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In vitro determinants of particulate toxicity: The dose-metric for poorly soluble dusts

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In vitro determinants of particulate toxicity: The dose-metric for poorly soluble dusts

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Rats exposed to high airborne mass concentrations of low toxicity poorly soluble particles (LTPSP) have developed lung disease such as fibrosis and lung cancer. These particles are regulated on a mass basis in occupational settings. However, animal studies have shown ultrafine particles producing a stronger inflammatory effect than fine particles per unit mass. The present study investigated whether the surface area of LTPSP is a better descriptor than mass of their ability to stimulate pro-inflammatory responses in vitro. In a human alveolar epithelial type II-like cell line, A549, we measured interleukin-8 (IL-8) mRNA, IL-8 protein release and glutathione (GSH) depletion as markers of pro-inflammatory effects and oxidative stress after treatment with a range of LTPSP (ultrafine and fine) and DQ12 quartz, a known cytotoxic dust In all the assays, ultrafine preparations of titanium dioxide [TiO2] and of Carbon Black [CB] produced much stronger inflammatory responses than the same mass dose of fine TiO2 and CB.

The results of the GSH assay confirmed that oxidative stress was involved in the response to all the particles, and two ultrafine metal dusts (cobalt and nickel) produced GSH depletion similar to ultrafine TiO2, with very similar surface areas. In all the assays, the fine and ultrafine CB produced a lower response than expected, given its high measured surface area. This may be because CB has a porous structure and therefore a very complex surface. As expected, DQ12 was more strongly inflammatory than the low toxicity dusts, on either a mass or surface area basis. Dose response relationships observed in the in vitro assays appeared to be directly comparable to dose response relationships in vivo when the doses were similarly standardised. Both sets of data suggested a threshold in dose measured as surface area relative to the area of the exposed cells, at around 1 to 10 cm2/cm2.

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SUMMARY

INTRODUCTION

High airborne mass concentrations of low toxicity poorly soluble particles (LTPSP) have been associated with the development of lung diseases, including fibrosis and cancer. These particles are regulated on a mass basis but recent rat inhalation studies have suggested that particle size is important and that surface area (of lung burden) is a better dose-metric than particle mass or number. The mechanisms behind the surface area phenomenon remain to be investigated. However, indications are that particle mediated oxidative stress in epithelial cells is an important mechanism for the initiation of inflammation, whereby epithelial cells produce pro-inflammatory mediators, such as interleukin-8 (IL-8), causing inflammatory cells (macrophages and neutrophils) to migrate to the site of deposition. The release of these mediators leads to pro-inflammatory effects and impairment of particle clearance.

HYPOTHESIS

Inhaled particles induce, through surface contact, an oxidative stress response in alveolar epithelial cells that causes the release of chemotaxins and this leads to pro-inflammatory effects and impairment of clearance. If this induction occurs through surface contact, then the oxidative stress and pro inflammatory effects should relate better to dose expressed as surface area than as mass. We test this hypothesis *in vitro* using a human alveolar epithelial type II cell line, A549, by measuring oxidative stress in the form of glutathione (GSH) depletion and pro-inflammatory cytokine gene expression as IL-8 mRNA expression and IL-8 protein release.

AIMS AND STUDY DESIGN

The aim of the present study was to investigate whether the surface area of LTPSP is a better dose metric than mass (or volume) for describing the particles' ability to induce pro-inflammatory effects *in vitro*. Additionally, it was hoped that the developed *in vitro* approach would become a reliable means of screening the toxicity of particles with the advantages of being less expensive and more ethical than animal testing.

The study was designed to examine a number of pro-inflammatory effects of the particles at concentrations that were not so high as to produce gross toxicity. Therefore, initially, the cytotoxicity of the particles at a range of doses was assessed by measuring LDH release. Then the oxidative stress (GSH depletion) and the chemokine IL-8 (as mRNA and as protein) were measured at an appropriate range of particle doses.

MATERIALS AND METHODS

Cells from the human alveolar epithelial type II cell line, A549, were exposed to cells at a range of measured mass doses. The specific surface areas of the dusts were measured using a gas adsorption technique, in order to convert mass doses to surface area doses. The doses were expressed as concentrations in two ways: first as amount per unit volume of *cell culture* fluid, and then as amount per unit area of exposed cells. With a confluent layer of cells covering the culture dish, this area was that of the dish.

The tested particles included ultrafine particles (carbon black [CB] and titanium dioxide [TiO_2]), fine particles CB and TiO_2, barium sulphate ($BaSO_4$) and DQ12 quartz for all the assays, and also ultra fine nickel (Ni) and ultrafine cobalt (Co) for some assays.

Cytotoxicity was measured in the form of lactate dehydrogenase (LDH) release at 24h, to obtain the percentage of total LDH that would be released in the event of cell death. We measured intracellular glutathione (GSH) at 4h, IL-8 mRNA at 6h and IL-8 protein release at 6h and 24h.

RESULTS

Cytotoxicity (LDH)

None of the LTPSP caused significant cytotoxicity in A549 cells in the form of LDH release into the medium; however, at higher concentrations the ultrafine CB interfered with the assay leading to some artificially low LDH readings. In contrast, DQ12 quartz at the highest concentrations of 192 and 384 μg/ml caused significant toxicity, with LDH at 27% and 54%, respectively.

Pro-inflammatory effects (IL-8 mRNA, IL-8 protein)

Levels of IL-8 mRNA were observed at 6h after exposure and 24h after exposure.

