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Free radical activity and pro-inflammatory effects of particulate air pollution (PM_{10}) in vivo and in vitro

X Y Li, P S Gilmour, K Donaldson, W MacNee

Abstract

Background **– Epidemiological evidence has implicated fine particulate air pollution, particularly particles less than 10 µm in diameter (PM_{10}), in the development of exacerbations of asthma and chronic obstructive pulmonary disease (COPD) although the mechanism is unknown. The hypothesis that PM_{10} particles induce oxidant stress, causing inflammation and injury to airway epithelium, was tested.**

Methods **– The effects of intratracheal instillation of PM_{10} was assessed in rat lungs (three per group). Inflammatory cell influx was measured by bronchoalveolar lavage (BAL) and air space epithelial permeability was assessed as the total protein in BAL fluid in vivo. The oxidant properties of PM_{10} particles were determined by their ability to cause damage to plasmid DNA and by changes in reduced (GSH) and oxidised (GSSG) glutathione. The effects of PM_{10} particles were compared in some experiments with those of fine (CB) and ultrafine (ufCB) carbon black particles.**

Results **– Six hours after intratracheal instillation of PM_{10} there was an influx of neutrophils (up to 15% of total cells in BAL fluid) into the alveolar space, increased epithelial permeability, the mean (SE) total protein in the BAL fluid increasing from 0.39 (0.01) to 0.62 (0.01) mg/ml, and increased lactate dehydrogenase (LDH) concentrations in the BAL fluid. An even greater inflammatory response was seen following intratracheal instillation of ufCB but not following CB instillation. PM_{10} particles had free radical activity in vivo, as shown by a decrease in GSH levels in the BAL fluid from 0.36 (0.05) to 0.25 (0.01) nmol/ml following instillation. The free radical activity of PM_{10} was confirmed in vitro by its ability to deplete supercoiled plasmid DNA, an effect which could be reversed by mannitol, a specific hydroxyl radical scavenger. BAL fluid leucocytes from rats treated with PM_{10} produced greater amounts of nitric oxide (NO), measured as nitrite (control 3.07 (0.33), treated 4.45 (0.23) µM/l $\times 10^6$ cells), and tumour necrosis factor α (control 21.0 (3.1), treated 179.2 (29.4) units/l $\times 10^6$ cells) in culture than those obtained from control animals. Since the PM_{10} preparation was contaminated with small amounts of filter fibres due to the extraction process, the effects of instillation of filter fibres alone was assessed. These studies showed that filter fibres did not account for the pro-inflammatory and injurious effects of the PM_{10} suspension.**

Conclusions **– These findings provide evidence that PM_{10} has free radical activity and causes lung inflammation and epithelial injury. These data support the proposed hypothesis for the mechanism by which particulate air pollution causes adverse effects in patients with airways diseases.**

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Keywords: PM_{10}, epithelium, oxidants.

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Particulate matter with an aerodiameter of ≤ 10 µm (PM_{10}) is an ubiquitous pollutant of urban air.[1] Numerous epidemiological studies have shown that particulate air pollution is associated with increased morbidity and mortality.[2] There is good evidence that PM_{10} has adverse effects on airways disease as shown by the association of PM_{10} levels with reduced lung function[3] and hospital admissions for asthma[4] and chronic obstructive pulmonary disease.[5] The association between PM_{10} and adverse pulmonary effects in diverse geographical locations[1] suggests that the exact composition of the particulate air pollution is not critical.

Although epidemiological evidence strongly supports the association between PM_{10} particles and adverse health effects, the mechanism is not understood.[6] Inhaled particles cause a spectrum of pulmonary responses. However, the development of adverse effects with PM_{10} at such low airborne mass concentrations remains a puzzle. We hypothesised that PM_{10} particles have oxidant properties, related to their size, which produce airway inflammation and increase air space permeability.[7] It has been proposed that other inhaled particles cause similar effects as a result of their oxidant properties.[8] The purpose of this study was to begin to test this hypothesis by measuring pro-inflammatory potential and the oxidant activity of PM_{10}.

Methods

PARTICLE SUSPENSIONS

PM_{10} particles were collected on filters from the Edinburgh monitoring station of the enhanced urban network. The filters were removed from

the tapered element oscillating microbalance in the monitoring station and stored for up to four months until used as described below.

