Adverse health effects of PM_{10} particles: involvement of iron in generation of hydroxyl radical

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Abstract

***Objectives*—Environmental particles < 10 μm average aerodynamic diameter (PM_{10}) are associated with mortality, exacerbation of airways diseases, and decrement in lung function. It is hypothesised that PM_{10} particles, along with other pathogenic particles, generate free radicals at their surface in reactions involving iron, and that this is a factor in the pathogenicity of PM_{10} particles. Identification of free radical activity in PM_{10} and examination of the content and role of iron in this process was undertaken.**

***Methods*—Free radical activity was detected with a supercoiled plasmid, φX174 RF1 DNA, and measured as scission of the supercoiled DNA (mediated by free radicals) by scanning laser densitometry. The role of the hydroxyl radical was confirmed by the use of the specific scavenger mannitol, and the role of iron investigated with the iron chelator desferrioxamine-B (DSF-B). Iron released from PM_{10} particles at pH 7·2 and pH 4·6 (to mimic conditions on the lung surface and in macrophage phagolysosomes, respectively) was assessed spectrophotometrically with the Fe^{++} chelator ferrozine and the Fe^{+++} chelator DSF-B.**

***Results*—PM_{10} particles showed significant free radical activity by their ability to degrade supercoiled DNA. A substantial part of this activity was due to the generation of hydroxyl radicals, as shown by partial protection with mannitol. Similarly, DSF-B also conferred protection against the damage caused to plasmid DNA indicating the role of iron in generation of hydroxyl radicals. Negligible Fe^{++} was released at either pH 7·2 or pH 4·6 by contrast with Fe^{+++}, which was released in substantial quantities at both pHs, although twice as much was released at pH 4·6.**

***Conclusions*—PM_{10} particles generate the hydroxyl radical, a highly deleterious free radical, in aqueous solution. This occurs by an iron dependent process and hydroxyl radicals could play a part in the pathogenicity of PM_{10} particles. Iron release was greatest at the pH of the lysosome (pH 4·6) indicating that iron may be mobilised inside macrophages after phagocytosis, leading to oxidative stress in the macrophages.**

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Considerable research attention has been devoted to the mechanism of lung injury caused by gaseous components of air pollution, but much less attention has been paid to the particulate matter. The adverse health impacts of particulate air pollution have been recently reviewed,[1 2] confirming the role of suspended particulate matter in acute mortality,[3 4] impairment of lung function,[5 7] and in exacerbating airways diseases such as chronic obstructive pulmonary disease and asthma.[8 9] PM_{10} particles, the commonly used indicator of respirable environmental suspended particulate matter, are measured with an automated particle sampling system that collects airborne PM_{10} particles with 50% efficiency and smaller particles with increasing efficiency.[10] A United Kingdom standard of 50 $\mu g/m^3$ was recently recommended by a Department of the Environment Committee.[10] The average concentration of PM_{10} particles in British cities ranges between 10 and 45 $\mu g/m^3$ with short term peaks extending to 60 $\mu g/m^3$; however, it is clear that concentrations of > 100 g/m^3 can be experienced.[10 11]

Few data are available on the detailed composition of PM_{10} particles and the composition is highly variable between locations.[10 11] The association between mortality and pulmonary symptoms with suspended particulate matter in such diverse geographic locations as New York, London, Philadelphia, and Utah, where the sources ranged from predominantly coal combustion to automobile emissions and to a steel mill,[12] suggests that the detailed composition of the suspended particulate matter is not critical but that the total mass of fine particles is important.

PM_{10} particles can be very small,[10] and particles in the 20 nm size range are common. We have hypothesised that direct interactions between the fine particles deposited and the pulmonary epithelium of the terminal bronchiolar or proximal alveolar regions is critical to the harmful effects of PM_{10} particles.[13] In an extension to the hypothesis we suggested that PM_{10} particles and other ultrafine material may have free radical activity that contributes to their toxicity and we provided provisional confirmatory evidence of this.[14]

In this paper we show that, similarly to findings from other particles,[15 16] free radicals may arise from PM_{10} by Fenton chemistry involving iron present on the particles.[17] Additionally, we investigated whether iron could be

mobilised from the PM_{10} particles to a greater degree at the acid pH found in phagolysosomes.[18]

Materials and methods

REAGENTS

The ϕX174 RF1 closed circular supercoiled DNA, and Pst1 were obtained from Gibco Europe (Paisley, UK). Mannitol and $NaHPO_2$ were purchased from BDH Chemicals (Poole, UK). Citric acid and iron chelators desferrioxamine (DSF-B) and 3-[2-pyridyl]-5,6-diphenyl-1,2,4-triazine-p,p-disulphonic acid (ferrozine) were purchased from Sigma Chemicals (Poole, UK).

