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Ultrafine particles: mechanisms of lung injury

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Many ultrafine particles comprised classically of low-toxicity, low-solubility materials such as carbon black and titanium dioxide have been found to have greater toxicity than larger, respirable particles made of the same material. The basis of the increased toxicity of the ultrafine form is not well understood and a programme of research has been carried out in Edinburgh on the toxicology of ultrafines aimed at understanding the mechanism. We used fine and ultrafine carbon black, TiO_2 and latex and showed that there was an approximately 10-fold increase in inflammation with the same mass of ultrafine compared with fine particles. Using latex particles in three sizes—64, 202 and 535 nm—revealed that the smallest particles (64 nm) were profoundly inflammogenic but that the 202 and 535 nm particles had much less activity, suggesting that the cut-off for ultrafine toxicity lies somewhere between 64 and 202 nm. Increased oxidative activity of the ultrafine particle surface was shown using the fluorescent molecule dichlorofluorescein confirming that oxidative stress is a likely process by which the ultrafines have their effects. However, studies with transition-metal chelators and soluble extracts showed that the oxidative stress of ultrafine carbon black is not necessarily due to transition metals. Changes in intracellular Ca^{2+} levels in macrophage-like cells after ultrafine particle exposure suggested one way by which ultrafines might have their pro-inflammogenic effects.

Keywords: ultrafine; particulate matter; lung; PM_{10}; inflammation; air pollution

1. Introduction

This paper summarizes research carried out collaboratively in the ELEGI Colt Laboratory at Edinburgh University and the Biomedicine Research Group at Napier University, Edinburgh. The research is focused on the mechanisms of pathogenicity of ultrafine particles in the lungs. Many toxicological studies over the last 10 years have confirmed earlier research indicating that the particles in the ultrafine size range (less than 100 nm) pose special problems to the lungs (reviewed in Donaldson *et al*. (1998)). Typically, ultrafine particles cause more inflammation in experimental studies than respirable particles above the ultrafine size range made from the same material (Donaldson *et al*. 1998).

Attention has focused on ultrafine particles lately because of

(1) increased application and use in industry with concomitant potential for occupational exposure (Pui & Chen 1997); and

Table 1. *Characteristics of particles used in the studies*

name	abbreviation	origin	1^0 size (nm)	surface area ($m^2\ g^{-1}$)
normal carbon black	CB	Haeffner	260.2	7.9
ultrafine carbon black	ufCB	Degussa	14.3	253.9
normal TiO_2	TiO_2	Degussa	250	6.5
ultrafine TiO_2	$UfTiO_2$	Degussa	20	50
normal latex	latex	Polysciences	202	28.3
ultrafine latex	uflatex	Polysciences	64	89.3

(2) research on particulate air pollution, $PM_{10}/PM_{2.5}$, has shown adverse effects at very low levels, resulting in a research thrust into which components of the PM_{10} particle mix might be responsible. The ultrafine particles have been hypothesized to be one component, amongst many, that could account for some of the adverse health effects of PM (MacNee & Donaldson 1999).

2. Materials and methods

The particle types used are shown in table 1.

We used bronchoalveolar lavage (see, for example, Li *et al*. 1996) to quantify inflammation following instillation of particles into rat lungs with or without the thiol antioxidant nacystelin, which was a kind gift from SMB Pharmaceuticals and which we have previously shown to be able to prevent pro-inflammatory responses by particles (Brown *et al*. 1999). The intracellular Ca^{2+} concentration was assessed using the dye Fura 2, which fluoresces in proportion to the amount of Ca^{2+} present in the cell, and thapsigargin as a stimulus for the release of endoplasmic reticulum stores of Ca^{2+} (Stone *et al*. 2000). mRNA for *IL-8* was measured by reverse transcriptase-polymerase chain reaction (RT-PCR), as described in Schins *et al*. (2000). The E1A positive A549 epithelial cell line was a gift from Professor J. C. Hogg, Vancouver, Canada.