The PM_{10} filter was cut into small pieces and 0.8 ml phosphate buffered saline (PBS) was added and vortexed for 20 seconds. The filter was then removed to avoid further contamination with filter fibres and the suspension was sonicated for 30 seconds (Ultrasonic Cleaner BP-1, Burkard Scientific Sales Ltd, New Jersey, USA). Since the extraction procedure produced a suspension of PM_{10} particles contaminated with small numbers of filter fibres (0–10 per light microscopic field at $\times 80$ magnification), filter fibre suspension (FFS) was prepared by sonicating an unused filter in PBS for 30 seconds. This suspension contained >300 fibres per light microscopic field (at $\times 80$ magnification).

We calculated the mean (SE) weight of the particles on eight filters as 996 (182) μg. The method of preparation removed 20–50% by weight of the particles, so we estimated that 199–498 μg of particles were present in 0.8 ml PBS. Since 0.2 ml of solution was instilled, this volume contained 50–125 μg of particles. In the UK the upper level according to the air quality standards is 50 $\mu g/m^3$.

The effects of instillation of PM_{10} into rat lungs was compared in some experiments with intratracheal instillation of 125 μg of fine carbon black (CB, Degussa Huber N990, diameter 200–250 nm) and ultrafine carbon black (ufCB, Degussa Printex 90, diameter 20 nm) suspensions in 0.2 ml of PBS.

INTRATRACHEAL INSTILLATION OF PARTICLE SUSPENSIONS

Syngeneic Wistar-derived rats of the HAN strain were anaesthetised with pentobarbitone and a volume of 0.2 ml of PBS particle suspension was instilled intratracheally. The controls for these experiments were both animals which did not receive any instillation and animals instilled with 0.2 ml PBS alone. Experiments were also carried out following intratracheal instillation of 0.2 ml filter fibre suspension.

BRONCHOALVEOLAR LAVAGE (BAL)

Six hours after intratracheal instillation of the particle suspensions the rats were killed and 4 ml PBS at 37°C was instilled and withdrawn from the lungs. After centrifugation this solution was referred to as BAL fluid. To obtain leucocytes from the BAL fluid 4×8 ml PBS was used to wash the lungs and collected in a Universal tube The cell suspension was then spun and cell pellets were resuspended in DMEM medium (Gibco, Paisley, UK) with 0.2% low endotoxin-bovine serum albumin (BSA, Sigma, Poole, UK) in which the cells from the first lavage were combined. The total and differential numbers of leucocytes in the BAL fluid were thus obtained; BAL leucocytes from control animals consisted of >99% macrophages.

To prepare soluble lung homogenate for measurement of reduced (GSH) and oxidised (GSSG) glutathione the lungs were resected after lavage and blotted dry. Since instillation experiments produce an uneven distribution of particles, and hence an uneven inflammatory response in the air spaces, we randomly sampled 1 g of lung tissue from all lung lobes. The samples were homogenised in 5% sulphosalicylic acid and the supernatant was then diluted in 0.1 M potassium phosphate buffer.

COLLECTION OF CELL CULTURE SUPERNATANT

BAL leucocytes from control rats and rats after intratracheal instillation of PM_{10} particles were cultured in DMEM (Gibco) + 0.2% BSA (Sigma) at a concentration of 1×10^6 per ml for 24 hours. The supernatant was then collected for the measurement of nitrite and tumour necrosis factor (TNF) as described below.

The A549 human type II alveolar epithelial cell line was purchased from ECACC (Salisbury, UK) and maintained in MEM medium containing 10% fetal calf serum. To determine particle induced A549 epithelial cell permeability and changes in glutathione levels the cells were co-incubated with PM_{10} suspension in MEM + 2% BSA for six hours.

MEASUREMENT OF EPITHELIAL PERMEABILITY IN VIVO AND IN VITRO

Rat lung epithelial permeability was assessed as the total protein concentration in BAL fluid. In a previous study[9] we confirmed that this technique produced similar results to measurements of air space epithelial permeability assessed as the passage of iodine-125 labelled BSA from air space to blood.[10] Protein concentrations were determined by incubating BAL fluid with Biorad solution (Big-Rap, Munchen, Germany) for 10 minutes at room temperature. The absorbance was read at 595 nm on a spectrophotometer (Unicam 8700 series, Cambridge, UK) and the concentration was determined by comparison with a standard curve for BSA.