- At 6h, the IL-8 mRNA increased significantly with dose, over the same range of mass dose, for DQ12 and all the LTSP except $BaSO_4$ and fine CB.
- At 24h after exposure, the DQ12 had still an elevated level of IL-8 mRNA. For all the LTPSP, the levels of IL-8 mRNA had dropped to background levels.

Il-8 protein release into the culture medium was also measured at 6h and 24 h after exposure.

- At 6h, there was minimal protein release for the LTPSP but significant release for the DQ12.
- At 24h, the levels of IL-8 protein mirrored the increases in IL-8 mRNA for the ultrafine LTPSP and DQ12. However, the ultrafine CB, at the higher concentrations, interfered with the measurement of IL-8 protein. Because of this interference (which might be relevant to some untested particles, not just CB), the IL-8 protein measurement appeared to be less reliable than the IL-8 mRNA assay. The DQ12 induced a higher release of IL-8 protein compared to the ultrafine particles and this may have been due to its greater surface reactivity.

For the same mass dose, the ultrafine particles (of TiO_2 or CB) produced a much higher level of IL-8 mRNA and IL-8 protein than the same dose of the same material as fine particles.

Oxidative Stress

All the LTPSP and metal (ultrafine Ni and Co) particles and DQ12 had the ability to deplete intracellular GSH at 4h suggesting the involvement of oxidative stress in the cellular responses.

Again, relative to mass dose, the ultrafine particles produced a greater response than fine particles of the same material.

Mass dose

For the markers of oxidative stress and pro-inflammatory effect, the ultrafine particles produce higher responses than the fine particles of the same material. However, the relationships with mass dose do not readily lend themselves to explaining the observed difference.

Surface area dose

Plotting the responses relative to surface area dose of LTPSP largely accounted for the difference between fine and ultrafine particles of the same material. Specifically, for the same material, the fine particles at low surface area dose gave results which joined onto the trends found for the ultrafine particles at high surface area dose. Furthermore, there were also some consistent patterns when comparing across materials.

For IL-8 mRNA plotted against particle surface area dose/surface area culture dish (cm^2/cm^2), there was an approximately common threshold but three relationships; DQ12 the steepest, then ultrafine TiO_2 and then ultrafine CB. The dose-threshold was approximately $1cm^2/cm^2$.

For IL-8 protein release, there appeared to be thresholds between 1 and 10 cm^2/cm^2 particle surface area per unit area of cell culture dish. The threshold appeared to be about 1 for the TiO_2 and most of the other LTPSP but closer to 10 for the CB. DQ12 followed a much steeper line than the LTPSP and appeared to have a lower threshold, below 1 cm^2/ cm^2.

For GSH depletion, there were significant dose response trends for all dusts, showing again three relationships: DQ12 having the highest activity, ultrafine TiO_2, fine TiO_2, fine CB and $BaSO_4$ in another group and ultrafine CB on its own.

When we normalised the IL-8 protein response as IL-8 protein per unit dose, the particles fell into two distinct groups. DQ12 stood on its own as producing far more IL-8 protein per unit dose than the LTPSP. All the LTPSP appeared to produce approximately the same response per unit surface area dose.

Relating in vitro and in vivo doses and results

To compare *in vitro* and *in vivo* data, we expressed the dose relative to the surface of the cells on which it was deposited. For the *in vivo,* this involved the area of the centri acinar region of the rat lung; for the *in vitro*, the area of the confluent layer of A549 calls. In both cases the cell type was (either entirely or mainly) Type II epithelial cells. Then, we compared the *in vitro* data on IL-8 mRNA expression in A549 cells from the present study with *in vivo* data on PMN levels in BAL fluid from rats exposed to ultrafine TiO_2, fine TiO_2 and $BaSO_4$. We found that the dose response curves were remarkably similar, with approximately the same threshold dose and similar slope. This dose threshold was about $1cm^2/cm^2$ (particle surface area per unit surface area of epithelial cells).

CONCLUSIONS

For a given dust type, we have observed differences related to particle size with finer particles (with greater specific surface area) having greater biological effects *in vitro* for the same mass dose.

The findings of the present *in vitro* study show that for LTPSP, surface area rather than mass is a better predictor for the pro-inflammatory effects *in vitro.* This is consistent with the previous observations from animal inhalation experiments that showed inflammation relating better to surface area than mass or number. Therefore, from these findings, we would predict that for other low toxicity, poorly soluble particles, exposure to an airborne respirable dust mass concentration of very small particles will cause more inflammation than the same mass concentration of larger particles of the same chemical composition. Furthermore, we would also suggest that it would be surprising if poorly soluble particles with more reactive surface area did not also show a similar dependence on particle size.

In this study, the specific surface area of particles was measured using a gas adsorption technique. For some particles, with complex or porous surfaces, the estimate obtained for surface area would depend on the technique used; for example smaller gas molecules penetrate deeper into pores or crevices making more surface available. This would affect particles like the carbon black. Nevertheless, the surface area measurement provided a useful dose metric. For particles with smooth surfaces, surface area is simpler.

Where airborne dust concentrations continue to be monitored in terms of mass, then it may be important to characterise the dust in terms of specific surface area, just as some dusts are commonly analysed for composition relevant to surface reactivity (e.g. quartz content).

DQ12 has a greater reactivity per unit surface area than the LTPSP. It produced a greater and/or more rapid and more persistent effect than the LTPSP relative to mass or surface area dose.