3. Results and discussion

(*a*) *Increased inflammation caused by ultrafine particles compared with fine particles of the same material*

As shown in figure 1, all three types of ultrafine particle were capable of causing more inflammation than their non-ultrafine counterparts. Note that there were differences in the dose used: 125 μg in the case of latex and TiO_2, and 500 μg in the case of CB. Fine or ultrafine particles were instilled into the rat lung at the same mass, and there are remarkably similar increases in inflammation compared with the non-ultrafine material in each case. There are differences in the proportional increase in polymorphonuclear neutrophils (PMN) between the particle types, that is, for about four times more particle by mass of ufCB at 125 μg, there is an approximately 10-fold increase in the extent of the inflammation. This suggests that the composition, or the surface area of the particles, is important (see table 1). We do not have data for CB and ufCB at the time of writing but these experiments are under way.

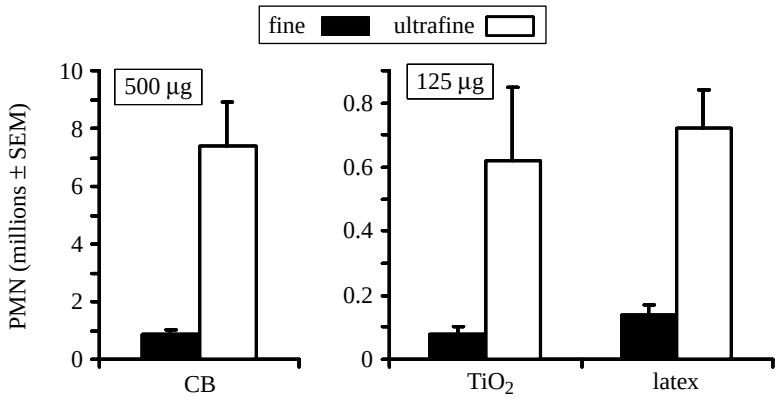

Figure 1. Inflammation, measured as the number (mean ± SEM of three rats) of neutrophils (PMN) in the lavage of rats instilled with either 125 or 500 µg of fine or ultrafine carbon black (CB), titanium dioxide (TiO_2) or latex 24 h previously.

(*b*) *Evidence for a size cut-off for ultrafine particle-mediated inflammation using latex particles*

The three different sizes of latex particle were used to shed light on the size at which the ultrafine particle effect appears (figure 2). The 64 nm latex caused much more inflammation than the 202 or the 535 nm latex at 125 mg. At the 500 µg dose, all of the particles caused more inflammation, but the 64 nm latex was markedly more inflammogenic than the other two and again there was little difference between the 202 and the 535 nm latex. This suggests that 64 nm particles show the ultrafine effect of producing enhanced inflammation and suggests that the cut-off for considering particles to be 'ultrafine' (less than 100 nm) may be approximately correct. More research with particles above and just below the 100 nm size are required to clarify this question of a size cut-off.

(*c*) *Role of transition metals in the inflammation caused by ultrafine carbon black*

Since several types of carbon-based particle, such as residual oil fly ash, have their effects via transition metals, we examined the role of transition metals in the inflammation caused by ultrafine carbon black. We used two strategies as follows.

(1) We treated CB and ufCB with the transition-metal chelator desferal (desferioxamine) before instilling: this chelates any transition metal present on, or released by, the particles. These chelated particles are then washed and instilled into the lungs of rats and inflammation assessed.

(2) We incubated the ufCB and CB in saline to collect transition metals or any other soluble material and then instilled these soluble components into rat lungs and assessed the inflammation.

As shown in figure 3*a*, the chelated particles were no different from the unchelated particles in their ability to cause lung inflammation. Figure 3*b* shows that there was no inflammogenic activity in the saline wash of the particles, demonstrating that no soluble transition metals, or other soluble components, were mediating the increased inflammation of the ufCB.