The permeability of A549 epithelial cell monolayers was determined using a modification of a technique which we developed previously.[10] However, instead of ^{125}I-BSA we used unlabelled BSA in the assay. In brief, A549 cells were cultured on Nunc tissue culture inserts (Gibco) in a 24-well plate to form cell monolayers which were incubated with particle suspensions for six hours. The medium in both inserts and wells was replaced with PBS followed by the addition of 1 mg of BSA into the insert. After 30 minutes the PBS in the wells was sampled and protein concentrations were determined.

TUMOUR NECROSIS FACTOR (TNF), LACTATE DEHYDROGENASE (LDH) AND NITRITE ASSAYS

TNF activity in BAL fluid and the supernatant from BAL leucocytes was measured using the L929 cell bioassay as described previously.[9]

LDH concentrations were assessed using the method of Bergmeyer *et al.*[11] Nitric oxide (NO) generation was determined as accumulated nitrite measured by a modified microplate assay using the Griess reagent.[12] In brief, samples were incubated with an equal volume of the Griess reagent at room temperature for 10 minutes. The absorbance at 540 nm was determined with an MR650 plate reader (Dynatech Laboratories Inc, USA) using sodium nitrite (Sigma) as the standard.

MEASUREMENT OF PARTICLE FREE RADICAL ACTIVITY

The free radical activity of the PM_{10} suspension was assessed by its ability to damage plasmid DNA as previously described.[13] In brief, φX174 RF plasmid DNA was incubated with 5 μl of particle suspensions at 37°C in a water bath for eight hours. Tracking dye was added to the DNA samples and the three plasmid forms (supercoiled, relaxed coil, and linearised plasmid) were separated by electrophoresis for 16 hours at 30 volts on a 0.8% agarose gel (Sigma). Photography of ethidium bromide-stained agarose gels under ultraviolet light showed the proportion of relaxed coil DNA, providing an indication of the free radical damage to the plasmid. This was then quantified by scanning laser densitometry (LKB Bromma, Uppsala, Sweden). To determine the nature of the free radical activity, mannitol (BDH Chemicals, Poole, UK) was added to the DNA-PM_{10} mixture in a final concentration of 4 mM before the assay.

MEASUREMENT OF GSH AND GSSG

The total cellular GSH concentration was assayed by the GSSG-reductase-DTNB recycling procedure as we have described previously.[10] To measure GSSG, the samples were first depleted of GSH by incubation with 2-vinylpyridine followed by the GSSG-reductase-DTNB recycling procedure. GSH concentrations were then calculated by subtracting the GSSG values from the total GSH values. The GSH and GSSG values were determined by comparison with GSH and GSSG (Sigma) standard curves.

STATISTICAL ANALYSIS

The results were expressed as mean (SE). Three rats per treatment group were used. Differences between mean values were assessed by analysis of variance.

Results

Intratracheal instillation of PM_{10} caused influx of neutrophils into rat lungs six hours after instillation, which accounted for 10–15% of the total numbers of BAL leucocytes (fig 1). Compared with control animals instilled with PBS, instillations of carbon black (CB) produced a small but significant neutrophil influx. However, the greatest inflammatory cell influx occurred following instillation of ultrafine carbon black (ufCB; fig 1) when neutrophils accounted for 40% of the total BAL leucocyte count. Leucocytes obtained from the BAL fluid six hours after PM_{10} instillation produced greater amounts of TNF and NO in culture than those from control animals (fig 2). Although inflammatory BAL leucocytes showed a greater potential to produce TNF and NO in culture, the levels were not significantly different six hours after PM_{10} instillation from those in PBS instilled animals (table 1).

PM_{10} particles increased air space epithelial permeability six hours after instillation as

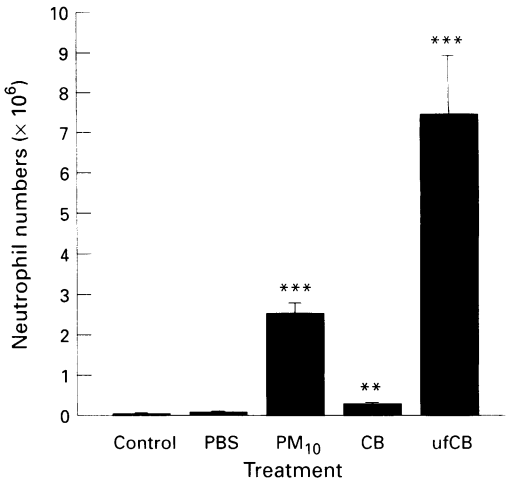

*Figure 1 Number of neutrophils in bronchoalveolar lavage (BAL) fluid from rats six hours after intratracheal instillation of PM_{10}, fine (CB) and ultrafine (ufCB) carbon black. The results in rats which had no instillation (control) or instillation with PBS are shown for comparison. Histograms and bars represent mean (SE) of 3–6 animals. ** p<0.01, *** p<0.001 compared with PBS.*