PREPARATION OF PM10 PARTICLES AND OTHER SAMPLES

The PM_{10} particles were taken directly from filters from the tapered element oscillating microbalance in the Edinburgh monitoring site of the United Kingdom enhanced urban network stationed in Prince's Street Gardens in Edinburgh, and kindly supplied by Mr James Hunter, Edinburgh District Council Department of Environmental Services.

Filters were quartered, and each quarter placed in 100 μl sterile H_2O, 4 mM mannitol, or 4 mM DSF-B solution as required. Each sample was sonicated in a sonicating water bath for 30 seconds and vortexed vigorously to remove PM_{10} particles from the filter into solution. Control filters containing no PM_{10} particles were similarly treated to control for filter particles which may effect the plasmid DNA.[19] The mean (SEM) weight of particulate matter on the filters was 996·2 (181·8) μg/filter and although it is impossible to measure the weight of particulates removed by sonication, we estimate that in our standard procedures 10%–30% of this is removed from the filters into solution. In some experiments, samples of PM_{10} particles prepared in either H_2O or DSF-B were centrifuged for 10 minutes at 13 000 rpm to remove large particles from solution, and the resulting clear supernatant was added to the DNA assay at the same concentrations as the uncentrifuged solutions of PM_{10} particles.

To measure iron from PM_{10} particles, each quarter filter was sonicated as before in 500 μl of buffer at either pH 4·6 or pH 7·2.

MEASUREMENT OF IRON RELEASED FROM PARTICLES

Iron released was measured under the two conditions pH 4·6 and pH 7·2. Samples of PM_{10} particles were suspended in either pH 4·6 or pH 7·2 citric acid/NaHPO2 buffer as already described, and after eight hours incubation at 37°C in a rotating incubator, larger visible particles were removed by centrifugation at 2500 rpm for 10 minutes. By our estimation, each quarter filter should provide a maximum of 74·7 mg. The iron concentration released after eight hours was measured by the addition of either 4 mM DSF-B for Fe^{++}, or 4 mM ferrozine for measurement of Fe^{+++}. Measurement of the iron Fe^{++} or Fe^{+++} leached from particles was by spectrophotometry at 562 nm and 430 nm respectively, and comparison with Fe^{++} or Fe^{+++} standards of $FeSO_4$ and $FeCl_3$. The amount of iron leached from 74·7 μg of particles after eight hours of incubation was expressed as nmol Fe^{++} or Fe^{+++}.

FREE RADICAL DAMAGE TO PLASMID DNA

ϕX174 RF1 DNA (290 ng) was incubated with PM_{10} particles and treated particles as previously described.[15] Briefly, all DNA control and treatment samples were incubated for eight hours at 37°C before electrophoretic separation on agarose gel of the supercoiled, relaxed coil, and linear fragments of the DNA. A linearised control sample was prepared with the use of the restriction endonuclease PstI. The amount of supercoiled DNA present after treatment, compared with the control, indicated the level of free radical damage to the plasmid. Five or 10 μl of solutions of PM_{10} particles, in either water, mannitol, or DSF-B solution, or control filter solution were added to the DNA for assay of free radicals. From our estimation of particles removed from each filter, the weight of particles in 5 μl should be 3·7 μg/assay. Acidified water buffered to pHs ranging from 7·0 to 5·0 was also added to the DNA assay in 10 μl aliquots, and was similarly incubated for eight hours at 37°C. Figure 1 is a typical agarose gel photograph of these treatments.