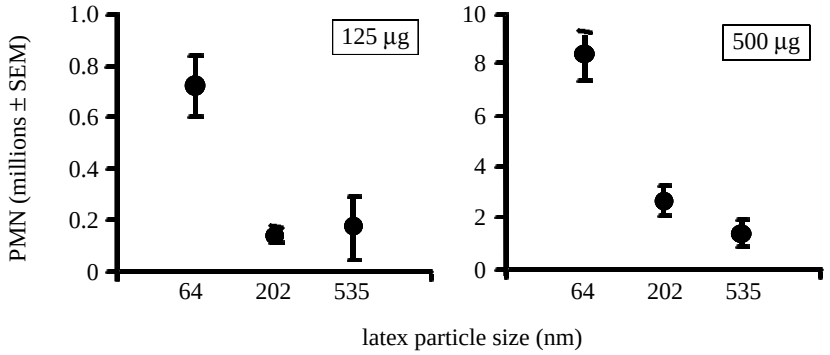

Figure 2. Inflammation, measured as the number (mean ± SEM of three rats) of neutrophils (PMN) in lavage after instillation of 125 or 500 µg of latex particles of various sizes 24 h previously.

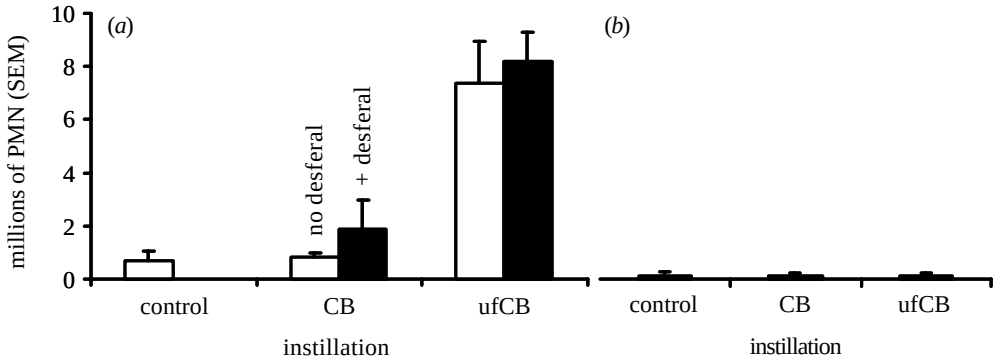

Figure 3. (*a*) Particles treated with iron chelator. (*b*) Diffusable material from the surface of particles. Inflammation, measured as millions of neutrophils (mean ± SEM PMN in three rats) in lavage. Treatments were (*a*) instillation of 125 or 500 µg of CB or ufCB that had been incubated in saline (no desferal (open bars) or desferal solution (black bars) prior to instillation); (*b*) lungs of rats instilled with 0.5 ml of saline alone (control) or saline that had been incubated with 1 mg ml^{-1} of CB or ufCB and the particles centrifuged out to leave the diffusable components.

(*d*) *Role of oxidative stress in the inflammation caused by ultrafine carbon black*

We have previously reported that ufCB has more oxidative stress-inducing activity than CB, as shown by ability to nick supercoiled DNA *in vitro* and deplete glutathione in epithelial cells in culture (Stone *et al*. 1998). We examined whether a thiol antioxidant could protect against the inflammation caused by ultrafine carbon black by instilling nacystelin (NAL, SMB Pharmaceuticals, Belgium) along with ufCB, ufTiO_2 and uflatex. As shown in figure 4, co-instillation of particles with NAL caused significant amelioration of the inflammation caused by the different types of ultrafine particle on their own. The protective effect of NAL was most substantial with ufCB and uflatex and much less marked with ufTiO_2.

(*e*) *Studies on the mechanism of lung inflammation caused by ultrafine particles*

We have examined the cellular and molecular basis of the increased inflammation caused by ultrafines. Calcium, as Ca^{2+}, is an important signalling mechanism for