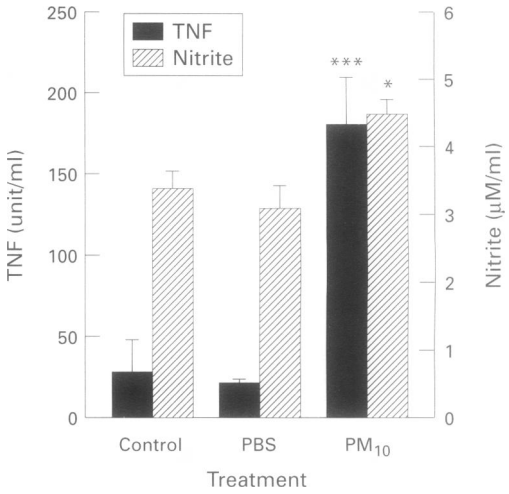

*Figure 2 Effect of intratracheal instillation of PM_{10} on tumour necrosis factor (TNF) and nitric oxide (NO) production by BAL leucocytes in culture. The results in rats which had no instillation (control) or instillation with PBS are shown for comparison. Histograms and bars represent mean (SE) of three experiments. * p<0.05, *** p<0.001 compared with PBS.*

Table 1 Levels of tumour necrosis factor (TNF), nitrite and lactate dehydrogenase (LDH) in bronchoalveolar lavage fluid from rat lung six hours after intratracheal instillation of PM_{10}

	TNF (unit/ml)	Nitrite (μM/ml)	LDH (unit/ml)
PBS (control)	0	12.9(3.9)	13.0(2.0)
PM_{10}	8.0(5.8)	10.5(1.0)	453.0(52.3)***

Values are mean (SE) of three rats.
*** $p<0.001$ compared with PBS control values.

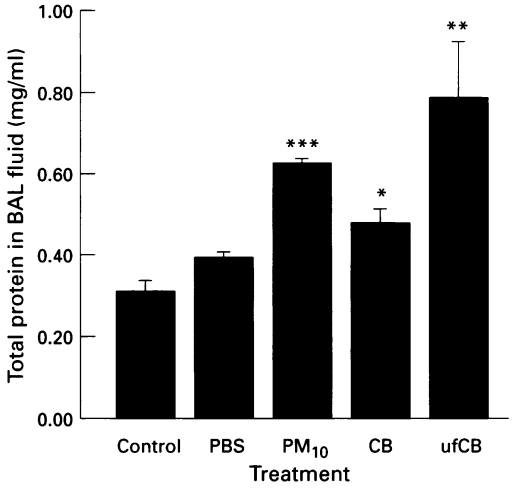

*Figure 3 Effect of intratracheal instillation of PM_{10}, fine (CB) and ultrafine (ufCB) carbon black on epithelial permeability of rat lung in vivo measured as total protein values in BAL fluid six hours after instillation. The results in rats which had no instillation (control) or instillation with PBS are shown for comparison. Histograms and bars represent mean (SE) of 3–6 animals. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with PBS.*

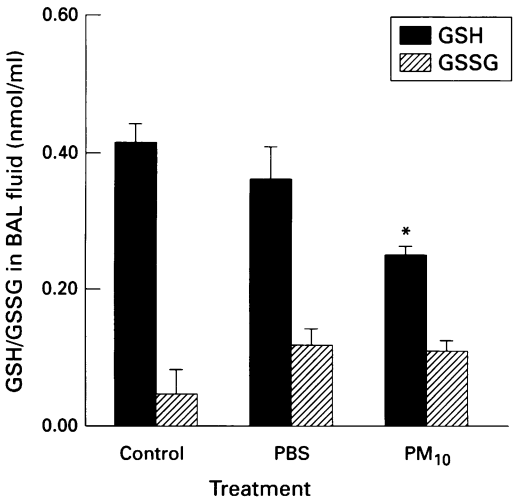

*Figure 4 Effect of intratracheal instillation of PM_{10} on oxidised (GSH) and reduced (GSSG) glutathione concentrations in BAL fluid six hours after instillation in rat lungs. Histograms and bars represent mean (SE) of three animals. * $p<0.05$ compared with PBS.*