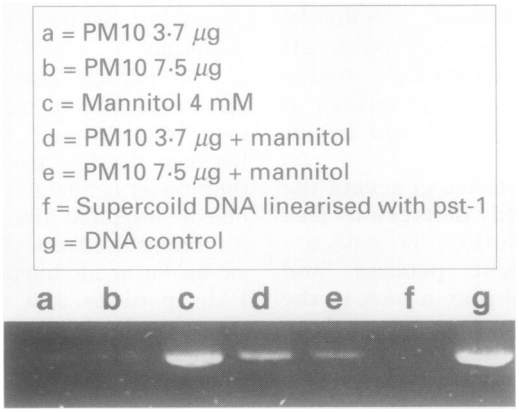

Figure 1 Photograph of the supercoiled DNA band of control plasmid DNA and after incubation with variously treated suspensions of PM_{10} particles. Note the depletion of supercoiled DNA with PM_{10} particles and protection of the depletion with mannitol.

x RAY FLUORESCENCE AND SCANNING ELECTRON MICROSCOPIC ENERGY DISPERSIVE *x* RAY ANALYSES

x Ray fluorescence analysis was carried out on an untreated PM_{10} filter with the Rigaku Model 2000 analyser of the Advanced Materials Centre, Napier University, exposing the entire central 10 mm of the filter to the incident *x* ray beam (generated on a rhodium target). The resulting element fluorescence emission from the sample was passed through collimators, diffracted by a lithium fluoride crystal to fall on a scintillation counter, which scanned through the 2 theta range 0 to 99 degrees. Only elements with an atomic num-

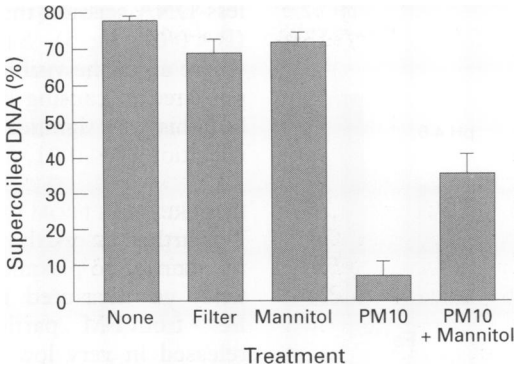

Figure 2 Percentage of supercoiled DNA after treatment with 3·7 μg PM_{10} particles with or without mannitol compared with untreated DNA control and unloaded filter control. Each bar represents the mean (SEM) values from four experiments.

ber ⩾ 9 (fluorine) were recorded.

After this, the same sample was examined in a Cambridge Stereoscan scanning electron microscope, and qualitative analysis was carried out through the attached link energy dispersive *x* ray analyser. By focusing the electron beam on the fibres of the filter it was possible to obtain an elemental analysis on the filter matrix material. Focusing on the clumps of particle visible between the filter fibres allowed analysis of the elements in the PM_{10} particles themselves. The spectra of the energy dispersive *x* ray analyser showed all elements from atomic number 11 (sodium) upwards.

STATISTICAL ANALYSIS

With the Minitab statistical analysis programme, one or two way analysis of variance was used to find the significance of treatment effects; the Tukey multiple comparison function was used when required.

Results

PARTICLE MEDIATED FREE RADICAL DAMAGE TO PLASMID DNA

The suspension of PM_{10} particles at an estimated 3·7 μg/assay caused considerable damage to the plasmid DNA as shown by the depletion of supercoiled plasmid DNA to relaxed coil or linearised DNA plasmid (fig 2). The PM_{10} particles depleted supercoiled DNA by an average of 70·6% (significant difference from untreated and control filter DNA samples, $P < 0·01$). The addition of mannitol, a specific hydroxyl radical scavenger, was used to measure the role of the hydroxyl radical in the degradation of the supercoiled DNA. The depletion of supercoiled DNA by PM_{10} particles was significantly ameliorated by the presence of mannitol (increase of 28·2% in supercoiled DNA compared with treatments without mannitol, $P < 0·01$). This clearly suggests the involvement of the hydroxyl radical in mediating a proportion of the DNA damage.

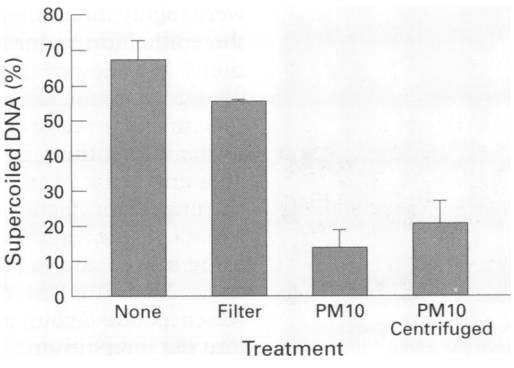

Figure 3 Percentage of supercoiled DNA after treatment with 3·7 μg PM_{10} particles of either normally prepared or centrifuged samples compared with untreated DNA control and unloaded filter control. Each bar represents the mean (SEM) values from five experiments.