The paradigm developed here represents a faster, more cost effective and more ethical approach to testing dusts in the future than animal experiments. The *in vitro* testing scheme would incorporate three assays: LDH to assess gross toxicity, GSH depletion to assess oxidative stress, and IL-8 mRNA to determine pro-inflammatory effects.

A dose-response relationship observed in *in vitro* assays appears to be directly comparable to a dose-response relationship *in vivo* when dose is expressed relative to the surface area of the exposed cells. This comparability facilitates extrapolation from *in vitro* test results, and it also supports the reliability and validity of the *in vitro* test system. Thus, this *in vitro* test system appears to have the advantages of being ethical, rapid, cost-effective, valid and reliable.

We recommend that:

- the dependence on surface area as a dose metric is potentially highly important and should therefore be substantiated by testing a wider range of dusts in this *in vitro* test system;
- the direct relationship between dose *in vitro* and dose *in vivo* is also potentially highly useful, and therefore should be strengthened to enable it to be used as a routine tool for risk assessment. In particular, it would be a major step to obtain data on the same response in both *in vivo* and *in vitro* systems. (This could be achieved without further *in vivo* studies by using currently available material from previous *in vivo* studies).

1. INTRODUCTION

1.1 BACKGROUND

The results from several animal inhalation experiments have shown that low toxicity poorly soluble particles (LTPSP), at high airborne concentrations, can produce an impairment of clearance and an inflammatory reaction (Driscoll *et al.,* 1996; Driscoll, 1996; McClellan, 1996). These observations led Morrow (1988) to hypothesise that inflammation is one consequence of the physical overloading of individual alveolar macrophages with particles (the others being impairment of macrophage-mediated clearance and a higher rate of particle translocation to the lymph nodes). This understanding has been central to the interpretation of toxicology studies of particles and has informed our understanding of pulmonary clearance (of particles and fibres) (Yu *et al*., 1994; Stöber *et al*., 1994; Katsnelson *et al*., 1992; Tran *et al*., 1994; 1999ab). The studies above relied on the assumption that clearance would become progressively impaired as the volumetric particle lung burden increased. However, relatively recent studies have shown that this is not the complete explanation of impairment of clearance associated with particle loading. For example, inhalation experiments with ultrafine particles (of the same mineral and chemical properties as their larger, low-toxicity fine counterparts) have produced inflammation without volume overload of macrophages (Oberdörster *et al*., 1994). In addition, studies with mineral fibre preparations, as opposed to particles, have shown a relationship between fibre surface area and disease, in the form of pulmonary fibrosis for a range of asbestos fibre types (Timbrell *et al.,*1988). Recent studies from our laboratory (Tran *et al.,* 1999ab; 2000ab) have shown that overload and inflammation did not occur in rats with large lung-burden in terms of mass or volume but a relatively small burden if expressed as surface area (due to a relatively coarse particle size). Moreover, the inflammation caused by ultrafine and fine particles appeared to correlate well with particle lung burden expressed as surface area. This evidence implies an important change to the current understanding of pulmonary clearance of particles and fibres.

1.2 MOLECULAR EVENTS LEADING TO INFLAMMATION IN THE LUNG BY LTPSP

The correlation between LTPSP surface area and the initiation of inflammation has been demonstrated in the studies mentioned above. However, the mechanisms behind the surface area phenomenon remain to be investigated. Recent advances in molecular biological techniques have indicated that inhaled particles can cause the production of reactive oxygen species (ROS) that leads to the depletion of antioxidants causing oxidative stress in the epithelial cells in the lung and that this is an important mechanism for the initiation of inflammation. Following this oxidative stress, the epithelial cells produce inflammatory mediators that cause infiltration of inflammatory cells, such as macrophages and neutrophils. The inflammatory cells that migrate to the lung may eventually contribute further to the oxidative stress at the site of particle deposition but they do not undergo oxidative stress themselves. The macrophages' functions are to phagocytose (ingest) pathogens and, during the phagocytosis, the macrophage generates ROS and proteases and regulates the extracellular environment through the release of cytokines. The oxidative stress that arises in epithelial cells from overproduction of ROS may initiate damage to cell membranes in the form of lipid peroxidation. Lipid radical species produced by this process may cause a depletion of the major antioxidant in the lung, glutathione (GSH). When this occurs, there is 'sensing' of the oxidative stress with activation of redox sensitive transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) that control the transcription of pro-inflammatory and proliferative genes such as interleukin-8 (IL-8) (Rahman and MacNee, 1998).