shown by increased levels of total protein in the BAL fluid compared with PBS instilled control animals (fig 3). At this time point LDH levels were higher than those in BAL fluid from control animals (table 1). As with the influx of inflammatory leucocytes, the greatest increase in air space epithelial permeability occurred following instillation of ufCB, CB producing a lesser increase in epithelial permeability than PM_{10} or ufCB (fig 3).

The addition of PM_{10} particles to A549 type II alveolar epithelial monolayers in vitro increased their permeability to BSA (table 2). This increased epithelial permeability was not due to cell death since monolayers of A549 cells incubated with PM_{10} for six hours did not release increased amounts of LDH (table 2).

Intratracheal instillation of PM_{10} decreased GSH levels without any significant change in GSSG levels in BAL fluid six hours after instillation compared with PBS instilled animals (fig 4). However, GSH and GSSG levels in lung tissue were the same in PBS and PM_{10} treated rats (table 3). Incubation of PM_{10} particles with A549 monolayers did not change intracellular GSH levels (table 2).

PM_{10} induced free radical mediated damage to plasmid DNA detected as depletion of supercoiled plasmid DNA bands. This effect could not be reproduced by using filter fibre suspension alone (data not shown). Mannitol, a specific hydroxyl radical scavenger, ameliorated the effect of PM_{10} (fig 5).

In order to clarify the role of fibre contamination in the activity of the PM_{10} suspension we compared the effects of PM_{10} particles, filter fibre suspension (FFS), and PBS instillations in the rat lung. Table 4 shows that FFS did not significantly alter BAL leucocyte components compared with PBS control six hours after intratracheal instillation, nor did it increase epithelial permeability measured as total protein concentration in the BAL fluid. Instillation of FFS did not decrease GSH levels in BAL fluid but, rather, increased GSH levels and did not increase the permeability of A549 cell monolayers to BSA in vitro (penetrated BSA: control, 0.06 (0.01) mg/ml versus FFS, 0.05 (0.01) mg/ml, $p>0.05$). One exception was that intratracheal instillation of FFS caused BAL leucocytes to produce increased NO in culture compared with PBS treated controls (table 4).

Table 2 Penetration of BSA through an epithelial cell monolayer, LDH in supernatant and intracellular GSH in A549 epithelial cells incubated with PM_{10} in vitro for six hours

Dose of PM_{10} (% dilution)	Penetrated BSA (mg/ml)	LDH (unit/2 million cells)	GSH (nmol/2 million cells)
0	0.10(0.02)	23.5(2.5)	2.70(0.13)
10	0.17(0.01)*	27.0(4.0)	2.76(0.05)
20	0.20(0.02)**	21.0(7.1)	2.68(0.03)

Values are mean (SE) of three experiments. The PM_{10} suspension used in the in vivo experiments was diluted to 10% and 20% of the original dose. There were no detectable levels of GSSG in A549 cells.
BSA = bovine serum albumin; LDH = lactate dehydrogenase; GSH = oxidised glutathione; GSSG = reduced glutathione.
* $p<0.05$, ** $p<0.01$ compared with control values.

Table 3 Concentrations of oxidised (GSH) and reduced (GSSG) glutathione in rat lung homogenates six hours after intratracheal instillation of PM_{10}

	GSH (nmol/g lung)	*GSSG (nmol/g lung)*
PBS (control)	234.9(29.2)	21.5(0.6)
PM_{10}	214.1(31.5)	19.8(5.1)

Values are mean (SE) of three rats.