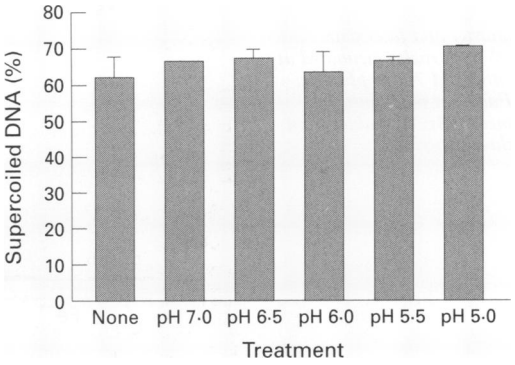

Figure 4 Percentage of supercoiled DNA after incubation at pH ranging from pH 7·0 to pH 5·0 compared with untreated DNA control. Each bar represents the mean (SEM) values from two experiments.

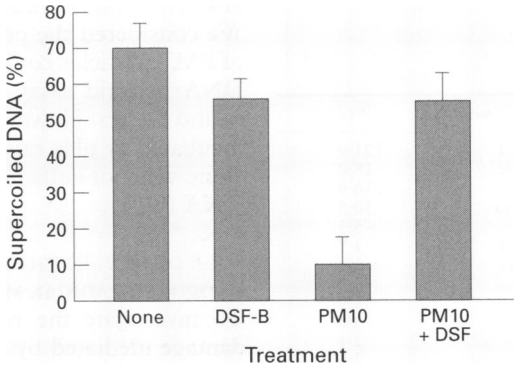

Figure 5 Percentage of supercoiled DNA after treatment with 3·7 μg PM_{10} particles with or without desferrioxamine (DSF-B) compared with untreated DNA. Each bar represents the mean (SEM) values from five experiments.

FREE RADICAL ACTIVITY OF SUPERNATANT OF CENTRIFUGED PM10 PARTICLES

We centrifuged the suspension of PM_{10} particles to remove the visible particles. Uncentrifuged samples of PM_{10} particles significantly ($P < 0·01$) degraded the supercoiled

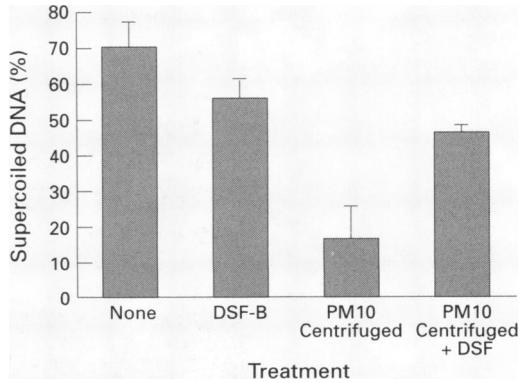

Figure 6 Percentage of supercoiled DNA after treatment with 3·7 μg PM_{10} particles and 3·7μg supernatant solution from centrifuged PM_{10} particles with or without desferrioxamine compared with untreated DNA. Each bar represents the mean (SEM) values from five experiments.

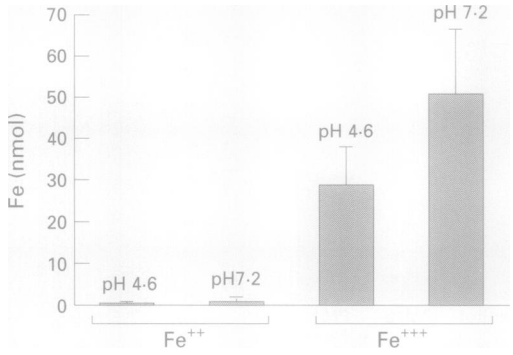

Figure 7 nmol Fe^{++} and Fe^{+++} released from one quarter of a filter with PM_{10} particles incubated at either pH 7·2 or pH 4·6. Each bar represents the mean (SEM) values from three experiments.

DNA (fig 3) and centrifugation to remove the largest PM_{10} particles did not significantly alter this activity (6·99% increase in supercoiled plasmid DNA compared with uncentrifuged samples ($P < 0·01$)).