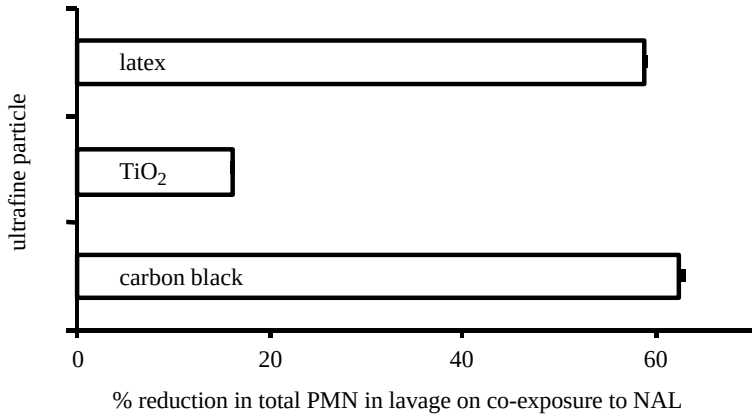

Figure 4. Percentage reduction in the inflammatory response (mean ± SEM of lavage neutrophils in three rats) caused by ultrafine particles instilled along with nacystelin compared with the particles alone.

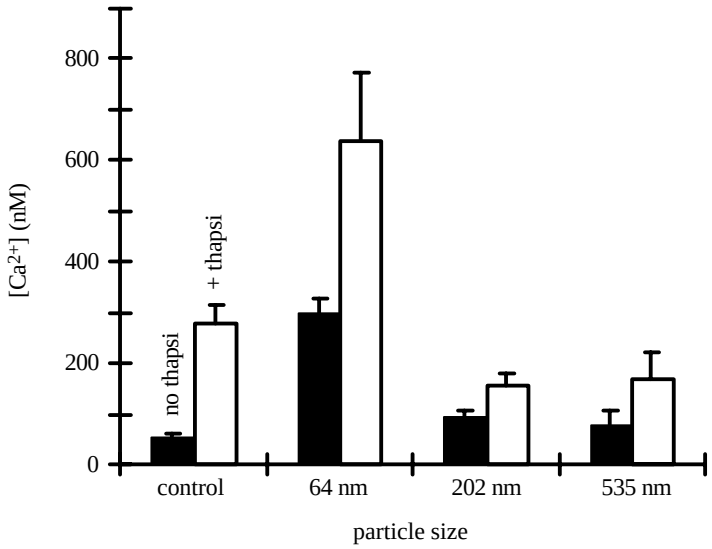

Figure 5. Resting ('no thapsi') and thapsigargin-stimulated ('+thapsi') intracellular Ca^{2+} levels (mean ± SEM of three separate experiments) in macrophage-like cells Monomac 6 exposed to different sized latex particles.

gene expression via activation of transcription factors (Dolmetsch *et al*. 1998). We have hypothesized that ultrafine particles may induce Ca^{2+}-mediated signalling for activation of transcription of the chemokine *IL-8*, which is highly chemotactic for PMN and could explain the inflammation produced by these particles. We used the dye Fura 2 that fluoresces in the presence of Ca^{2+} to assess the levels of Ca^{2+} in macrophage-like cells. As shown in figure 5, the resting intracellular Ca^{2+} levels, and the thapsigargin-stimulated intracellular Ca^{2+} levels, are rapidly and significantly increased on treatment with the ultrafine latex but not with the larger sizes of latex particle. We reported this previously for ufCB and CB and consider this to be an important 'priming' effect for gene expression that results from a direct or indirect