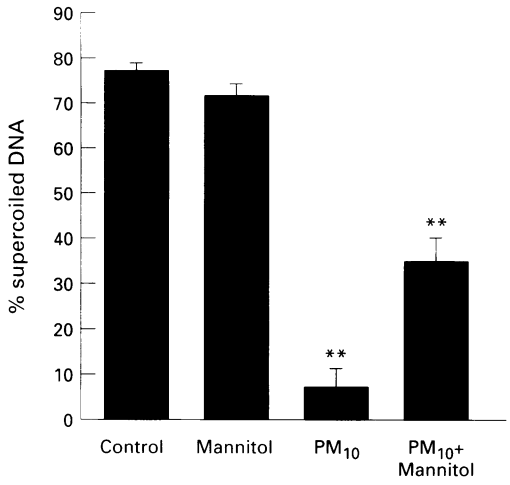

*Figure 5 Free radical injury to plasmid DNA by PM_{10} and protection by mannitol. Histograms and bars represent mean (SE) of 3–4 experiments. ** $p<0.01$ compared with control values. A significant difference was seen between PM_{10} alone and PM_{10}+mannitol ($p<0.001$).*

Discussion

This study examined our hypothesis[7] that PM_{10} causes airway inflammation and that this occurs partly because of the oxidant properties of the numerically predominant ultrafine particle component.[14]

Our observations show that six hours after intratracheal instillation PM_{10} produced an acute neutrophil influx into the air spaces with accompanying increased epithelial permeability. This inflammation and increase in epithelial permeability could have been partly due to a direct PM_{10} induced epithelial injury reflected in the raised LDH concentrations in the BAL fluid. The ability of PM_{10} to cause epithelial permeability was confirmed in vitro by the increased transfer of albumin across epithelial monolayers in the presence of PM_{10}. However, this was not associated with cell injury as measured by increased LDH release.

In preparing PM_{10} suspensions we found that there was contamination with a small number of fibres derived from the filter during the preparation procedure. The potential for these fibres to contribute to the biological activities of the PM_{10} preparation was therefore addressed. We assessed the ability of filter fibre suspension (FFS) alone, which contained at least 30 times as many fibres as the PM_{10} suspension, to cause pro-inflammatory effects when instilled into rat lungs. These studies showed that the fibres alone do not cause a neutrophil influx into the air spaces, nor do they increase epithelial permeability or decrease GSH levels in BAL fluid. We therefore conclude that PM_{10} is responsible for the observed inflammatory effects in the lungs and not the presence of a small amount of contamination by filter fibres. Further studies are planned when larger quantities of PM_{10} particles collected by high volume sampler without the use of filters become available.

The hypothesis that the epithelial injury was due to the oxidative properties of PM_{10} is supported by our observations that the PM_{10} particles produce plasmid DNA scission, a sensitive assay for detecting the ability of particles to cause free radical injury.[15] The free radical injury was due in part to hydroxyl radicals since it was substantially inhibited by mannitol. It is not surprising that only some of the free radical damage was caused by hydroxyl radical since PM_{10} is a complex mixture of particles from many sources. These include polyaromatic hydrocarbon coated diesel particles and organically derived particles such as fungal spores which contain diverse electrophilic or redox active species which could damage DNA.

Many kinds of particles which cause pathological effects in the lungs have detectable but variable amounts of free radical activity at their surfaces – for example, quartz,[16] coalmine dust,[17] asbestos,[18] glass/refectory fibres,[13] and ultrafine titanium dioxide (TiO_2).[8] Furthermore, the role of iron in producing the highly injurious hydroxyl radical has been suggested to be a unifying theme in particle toxicity.[19] Support for a role for iron in the oxidant effects of PM_{10} comes from studies from our laboratory which indicate that the free radical activity of PM_{10} is abolished in the presence of the iron chelator desferrioxamine.[20]

This is the first study to demonstrate the oxidant potential of PM_{10} which may reflect the surface chemistry of the ultrafine component.[14] To this end, in preliminary studies we noted that the supernatant from high speed centrifugation of PM_{10} suspension to clarity, which presumably contained only the ultrafine components, still caused the same degree of plasmid DNA scission.[20] This strongly suggests that ultrafine particles provided the bulk of the free radical activity of PM_{10}.