EFFECT OF pH ON DNA PLASMID

We considered the possibility that suspensions of PM_{10} particles could acidify the buffer in the DNA plasmid assay which could itself cause strand breaks. However, when the plasmid was incubated at pHs ranging from 7·0 to 5·0, pH alone was not sufficient to deplete supercoiled DNA (fig 4).

ROLE OF IRON IN THE PRODUCTION OF HYDROXYL RADICAL MEDIATED BY PM10

To investigate the role of iron in the DNA damage mediated by PM_{10} particles, the filters were prepared and sonicated in the Fe^{+++} iron chelator desferrioxamine. Our previous work on asbestos fibres has shown that DSF-B is an effective chelator of iron associated with the surface of particles, and is effective in protecting against plasmid damage mediated by iron on the surface of particles.[15] Samples of iron chelated PM_{10} particles caused significantly less DNA scission than control PM_{10} particles ($P < 0·01$, fig 5). Samples of PM_{10} particles where all of the visible particles were spun out persisted in causing damage to plasmid DNA but this was significantly ameliorated by iron chelation ($P < 0·01$, fig 6).

IRON RELEASE FROM PM10 PARTICLES

To further assess the potential role of iron in the damage to plasmid DNA mediated by particles we measured the release of Fe^{++} and Fe^{+++} from PM_{10} particles in solution. Fe^{++} was released in very low or negligible concentrations at both pHs (fig 7). In comparison, Fe^{+++} was released in comparatively high concentrations at both pHs (fig 7), with almost twice the release at pH 4·6.

Table 1 Semi-quantitative results from x ray fluorescence of a filter of PM_{10} particles

Element	*Wt %*
Cl	21·9
Fe	18·0
Si	14·9
Na	10·5
F	5·3
Cr	4·7
Ti	4·3
Ca	3·9
Ba	3·9
S	3·0
K	2·4
Al	2·4
Zn	1·9
Mg	1·4
Ni	1·3
Mn	0·3
Mo	0·03

x RAY FLUORESCENCE AND ENERGY DISPERSIVE *x* RAY ANALYSIS

Table 1 shows the results from the *x* ray fluorescence analysis. These results are only semi-quantitative and are likely to be accurate to only ± 10% of the true value. In the scanning electron microscope the surface of the filter is seen to comprise the fibrous membrane of the filter with embedded fine particles and clumps of particles (fig 8). The energy dispersive *x* ray analyser results were obtained firstly from the glass fibres of the filter itself, and showed peaks principally for silicon, zinc, sodium, potassium, and barium (fig 9A). Examination of the PM_{10} particles, away from the fibres, showed strong peaks for iron, silicon, sulphur, chlorine, sodium, potassium, and calcium (fig 9B). Because of the nature of the analysis, it is impossible to be sure that the spectral peaks found arise solely from the area being viewed, as the material below and to the side will "colour" the results. However, the striking difference in elemental analysis between the filter fibres and particulate peaks confirm that the two distinct entities were being separately resolved.

Figure 8 Scanning electron micrograph of a collection filter loaded with PM_{10} particles. Note the fibres of the filter and the presence of PM_{10} particles between the fibres.

Discussion

The adverse health effects of PM_{10} particles are exacerbations of airways disease, diminished lung function, and increased cardiovascular mortality.[1,2] We recently advanced a unifying hypothesis to explain these responses[13] centred around the ability of PM_{10} particles to penetrate the pulmonary interstitium and cause interstitial inflammation. This hypothesis evolved from studies with ultrafine titanium dioxide particles and the finding that these were highly inflammogenic and rapidly crossed the epithelium to initiate interstitial inflammation.[20] We showed that ultrafine titanium dioxide has free radical activity,[14] and hypothesised that this is a factor that enhances penetration to the interstitium. The relatively huge surface area/unit mass of ultrafine material[20] could be an important factor in leading to oxidative stress of the epithelium.[17] We have shown using in vivo and in vitro models that oxidative stress leads to loss of epithelial integrity,[21,22] which could favour incorporation of particles into the interstitium.