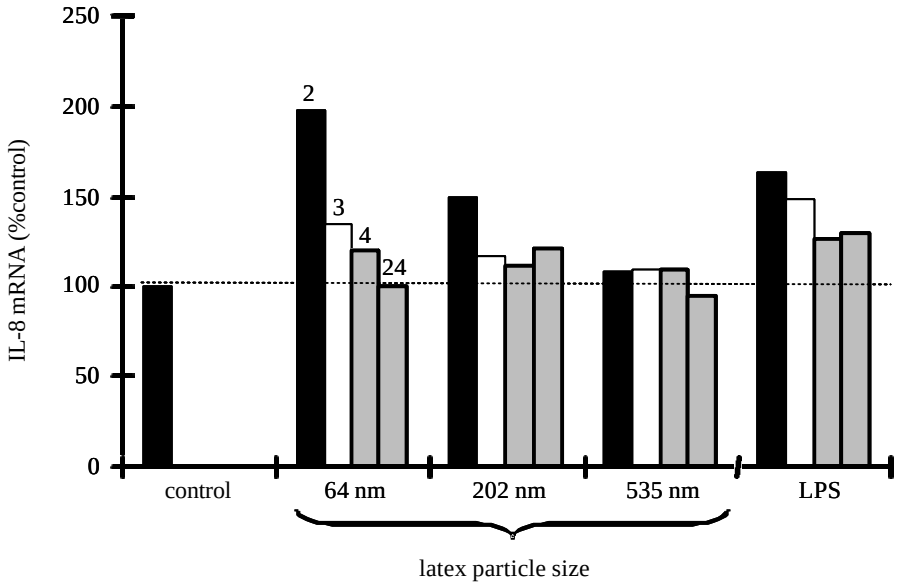

Figure 6. *IL-8* mRNA levels in A549 epithelial cells exposed to different sizes of latex or lipopolysaccharide. Different shades of bar represent time after exposure in hours as indicated on the 64 nm group. Data from a single experiment.

effect of ultrafine particles on the membrane Ca^{2+} channels (Stone *et al.* 2000). This effect can be inhibited by antioxidants such as NAL, and so a role for oxidative stress is suggested in the Ca^{2+} effect. Figure 6 supports these findings by showing a particle-size-related effect of the latex particles on levels of mRNA for *IL-8*, showing that transcription of this important chemokine is indeed increased more by uflatex particles than the other sizes of latex studied.

(*f*) *Pulmonary adenoviral infection as susceptibility factor in inflammation caused by ultrafines*

The adverse health effects of PM_{10} are seen only in certain susceptible populations and little is understood of the factors that underlie this susceptibility. We hypothesized that cells transfected with the adenoviral gene E1A might show increased susceptibility to ultrafine particles in terms of the pro-inflammatory effects these particles induce. This is based on reports that cells expressing E1A showed hyperresponsivity of the NF-κB pathway, an oxidative stress-responsive transcription pathway that we have shown to be important to the pro-inflammatory effects of PM_{10} (K. Donaldson *et al.*, unpublished data). Figure 7 shows preliminary data from a single experiment showing the *IL-8* protein release from control A549 cells (E1A− (negative)) and A549 cells that have been stably transfected with the E1A gene (E1A+ (positive)), in response to exposure to CB and ufCB. The white columns show that normal A549 cells show no discrimination between CB and ufCB but that the E1A+ cells release approximately threefold more. The data show that the ufCB causes more release of *IL-8* than CB on a mass basis and that the E1A+ cells release more *IL-8* in response to ufCB than the E1A− cells.

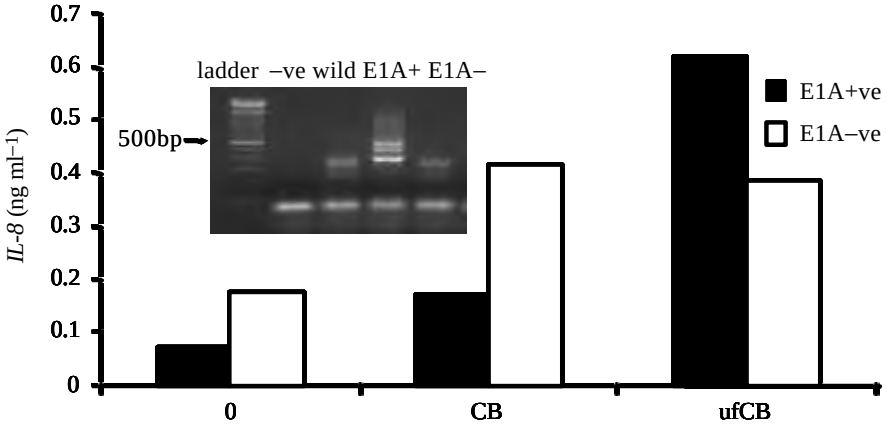

Figure 7. Result of a single experiment showing release of *IL-8* protein by A549 epithelial cells that are stably transfected with the E1A gene (E1A+) or not (E1A−) in response to exposure to CB and ufCB. Inset shows RT-PCR product of the E1A gene.