Support for the hypothesis that ultrafine particles have free radical activity is derived from our previous studies showing the oxidant po-

Table 4 Effect of intratracheal instillation of phosphate buffered saline (PBS) or filter fibre suspension (FFS) on leucocyte components, epithelial permeability, and GSH/GSSG in bronchoalveolar lavage (BAL) fluid six hours after intratracheal instillation in rat lungs

Treatment	*Neutrophil (%)*	*Neutrophil number ($\times 10^6$)*	*Protein in BAL fluid (mg/ml)*	*GSH in BAL fluid (nmol/ml)*	*GSSG in BAL fluid (nmol/ml)*	*NO produced by BAL leucocytes ($\mu M/10^6$ cells)*
PBS	2.67(0.88)	0.68(0.28)	0.57(0.03)	0.44(0.08)	0.06(0.02)	1.37(0.23)
FFS	4.00(0.58)	0.58(0.15)	0.55(0.11)	0.96(0.08)**	0.09(0.05)	2.19(0.37)*

Values are mean (SE) of three rats for each group.
GSH = oxidised glutathione; GSSG = reduced glutathione.
* $p<0.05$, ** $p<0.01$ compared with PBS control.

tential of another ultrafine particle, TiO_2. This material has free radical activity in the ultrafine form (20 nm in diameter), but is inert as larger sized particles (250 nm).[8]

Further evidence in support of the contention that it is the free radical activity of PM_{10} that is responsible for its biological activity in vivo is shown by changes in the important lung antioxidant glutathione. PM_{10} decreased reduced glutathione (GSH) but had no effect on the levels of oxidised glutathione (GSSG) in the BAL fluid. Furthermore, intracellular GSSG/GSH ratios were not affected by PM_{10} either in lung tissue in vivo or in epithelial cells in vitro, at least at the single time point when measurements were made. It is possible that such changes may have occurred at earlier time points resulting in compensatory mechanisms such as upregulation of the genes involved in GSH synthesis as we have reported following exposure to cigarette smoke.[21] Future studies which examine the time course of GSH homeostasis in PM_{10} treated cells and lung tissue should elucidate this hypothesis.

The exact composition of the PM_{10} sample which we used is not yet available. However, analysis of the composition of PM_{10} particles obtained from other sources indicates that carbonaceous material makes up 50% of their mass.[22] We therefore compared the effects of instillation of both fine and ultrafine carbon black particles with those of PM_{10} particles in similar doses in the rat lung. These studies showed that ultrafine carbon black, rather than fine carbon black particles, produce similar qualitative although greater quantitative pro-inflammatory effects to those of PM_{10}. The greater inflammatory effect of ultrafine carbon black could have been anticipated from our hypothesis, since ultrafine carbon black is composed entirely of ultrafine particles, which is not the case for PM_{10} which, by definition, has only 50% of its particles $<10\,\mu m$ in aerodiameter. The fact that the greater quantity of ultrafine particles in the carbon black preparation produced more lung inflammation supports our contention that the ultrafine component of PM_{10} has the greatest inflammatory potential.

We have previously shown that the increased epithelial permeability caused by cigarette smoke, which has an enormous oxidant potential, may be due to a fall in intracellular GSH.[10] Although a similar mechanism has not been shown with PM_{10} in the present study, the effect of PM_{10} on GSH homeostasis will be the target of future studies. Other candidate inflammatory mediators may be involved in increasing epithelial permeability, such as TNF[9] and nitric oxide.[23] Data from the present study do not show increased NO or TNF levels in BAL fluid in association with a neutrophil influx into the air spaces following instillation of PM_{10}. However, in vitro BAL leucocytes from PM_{10} treated rats produced significantly more NO and TNF in culture than control BAL cells. We believe that the release of these mediators results from the effect of loading the cells with particles,[24] but also from PM_{10} induced oxidant stress.[25] In addition, there is an interaction between these two inflammatory mediators such that TNF can stimulate NO production.[26,27] The absence of detectable TNF and NO levels in BAL fluid from PM_{10} treated rats compared with control animals is likely to result from the presence of inhibitors in the BAL fluid.

The results of this study are preliminary and are limited due to the lack of the availability of large quantities of PM_{10}. We have therefore only been able to study an animal model of instillation rather than the preferred inhalational model which would be more relevant to environmental exposures. As a result, comparative calculation of the doses relative to environmental exposures are difficult. Furthermore, it is possible that the characteristics of the PM_{10} may be altered by storage and the process of removing it from the filters. In this preliminary study, where only one dose and the effects at one time point could be achieved due to the availability of PM_{10}, we therefore opted to study a dose which was higher than environmentally plausible.

However, with all of these constraints, this study provides evidence that PM_{10} has free radical activity and causes an inflammatory response and epithelial injury in the lungs. These data provide support for our hypothesis[7] of the mechanism of the harmful effects of PM_{10} in exacerbating airways diseases.

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