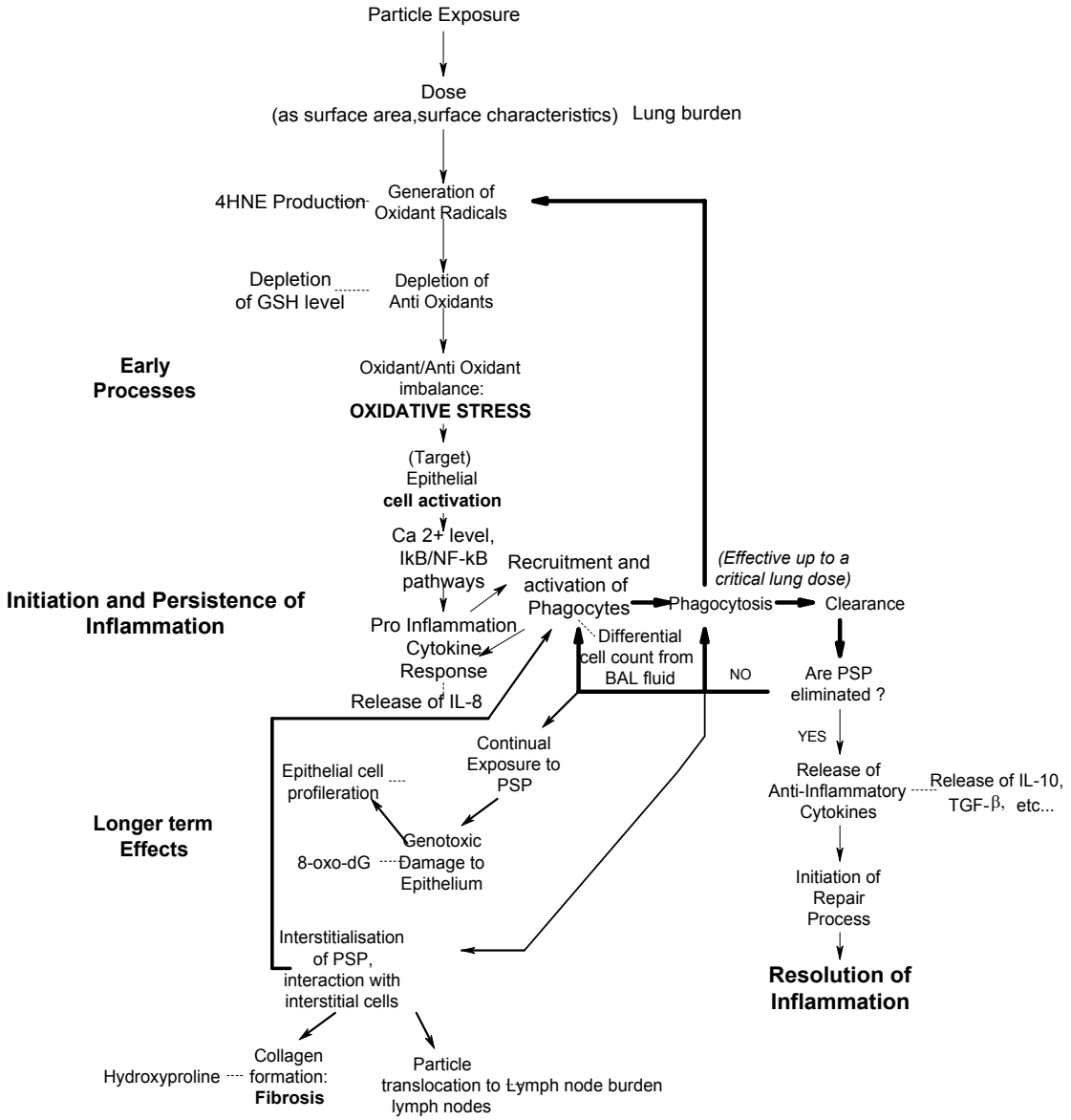


Figure 1.1

The hypothesised exposure-dose-response relationship to inhaled LTPSP and the early processes leading to inflammation and the longer term effects, such as fibrosis and cancer

There is little work on the molecular signalling events following LTPSP exposure thats leads to inflammation but, for crystalline silica, there is clear evidence of oxidative stress at the particle surface (Donaldson and Borm, 1998) and activation of NF-κB (Sacks *et al.*, 1998). The steady state depletion of I-κB in LTPSP-exposed cells very likely underlies the chronic switching on of IL-8 which is under the transcriptional control of NF-κB (Schins *et al.,* 2000). Oxidative stress leading to the activation of cell signalling cascades may produce inflammation that contributes to fibrosis and cancer. These events are summarised in Figure 1.1.

A growing amount of evidence indicates that cytokines produced locally by inflammatory cells in the lung play a pivotal role in the control of the toxic response. In particular, the number of cytokines involved in the pulmonary response to inhaled particles is rapidly increasing and includes chemokines (IL-1, IL-6, IL-10) and cytokines (TNF-α, TGF-ß). In addition, recent studies show that cytokines affect fibroblast activation and proliferation, as well as collagen deposition during evolution of chronic fibrotic lung disease. In particular, γ-interferon suppresses fibroblast activities such as proliferation and collagen production, while interleukin-4 augments fibroblast growth and collagen production (Sempowski *et al*., 1996). These two mediators are the prototypic cytokines that functionally define either a T helper 1 (Th1) or a T helper 2 (Th2) response in the lung (Mossman *et al.,* 1986). Clinical studies have also revealed a predominance of the Th2 cytokine pattern of inflammatory response in the pulmonary interstitium in fibrotic diseases (Wallace *et al.,* 1995). Recent findings indicate that anti-inflammatory cytokines (IL-4, IL-10, IL-13, TGF-β) which accompany and tend to activate the resolution of the inflammatory response induced in the lung by inhaled particles, all belong to the Th2 cytokine group. This suggests that the sustained attempt by the lung to resolve the inflammatory reaction induced by PSP could contribute to the pathogenesis of the fibrotic reaction through the protracted secretion of pro-fibrotic cytokines (Huaux *et al.,* 1999).

1.3 THIS STUDY

In this study we use in vitro assays to examine the response of human alveolar type II epithelial cells to LTPSP. The specific hypotheses to be tested are described in the next chapter.