4. Conclusions

The research described here is ongoing but suggests important new understandings of the likely mechanism of lung injury caused by ultrafine particles. By using a range of different ultrafine particles and their fine counterparts we have sought to learn about the generic effects of ultrafines. However, there are likely to be differences in toxicological effects of different materials presented as ultrafine particles, depending, for example, on the solubility, etc., of the particles (see Donaldson *et al*. 1998). However, based on the insoluble, low-toxicity ultrafine particle types used here, we suggest the following.

(1) Ultrafine particles are more inflammogenic than their fine but still respirable counterparts made from the same material.

(2) The cut-off size for this increased toxicity lies somewhere between 65 and 200 nm, although the cut-off may not be sharp.

(3) Ultrafine particles can cause inflammation via processes independent of the release of transition metals. The property that drives this toxicity is unknown but very likely relates to particle number or particle surface area and involves oxidative stress.

(4) Although transition metals are not necessarily involved in the initiation of inflammation, oxidative stress is important, as shown by the ability of an antioxidant to protect against the inflammatory effects of all three ultrafines used here. If transition metals were present along with the ultrafine particles, the effects could be additive or synergistic.

(5) Increases in the intracellular Ca^{2+} may underlie the cellular effects of ultrafines by a mechanism not yet understood but involving increased influx of Ca^{2+} via the membrane Ca^{2+} channels following contact with particles and probably involving oxidative stress. Increased Ca^{2+} in cells exposed to ultrafines can lead to the transcription of key pro-inflammatory genes such as *IL-8*.

(6) Infection with adenovirus, a virus that causes the common cold, may serve to render lung cells susceptible to the production of increased amounts of inflammatory mediators. This could occur via interaction of the E1A protein with oxidative stress-responsive transcription pathways rendering the cells more susceptible to the oxidative effects of particles and leading to enhanced expression of pro-inflammatory genes such as *IL-8*. There may also be a role for Ca^{2+} in this phenomenon.

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Discussion

C. V. Howard (*Foetal Toxico-Pathology, University of Liverpool, UK*). Do you have the basis in your assays for comparing different substances at equal dosage and equal particle size ranges by inhalation, to construct a table of relative toxicities? It may be that this would be of use to policy makers to help them design strategies for trying to control those processes that produce the most toxic particles.

K. Donaldson. We agree that some measure of relative potency by inhalation is desirable but this would be costly.

M. Williams (*DETR, London, UK*). Professor Oberdörster plotted PMN response against *mass* and showed that ultrafine TiO_2 had a larger response than fine TiO_2, but, when plotted against *surface area*, all points fell on one curve. The graphs early on in your talk showed similar behaviour, and in many of your later histograms, the responses seemed to scale with surface area. Would one expect this if (cf. Dr Jefferson's paper) there was something special about ultrafines and it was not just a surface area effect?

K. Donaldson. This is an important question that needs to be addressed by well-defined dose–response studies. However, our impression, from limited data, is that ultrafine particles have extra surface reactivity as well as extra surface, compared with non-uniformities.

A. D. Maynard (*NIOSH, Cincinatti, OH, USA*). There has been significant emphasis on the contribution that low-solubility particle surface area may make to the nature, magnitude and rate of biological interactions. However, characterization of 'biologically relevant' surface area will depend on the length-scale over which these interactions occur. Could you speculate on the order of magnitude of length-scale that is likely to be of greatest relevance in determining interaction mechanisms?

K. Donaldson. The only information that I know about regarding the length or distance that cells can resolve in a paper by Wojciak-Stothard *et al*. (1996). This paper shows that macrophage-like cells align their cytoskeleton along grooves 44 nm in depth. This suggests that cells can discriminate, via one assumes surface receptors, well down into the ultrafine size range. The physiological responses that such a cytoskeletal re-organization might cause are of great potential interest.

Additional reference

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