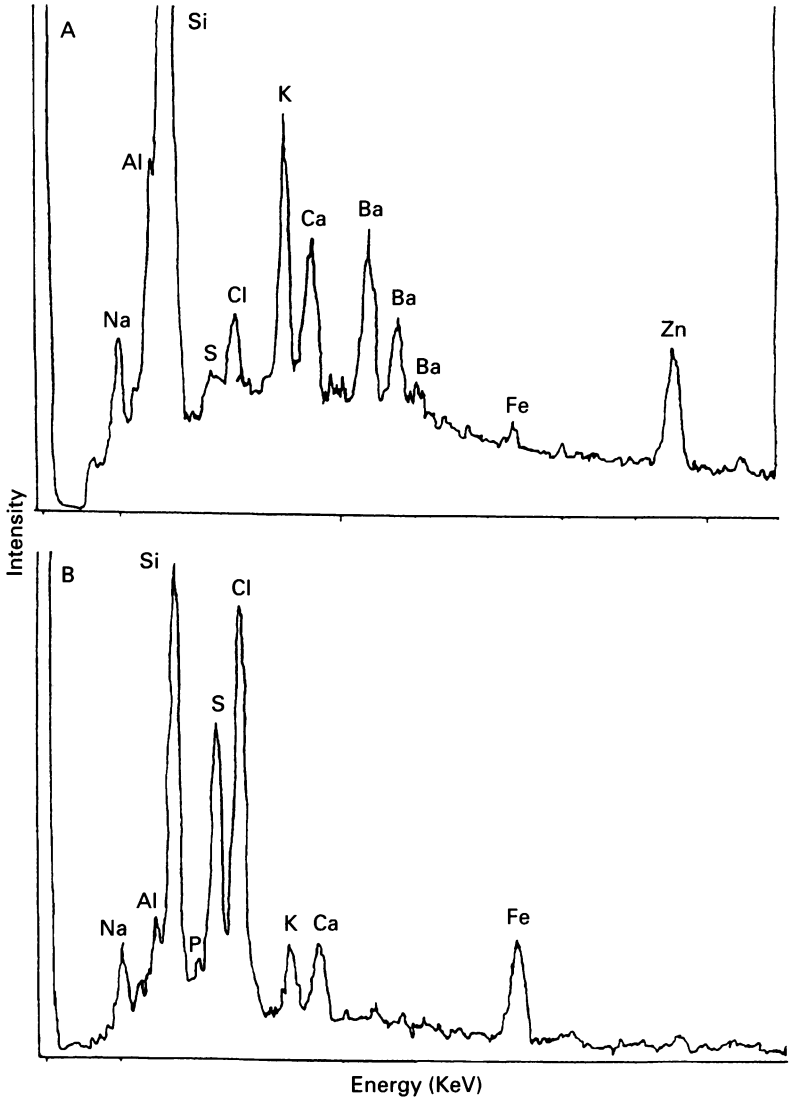

Figure 9 Spectra from energy dispersive x ray analyser analysis of (A) filter fibres and (B) particulate material.

For the first time we show here that PM_{10} particles have free radical activity, as they are able to deplete supercoiled plasmid DNA. Furthermore, the main mediator of the injury was the hydroxyl free radical, as shown by the protection afforded by mannitol. Although this is the first time this phenomenon has been found with PM_{10} particles, a range of other pathogenic particles has been shown to have the capacity to generate hydroxyl radicles at their surface and this has been implicated in their pathogenicity.[15 16] However, the potentially huge surface area presented by ultrafine PM_{10} particles may cause them to have enhanced potential for delivering an oxidative stress to cells or fluid of the lung lining that they encounter. Not all of the free radical DNA scission could be inhibited by mannitol, showing that other mechanisms for generating free radicals are present. This is not surprising, given the heterogeneous nature of PM_{10} particles and the likely presence of numerous other redox cycling molecules. The use of desferrioxamine to chelate iron in the PM_{10} particles resulted in the abolition of the DNA damage showing that iron was responsible for hydroxyl radical activity. Desferrioxamine was not likely to have reduced free radicals by simply coating the particles, as with ultrafine titanium dioxide, free radical injury continued to be expressed in the presence of desferrioxamine-B (unpublished data).