2. HYPOTHESIS

From all the recent evidence we form a new hypothesis. Inhaled particles *via* properties of their surface induce an oxidative stress response in epithelial cells. The stress response induced is likely to develop substantially as lung burden rises above a particulate dose-threshold, expressed as particulate surface area. This leads to an increase in inflammation, observed as an increase in the level of neutrophils into the lung. In this scheme of events, as long as the epithelial cells continue to release chemotaxins, macrophages, which play an important role in the resolution of inflammation, are less likely to migrate from the affected area and that impairs clearance. Thus clearance may be retarded without the macrophages necessarily being overloaded. However, eventually, the continued phagocytosis by retained macrophages may lead to the volumetric overloading of these cells with particles, and then the volumetric overloading will in itself cause retardation of clearance as described by Morrow (1988).

The new hypothesis differs markedly from the original hypothesis of volumetric overload as it proposes that impairment of particle clearance is caused in the first instance by epithelial cell chemotaxin release rather than by the overloading of macrophages by particles. In addition, if the inflammation is related to the total particle surface area of lung burden as suggested by our recent findings (Tran *et al.,* 1999ab; 2000ab) then this has far reaching practical implications. Adverse effects, *i.e.* inflammation and impairment of clearance, can no longer be associated with high exposures as measured in terms of mass or volume concentration and are more likely to be related to surface area dose. These findings have important implications for workplace exposures to ultrafine particles, and for regulatory policy development such as setting occupational exposure limits.

The structure of the lung reduces the likelihood of inhaled particles reaching the delicate tissue of the alveolar space. The deposition of particles in the lower respiratory tract is influenced by (a) the branching structure of the bronchi and (b) particle size. The alveolar surface are covered by three epithelial cell types, alveolar type I, type II and type III. Type I cells cover approximately 95%, type II cover approximately 3-4%. However, the type II cells are the main cell type in the central acinar region, and that is where most of the alveolar lung burden deposits initially (Brody *et al*, 1981). The nature of the stress response elicited on the type II epithelial cells in the lung by particles may be key to understanding the influence of particle characteristics.

Recent investigations have suggested that the responses observed from type II epithelial cells are likely to be mediated by ROS generated by the particles themselves and by the inflammatory cells recruited to the lung during particles phagocytosis (Schins *et al.,* 2000). A corollary to this is that if a particle has a reactive surface, a property that will generate ROS, then its toxicity would be higher but still would be expected to be dependent on its surface area. Following the generation of ROS, an oxidant (ROS)/antioxidant imbalance (GSH depletion) occurs leading to the activation of transcription factors, such as NF-κB. Following activation of NF-κB and binding to the promotor region in pro-inflammatory genes, induction of gene expression occurs. One of these is the pro-inflammatory cytokine, IL-8 and this protein is released from epithelial cells leading to neutrophil influx and inflammation.

We test this hypothesis in the present studies by measuring oxidative stress in the form of GSH depletion at 4h, NF-κB activation and IL-8 mRNA induction at 6h and IL-8 protein release at 12 and 24h in A549 human alveolar type II epithelial cells *in vitro*. The time points were selected to follow the anticipated sequence of events: depletion of GSH is the first effect of the presence of particles, the NF-κB activation and IL-8 mRNA induction follow and in turn lead to IL-8 protein release. GSH depletion is expected to be the initial event that

triggers gene transcription, and pro inflammatory gene expression is generally maximal at 4-6 hours after treatment. Correspondingly GSH levels are best measured at 4 hours, and the mRNA at 6 hours. The 12 and 24 hours time points are chosen to allow translation, secretion and accumulation of IL-8 protein without allowing time for the excessive accumulation cell products in the culture which might produce effects on the cells that are secondary to treatment effects.

At the outset, we had also intended to look at the interaction between macrophages challenged with particles and epithelial cells. However, the project resources were needed to address just the epithelial cells. However, the outcomes of this study (on the correspondence between doses *in vitro* and *in vivo*) should enable an optimal design for any subsequent *in vitro* experimentation on the interaction of macrophages and epithelial cells.

3 AIMS AND STUDY DESIGN

3.1 AIMS

The research presented here continues from our previous studies on low toxicity poorly soluble particles (LTPSP) (Tran *et al.,* 1999ab; 2000ab). The present studies seek to make an important contribution to our understanding of the mechanism(s) of the adverse pulmonary effects of inhaled particles. The aim of this work is to evaluate the relative importance of particle size, surface area and surface chemistry and reactivity in stimulating chemotactic signals from epithelial cells *in vitro.*

The specific research objectives that are addressed in the research are:

(a) to test the hypothesis that the biomarkers of effects of LTPSP observed *in vitro* (oxidative stress and IL-8 release), can be related to particle surface area for a range of LTPSP,

(b) to compare the mechanisms for particles of low intrinsic toxicity to one with a reactive surface, *e.g.* DQ12 quartz, and thereby extend the above hypothesis; and

(c) to develop a way of relating *in vitro* dose response relationships to *in vivo* dose response relationships.

3.2 STUDY DESIGN

The present study has been designed to examine a number of parameters important in pro-inflammatory effects *in vitro* in a human alveolar type II-like epithelial cell line, A549, following exposure to LTPSP and DQ12 quartz. Initially, cellular toxicity was investigated by LDH release from the cells to determine the gross toxicity of these particles. The rationale for performing these initial experiments was to ensure that the effects on more sophisticated end-points were not produced secondary to cellular toxicity, and to confirm that the panel of ultrafine and fine particles have low intrinsic toxicity in A549 cells.

