* **88,** 293–298.

Donaldson, K., and Borm, P. J. A. (1998). The quartz hazard: A variable entity. *Ann. Occup. Hyg.* **42,** 287–294.

Donaldson, K., Gilmour, P. S., MacNee, W., and Oberdorster, G. (1995). Free radical activity associated with ultrafine titanium dioxide particles and PM_{10} material. *Am. J. Respir. Crit. Care Med.* **151,** A64.

Donaldson, K., Li, X. Y., and MacNee, W. (1998). Ultrafine (nanometer) particle mediated lung injury. *J. Aerosol Sci.* **29,** 553–560.

Dorries, A. M., and Valberg, P. A. (1992). Heterogeneity of phagocytosis for inhaled versus instilled material. *Am. Rev. Respir. Dis.* **146,** 831–837.

Driscoll, K. E. (1996). Role of inflammation in the development of rat lung tumours in response to chronic particle exposure. *Inhalation Toxicol.* **8,** 139–153.

Driscoll, K. E., Carter, J. M., Howard, B. W., Hassenbein, D. G., Pepelko, W., Baggs, R. B., and Oberdorster, G. (1996). Pulmonary inflammatory, chemokine, and mutagenic responses in rats after subchronic inhalation of carbon black. *Toxicol. Appl. Pharmacol.* **136,** 372–380.

Driscoll, K. E., Deyo, L. C., Carter, J. M., Howard, B. W., Hassenbein, D.G., and Bertram, T.A. (1997). Effects of particle exposure and particle-elicited inflammatory cells on mutation in rat alveolar epithelial cells. *Carcinogenesis* **18,** 423–430.

Driscoll, K. E., Deyo, L. C., Howard, B. W., Poynter, J., and Carter, J. M. (1995). Characterising mutagenesis in the *hprt* gene of rat alveolar epithelial cells. *Exp. Lung Res.* **21,** 941–956.

Driscoll, K. E., Lindenschmidt, R. C., Maurer, J. K., Higgins, J. M., and Ridder, G. (1990a). Pulmonary response to silica and titanium dioxide: Inflammatory cells, alveolar macrophage-derived cytokines, and histopathology. *Am. J. Respir. Cell Mol. Biol.* **2,** 381–390.

Driscoll, K. E., and Maurer, J. K. (1991). Cytokine and growth factor release by alveolar macrophages: Potential biomarkers of pulmonary toxicity. *Toxicol. Pathol.* **19,** 398–405.

Driscoll, K. E., Maurer, J. K., Lindenschmidt, R. C., Romberger, D., Rennard, S. I., and Crosby, L. (1990b). Respiratory tract responses to dust: Relationship between dust burden, lung injury, alveolar macrophage fibronectin release, and the development of pulmonary fibrosis. *Toxicol. Appl. Pharmacol.* **106,** 88–101.

Ferin, J., Oberdorster, G., and Penney, D. P. (1992). Pulmonary retention of ultrafine and fine particles in rats. *Am. J. Respir. Cell Mol. Biol.* **6,** 535–542.

Ferin, J., Oberdorster, G., Penney, D. P., Soderholm, S. C., Gelein, R., and Piper, H. C. (1990). Increased pulmonary toxicity of ultrafines?. I. Particle clearance, translocation, morphology. *J. Aerosol Sci.* **21,** 381–384.

Ferin, J., Oberdorster, G., Soderholm, S. C., and Gelein, R. (1991). Pulmonary tissue access of ultrafine particles. *J. Aerosol. Med.* **4,** 57–68.

Gilmour, P. S., Brown, D. M., Beswick, P. H., Benton, E., MacNee, W., and Donaldson, K. (1997). Surface free radical activity of PM_{10} and ultrafine titanium dioxide: A unifying factor in their toxicity?. *Ann. Occup. Hyg.* **41,** 32–38.

Gilmour, P. S., Brown, D. M., Lindsay, G. T., Beswick, P. H., MacNee, W., and Donaldson, K. (1996). Adverse health effects of PM_{10} particles: Involvement of iron in generation of hydroxyl radicals. *Occup. Environ. Med.* **53,** 817–822.

Harper, R. A., Stirling, C., Towsend, K. M. S., Kreyling, W. G., and Patrick, G. (1994). Intracellular particle dissolution in macrophages isolated from the lung of the Fischer (F-344) rat. *Exp. Lung Res.* **20,** 143–156.

Heinrich, U., Fuhst, R., Rittinghausen, S., Creutzenberg, O., Bellman, B., Koch, W., and Levensen, K. (1995). Chronic inhalation exposure of Wistar rats and two different strains of mice to diesel engine exhaust, carbon black, and titanium dioxide. *Inhalation Toxicol.* **7,** 533–556.

Kato, H., Horino, A., Taneichi, M., Fukuchi, N., Eto, Y., Ushijima, H., Komuro, K., and Uchida, T. (1998). Macrophage inhibition of lymphocyte and tumor cell growth is mediated by 25-hydroxycholesterol in the cell membrane. *Int. Arch. Allergy Immunol.* **117,** 78–84.

Lee, K. P., Trochimocwicz, H. J., and Reinhardt, C. F. (1985). Pulmonary response of rats exposed to titanium dioxide (TiO_2) by inhalation for two years. *Toxicol. Appl. Pharmacol.* **79,** 179–192.

Lehnert, B. (1990). Alveolar macrophages in a particle "overload" condition. *J. Aerosol Med.* **3**(Suppl. 1), S9–S42.

Lehnert, B. E., Ortiz, J. B., London, J. E., Valdez, Y. E., Cline, A. F., Sebring, R. J., and Tietjen, G. L. (1990). Migratory behaviours of alveolar macrophages during the alveolar clearance of light to heavy burdens. *Exp. Lung Res.* **16,** 451–479.