Centrifugation of the suspension of PM_{10} particles removed all visible particles but did not affect the free radical activity. This suggests that the free radical activity resides in ultrafine particles that cannot be centrifuged or that the active components that generate the hydroxyl radical diffuse off the particles into solution. The fact that subsequent chelator treatment of the supernatant from PM_{10} particles ameliorated the free radical activity showed the involvement of iron in this soluble or ultrafine component. In fact the whole of the free radical damage could be abolished with the iron chelator and the centrifuged supernatant was capable of producing all of the free radical activity of the suspension. It can therefore be assumed that all of the iron that leached from the particles during a normal assay was released in the short time that it took to prepare a supernatant. The PM_{10} particles released substantial amounts of iron, principally Fe^{+++}, confirming that iron could be released into the fluid of the lung lining and in increased amounts in the macrophage phagolysosome. We assume that there was a reducing source in the PM_{10} particles that led to the redox cycling with production of Fe^{++} and hydroxyl radical, but we are unaware of the identity of this agent.

To further measure the concentrations of iron in the PM_{10} particles we used the techniques of *x* ray fluorescence and analytical scanning electron microscopy. These methods detect only the element, and not the combination state—that is, whether present as chloride, sulphide, oxide, etc. The use of scanning electron microscopy showed that the *x* ray fluorescence analysis was likely to be an average of fibres that are part of the filter and the PM_{10} particles themselves. Energy dispersive *x* ray analyser analysis of the filter fibres confirmed that they are glass, as shown by barium and zinc peaks; the iron peak was very modest. The PM_{10} particles were, by contrast, dominated by substantial silicon, iron, sulphur, chlorine, and modest aluminium, potassium, and calcium peaks; the absence of barium and zinc peaks confirmed that there was no signal from the fibres being analysed, only the signal from particles. The high sulphur peak in the energy dispersive *x* ray analyser spectrum of the PM_{10} particles suggests that the iron may be present as its sulphate or sulphide. The chloride may also be associated with the iron, but is more likely to be allied to the light elements (sodium, calcium, potassium, and magnesium) and may have arisen from sea spray.

Iron, as various salts, has been reported to be present in suspended particulate matter samples[23] to the calculated concentration of 0·5 $\mu g/m^3$ of air[24] but the size of the fraction that it is associated with is unknown.

PM_{10} particles thus join a list of particles with adverse health effects considered to be mediated through iron and free radicals—namely, asbestos,[25] coal,[26] and quartz.[27] Iron

from asbestos[28 29] and asbestos bodies[30] have been shown to have a role in the generation of free radicals. We have also shown that amphibole asbestos has free radical activity that can be ameliorated by treatment with an iron chelator[15] in the same supercoiled DNA scission assay used here. The ability of asbestos to stimulate macrophages to release tumour necrosis factor-α can be abolished by chelation of iron on the fibres[31] highlighting the importance of oxidative stress in cytokine gene transcription mediated by particles.[32] Asbestos stimulated transcription of cellular oncogenes c-fos and c-jun is inhibited by the thiol antioxidant NAC[33] in mesothelial cells, and other redox sensitive transcription factors are involved in the transcription of genes for proinflammatory cytokines.[32] It is possible that PM_{10} particles, through the free radical activity shown here, may have similar effects on the redox balance of key lung cells leading to the transcription of proinflammatory cytokines.

The data here provide a plausible hypothesis for the adverse health effects of PM_{10} particles that needs to be further tested. If iron is present in substantial quantities on the ultrafine fraction, then the large surface area presented by this fraction[20] could lead to severe oxidative stress in the fluid of the lung lining and at contact between particles and cells. In the macrophage phagolysosome where the low pH favours mobilisation of iron there could also be opportunity for enhanced generation of free radicals with predictable consequences for gene expression in these cells.

The effects of PM_{10} particles in causing exacerbations of airways disease by an oxidative mechanism, as suggested here, is particularly plausible in view of the authors' evidence that these susceptible groups have a measurable deficit in their antioxidant defences.[34] We have shown that smokers and patients with exacerbations of chronic obstructive pulmonary disease and asthma have a systemic deficit in their antioxidant defences as measured with a test that detects total antioxidant activity in plasma.[34] The deficit in antioxidant defences shown in plasma is likely to be originating from the lung and so these patients are likely to have oxidative stress in the epithelium and the fluid of the lung lining. Further exposure of the oxidatively stressed lung to PM_{10} particles with the free radical activity shown here, could be a significant additive oxidative stress that could lead to aggravation of their condition. The resulting transfer of particles into the interstitium and pulmonary inflammation could be a significant factor in promoting cerebrovascular mortality.[13]

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