To investigate the pro-inflammatory effects of these particles *in vitro*, at non-cytotoxic concentrations, we chose to measure oxidative stress in the form of GSH depletion. In addition, we selected to measure the chemokine IL-8, which signals for neutrophil influx *in vivo*, at both the mRNA and protein level. Finally, all the results were expressed relative to surface area dose to determine if this was the best dose-metric for effects *in vitro*.

4 MATERIALS AND METHODS

The first paragraph for each of the methodology sections gives a brief outline of the method used and subsequent paragraphs give a detailed account of the method.

4.1 CELL CULTURE

Cells from a human alveolar type II-like epithelial cell line, A549, obtained from the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK) were maintained in continuous culture at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Company, Poole, UK) containing 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine (Gibco, Paisley, UK) and 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco, Paisley, UK). For the *in vitro* assays, the cells were plated in 96 well or 6 well plates at a density of 9×10^4 cells/ml or 0.2×10^6 cells/ml, respectively. The cells were grown to 80% confluency in 10% FCS for 24 h, washed with calcium and magnesium free phosphate buffered saline (CMFPBS) and serum starved for 24 h prior to treatment.

4.2 TREATMENT OF A549 CELLS WITH PARTICLES

Table 4.1
Particle samples used in the study

Particles	Surface area (m^2/g)
Ultrafine carbon black (CB)	253.92
Fine CB	7.92
Ultrafine titanium dioxide (TiO_2)	50.64
Fine TiO_2	6.64
Barium sulphate ($BaSO_4$)	3.68
DQ12 quartz	10.12
Ultrafine nickel (Ni)	36.88
Ultrafine cobalt (Co)	36.24

A range of LTPSP and DQ12 with differing surface areas as indicated in Table 4.1 were used in these studies (the ultrafine Ni and Co were only used in the GSH depletion experiments). The specific surface areas of the particles were measured using the Brunauer Emmett Teller (BET) gas adsorption method (Brunauer *et al*, 1938) with nitrogen at Morgan Materials Technology (Stourport on Severn, UK) from a sample of about 5 g of dust.

A549 cells were treated for 4, 6, 12 or 24 hours, with fine and ultrafine LTPSP at final concentrations of 15, 31, 62, 125 and 250 μg/ml and final concentrations of 48, 96, 192 and 384 μg/ml for DQ12 quartz. For cell treatments, the particles were suspended in DMEM without FCS for 24 hours prior to treatment to starve them of serum and arrest their growth. The particle suspensions were sonicated for 5 min and then added to the wells to produce the final concentrations indicated above. Tumour necrosis factor-alpha (TNF-α) was used as a positive control at a concentration of 10 ng/ml.

4.3 CELLULAR TOXICITY IN A549 CELLS BY MEASUREMENT OF LDH

The LDH (lactate dehydrogenase) assay is a reliable technique to investigate cytotoxicity in the form of loss of membrane integrity by measuring LDH release into the cellular media. The assay was performed in triplicate with all of the LTPSP and at all concentrations.

Cellular toxicity of the particles in Table 4.1 was assessed by measurement of LDH leakage from the A549 cells into the cell culture media and LDH activity measured using the Cytotoxicity detection Kit (LDH) (Roche Molecular Biochemicals, Lewes, UK) according to the manufacturers instructions. Briefly, cells were cultured in 96 well plates and treated with particles for 24h in serum free media. Following exposure, the culture media was analysed for LDH activity. There are two enzymatic reactions. The cell culture medium is incubated with the pyridine nucleotide NAD^+, lactate substrate, catalyst diaphorase and the tetrazolium salt, INT. In the first reaction, the LDH reduces NAD^+ to NADH and H^+ by oxidation of lactate to pyruvate. In the second reaction, the catalyst transfers H/H^+ from NADH + H^+ to the tetrazolium salt INT, reducing INT to formazan. The formazan dye formed is water-soluble and is quantitated on an ELISA plate reader at 500nm. The INT shows no significant absorption at this wavelength. The release of LDH from the cells is directly correlated to the amount of formazan produced in this reaction. The amount of cytotoxicity is related to 100% cell death, where A549 cells are treated with Triton-X 100 that will release all of the cellular LDH.

4.4 INTERLEUKIN-8 (IL-8) MRNA IN A549 CELLS MEASURED BY REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

To measure IL-8 gene expression, mRNA is isolated from treated cells and subjected to RT-PCR with primers specific to IL-8. The PCR products are then run on a polyacrylamide gel and intensity bands quantitated. Only a small number of samples can be run on any one gel, but the fine and ultrafine samples within a dust were run on the same gel.

4.4.1 RNA isolation

Following 6h or 24h treatment with particles, human IL-8 was detected using semi-quantitative RT-PCR. RNA was extracted from treated cells using the TriZol reagent, which is a mono-phasic solution of phenol and guanidine isothiocyanate, followed by the addition of chloroform. Then, centrifugation separated the solution into an aqueous phase and an organic phase, with the RNA present in the aqueous solution and the DNA in the organic portion. This separation prevented any problems with DNA precipitation.