Li, X. Y., Gilmour, P. S., Donaldson, K., and MacNee, W. (1996). Free radical activity and pro-inflammatory effects of particulate air pollution (PM_{10}) *in vivo* and *in vitro*. *Thorax* **51,** 1216–1222.

MacNee, W., and Donaldson, K. (1999). Particulate air pollution: Injurious and protective mechanisms. In *Air Pollution and Health* (S. T. Holgate, J. M. Samet, M. S. Koren, and R. L. Maynard, Eds.), pp. 653–672, Academic Press, San Diego.

Mauderly, J. L. (1996). Lung overload: The dilemma and opportunities for resolution. *Inhalation Toxicol.* **8**(Suppl.), 1–28.

Morrow, P. E. (1988). Possible mechanisms to explain dust overloading of the lungs. *Fundam. Appl. Pharmacol.* **10,** 369–384.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65,** 55–63.

Muhle, H., Bellmann, B., Creutzberg, O., Dansenbrock, C., Ernst, H., Kilpper, R., MacKenzie, J. C., Morrow, P., Mohr, U., Takenaka, S., and Mermelstein, R. (1991). Pulmonary response to toner upon chronic inhalation exposure in rats. *Fundam. Appl. Toxicol.* **17,** 280–299.

Nadeau, D., Vincent, R., Kumarathasan, P., Brook, J., and Dufresne, A. (1996). Cytotoxicity of ambient air particles to rat lung macrophages: Comparison of cellular and functional assays. *Toxicol. in Vitro* **10,** 161–172.

Nikula, K. J., Snipes, M. B., Griffith, W. C., Henderson, R. F., and Mauderly, J. L. (1995). Comparative pulmonary toxicities and carcinogenicities of chronically inhaled diesel exhaust and carbon black in F344 rats. *Fundam. Appl. Toxicol.* **6,** 459–483.

Oberdorster, G. (1996). Significance of particle parameters in the evaluation of exposure-doseresponse relationships of inhaled particles. *Inhalation Toxicol.* **8,** 73–89.

Oberdorster, G., Ferin, J., Gelein, R., Soderholm, S. C., and Finkelstein, J. (1992a). Role of the alveolar macrophage in lung injury: Studies with ultrafine particles. *Eniviron. Health Perspect.* **97,** 193–199.

Oberdorster, G., Ferin, J., and Lehnert, B. E. (1994). Correlation between particle size, *in vivo* particle persistence, and lung injury. *Environ. Health Perspect.* **102,** 173–179.

Oberdorster, G., Ferin, J., and Morrow, P. E. (1992b). Volumetric loading of alveolar macrophages (AM): A possible basis for diminished AM-mediated particle clearance. *Exp. Lung Res.* **18,** 87–104.

Pope, C. A., Dockery, D. W., and Schwartz, J. (1995). Review of epidemiological evidence of health-effects of particulate air-pollution. *Inhalation Toxicol.* **7,** 1–18.

Rahman, Q., Norwood, J., and Hatch, G. (1997). Evidence that exposure of particulate air pollutants to human and rat alveolar macrophages leads to differential oxidative response. *Biochem. Biophys. Res. Commun.* **204,** 669–672.

Rom, W. N. (1991). Relationship of inflammatory cell cytokines to disease severity in individuals with occupational inorganic dust exposure. *Am. J. Ind. Med.* **19,** 15–27.

Sauty, A., Mauel, J., Philippeaux, M. M., and Leuenberger, P. (1994). Cy-

tostactic activity of alveolar macrophages from smokers and nonsmokers: Role of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha. *Am. J. Respir. Cell Mol. Biol.* **11,** 631–637.

Stallcup, K. C., Liu, Y. N., Dorf, M. E., and Mescher, M. F. (1986). Inhibition of lymphoid cell growth by a lipid-like component of macrophage hybridoma cells. *J. Immunol.* **136,** 2723–2728.

Stone, V., Shaw, J., Brown, D. M., MacNee, W., Faux, S. P., and Donaldson, K. (1998). The role of oxidative stress in the prolonged inhbitory effect of ultrafine carbon black on epithelial cell function. *Toxicol. in Vitro* **12,** 649–659.

Tran, C. L., Buchanan, D., Cullen, R. T., Searl, A., Jones, A. D., and Donaldson, K. (2000). Inhalation of poorly soluble particles II Influence of particle surface area on inflammation and clearance. *Inhalation Toxicol.* **12,** 101–115.

Vallyathan, V., Shi, X., Dala, N. S., Irr, W., and Castranova, V. (1988). Generation of free radicals from freshly fractured silica dust: Potential role in acute silica-induced lung injury. *Am. Rev. Respir. Dis.* **138,** 1213–1219.

Vanhee, D., Gossett, P., Boitelle, A., Wallert, B., and Tonnel, A. B. (1995). Cytokines and cytokine network in silicosis and coal workers' pneumoconiosis. *Eur. Respir. J.* **8,** 834–842.

Vincent, J. H., and Donaldson, K. (1990). A dosimetric approach for relating the biological response of the lung to the accumulation of inhaled mineral dust. *Br. J. Ind. Med.* **47,** 302–307.

Warheit, D. B., Hansen, J. F., Yuen, I. S., Kelly, D. P., Snajdr, S. I., and Harsky, M. A. (1997). Inhalation of high concentrations of low toxicity dusts in rats results in impaired pulmonary clearance mechanisms and persistent inflammation. *Toxicol. Appl. Pharmacol.* **145,** 10–22.