Following treatment, the culture medium was removed and the cells washed twice with cold calcium/magnesium free phosphate buffered saline (CMFPBS). Cold TRIzol (Life Technologies, Paisley, UK) was added to each well and triplicates pooled to give a final volume of 1ml. Samples were transferred into sterile tubes and placed on ice. 100μl of chloroform was added to each tube, vortexed and incubated on ice for 5min, before

centrifugation at 13,000g for 15min at 4°C. The top layer (aqueous phase) from each tube was transferred to a new tube and 0.5ml of cold isopropanol was added and samples incubated on ice for 15min, vortexed and again centrifuged at 13,000g for 15min. The supernatants were then discarded and pellets washed in 1ml of cold 75% ethanol, vortexed and centrifuged at 7,000g at 4°C. The supernatants were discarded and pellets dissolved in 40µl of diethylpyrocarbonate (DEPC)-treated water. Samples were stored at -70°C until used.

RNA concentration was determined spectrophotomically by reading the optical densities at 260 and 280nm. RNA samples having an $absorbance_{260/280}$ ratio of > 1.6 were used The concentration was calculated form the absorbance measured at 260 nm. Beer Lambert's Law states that absorbance (A) is equal to the molar extinction coefficient of the substance of interest (ε) multiplied by the concentration of the substance (C): $A = \varepsilon \times C$. Therefore, $C = A/\varepsilon$.

For spectrophotometrical analysis, the RNA sample was diluted 250 fold, so the calculation included a dilution factor (D=250). The molar extinction coefficient (ε) for RNA is 0.025. To report the RNA in units of µg/µl, a conversion factor of 1000 was included in the formula for RNA concentration:

$C = A \times D/\varepsilon \times 1000$

Hence:

RNA concentration = absorbance x 10

4.4.2 Assessment of IL-8 mRNA by RT-PCR

Two µg of RNA were added to a solution containing 5X reverse transcriptase buffer (Promega, Southampton, UK), 100µg/ml oligo deoxythymidine (dT), 100mM dithiothreitol (DTT), 10mM deoxynucleotide triphosphates (dNTPs), and RNAse inhibitor, reverse transcribed into complementary DNA (cDNA) at 37°C for 1h using Moloney-Murine Leukemia Virus Reverse Transcriptase (200U/µl) and incubated at 94°C for 10min (all components from Gibco, Paisley, UK). Using a thermal cycler (Hybaid, Ashford, UK). Aliquots of cDNA (2 and 5µl) were PCR amplified in 47µl and 50µl reaction volumes containing PCR mix (1X TAQ polymerase buffer, 2.5mM $MgCl_2$, 0.2mM dNTPs) (Promega, Southampton, UK) and TAQ polymerase (1U/µl), for human glyceraldehyde-3-phosphate dehydrogenase (GADPH) and IL-8, respectively. Conditions for PCR were as follows: for IL-8, 35 cycles of denaturation (92°C for 1min), annealing (60°C for 1min), and extension (72°C for 1min), final extension (5min at 72°C); for GAPDH, 35 cycles of denaturation (94°C for 45sec), annealing (60°C for 45sec), and extension (72°C for 90sec), and final extension (72°C for 10min). Oligonucleotide primers used in the PCR reactions were chosen using the published sequence of human IL-8 cDNA (Lindley *et al.,* 1988) and GAPDH (Maier *et al.,* 1990). The primers for IL-8 and GAPDH were synthesized by MWG-Biotech (Milton Keynes, UK). The sequence of the primers used in the PCR were as follows: IL-8 (sense 5'–ATG ACT TCC AAG CTG GCC GTG GCT-3') and (anti-sense 5'-TCT CAG CCC TCT TCA AAA ACT TCT C-3'); GAPDH (sense 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3') and (anti-sense 5'-TCT AGA CGG CAG GTC AGG TCA ACC-3'). PCR products were electrophoresed in 1.5% agarose containing ethidium bromide, scanned using a white/UV transilluminator (Ultra Violet Products, Cambridge, UK) and quantified by densitometry. IL-8 bands were expressed as a percentage of the intensity of the GAPDH bands, which was used as a housekeeping gene.

4.5 IL-8 PROTEIN RELEASE FROM A549 CELLS MEASURED BY ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

The ELISA assay measures IL-8 protein that has been released in the cellular medium. As with the LDH assay, all of the LTPSP were analysed on the same day in triplicate.

Following incubation of A549 cells with the particles for 6h or 24h, cell supernatants were recovered and centrifuged at 13,000g for 10min and the particulate-free supernatant stored at -80°C until analysis. The presence of IL-8 protein in the cell culture medium was measured by ELISA. Briefly, monoclonal and biotinylated anti-human IL-8 antibodies (R&D Systems, Abingdon, UK) were used in these studies. Flat-bottomed 96-well microtitre plates were coated overnight at room temperature with 100µl of monoclonal capture antibody (4µg/ml in CMFPBS). Plates were rinsed (3X) with wash buffer (0.05% Tween 20 in Tris-buffered saline, pH 7.4) and incubated for 1h with diluent (0.1% BSA, Tris-buffered saline, pH 7.3; 20mM Trizma base, 15mM NaCl) to block non-specific binding sites. Wells were aspirated and rinsed with wash buffer (3X) and 100µl of supernatant or standards were added to the wells. Plates were incubated for 1h on the shaker, rinsed and 100µl of the appropriate biotinylated detection antibody added (20ng/ml) and incubated on the plate shaker for 1h. Following rinsing with wash buffer, 100µl of streptavidin-horse radish peroxidase (Dako, Denmark; diluted 1:20,000) was added to each well and incubated on the shaker for 20min.. A substrate stock of 3,3',5,5,-tetramethylbenzidine (TMB) was prepared by dissolving 10mg/ml in dimethysulphoxide (DMSO). To prepare the substrate buffer, 100µl of TMB was added per 10ml of a 100mM solution of sodium acetate/citrate (pH 4.9) with 5µl of 30% H_2O_2. After washing, 100µl of substrate buffer was added to each well and allowed to incubate for 20min. Plates were wrapped in aluminium foil (TMB light sensitive) and the reaction was terminated by adding 100µl of sulphuric acid (1mM). The optical density (OD) was measured at 450nm in a spectrophotometric plate reader. The values were determined from a standard curve using recombinant IL-8 protein (R&D, Abingdon, UK) and expressed as pg/ml.

4.6 ASSESSMENT OF TOTAL GLUTATHIONE (GSH AND GSSG)

This technique measures oxidation stress in the form of GSH depletion. GSH was isolated from the cells and the samples were analysed in triplicate for all of the LTPSP on the same day. GSH was measured using the Tietze method (1969) which involves the recycling of GSH by glutathione reductase and Beta-nicotinamide adenine dinucleotide phosphate as occurs *in vivo*. The procedure measures total GSH (oxidised and reduced). In the presence of 5'5' dithio-bis-2-nitrobenzoic acid, a compound which binds to thiol groups and forms a complex, the amount of GSH can be assessed by measuring the absorbance at 412 nm.

A549 cells were cultured in 6 well plates and treated for 4h with particles. (Where sufficient cells were available, the 6 well plate was used; where fewer cells were available, then the miniaturised version of the same assay in 96 well plates was used.) Following incubation, the cells were suspended in 1ml of cold 0.6% sulfosalicylic acid, sonicated and vortexed several times. The cells were then centrifuged at 5,000g for 5min at 4°C. The supernatant was immediately used to measure total intracellular glutathione by the glutathione-reductase-DNTB recycling assay adapted for microtitre plates (Tietze, 1969). A glutathione stock solution (1mg/ml) was diluted (1:100) to make a working solution of 100µg/ml. 800µl of working solution was used and diluted to make a top standard concentration of 8µg/ml. A range of standards were prepared by serial dilution (0.125 - 4µg/ml) and kept on ice. In a 96 wel ELISA plate, 20µl of buffer was added to well A (blank), 20µl of each standard to wells B-H in column 1, and 20µl of sample to wells A-H in columns 2 and 3. The following

reagents were prepared as indicated: 2mg of DTNB in 3ml, 2mg of β-NADPH in 3ml and 40µl of glutathione reductase (GR) in 3ml. All reagents were covered in foil. Equal volumes of DNTB and GR were mixed and 120µl of the combined solution added to each well for 30sec to allow for the conversion of GSSG to GSH, followed by addition of 60µl β-NADPH. The plate was then immediately analysed in a microplate reader at 412nm. Measurements were taken every 30sec for 2min and repeated twice more for each sample. The rate of 2-nitro-5-thiobenzoic acid formation (change in absorbance/min) was calculated and the total glutathione concentration in the samples was determined by using linear regression to calculate the values obtained from the standard curve. The total glutathione concentration was expressed as nmoles/mg protein.

4.7 MEASUREMENT OF NUCLEAR FACTOR-κB (NF-κB) DNA BINDING ACTIVITY

NF-κB is a transcription factor that is under the control of oxidative stress. This technique measures the ability of the NF-κB protein to bind to DNA having a specific binding sequence for NF-κB. The DNA binding is resolved by gel electrophoresis. Only a limited number of samples could be analysed on any one day.

4.7.1 Preparation of nuclear protein extracts

A549 cells were incubated for 6h with particles and nuclear protein extracts were prepared according to the procedure of Staal *et al.* (1990). Following exposure, cells were washed twice in CMFPBS, lysed and scraped into 25µl buffer B (10% NP40) and 400µl buffer A (10mM Hepes, 10mM KCl, 2mM $MgCl_2$, 1mM DTT, 0.1mM EDTA, 0.4mM PMSF, 0.2mM NaF, 0.2mM $NaVO_3$, 1µg/ml leupeptin) into tubes and centrifuged at 4°C for 1min at 13,000g. The supernatant was decanted off and the pellet containing nuclei was resuspended in 50µl of buffer C (50mM Hepes, 50mM KCl, 300mM NaCl, 0.1mM EDTA, 1mM DTT, 0.4mM PMSF, 105 glycerol, 0.2mM NaF, 0.2mM $NaVO_3$). Samples were agitated for 20min at 4°C followed by centrifugation at 13,000g for 5min at 4°C. The supernatant was removed and stored at -20°C until analysis.

4.7.2 Protein determination by Bradford assay

The Bio-Rad protein assay, based on the Bradford method (Bradford, 1976) was used to measure nuclear protein concentration. The assay is a dye-binding method utilising the differential colour change of a dye in response to various concentrations of protein. In this study, the commercial Bio-Rad reagent, which works on the principle of the Bradford assay, contains Coomassie Brilliant Blue G-250 dye. This dye binds to protein and forms a complex that is subsequently measured spectrophotometrically at an absorbance of 595 nm as the optimum for the dye.

The reagent contains Coomassie Brilliant Blue G-250, phosphoric acid, and methanol. Coomassie Brilliant Blue G-250 protein-binding dye exists in three forms: cationic, neutral and anionic. Although the anion is not freely present in the dye reagent pH, it is this form that complexes with the protein. Dye binding requires a macromolecular form and interacts with arginine in the protein rather than primary amino groups. The binding behaviour is attributed to Van der Waals forces and hydrophobic interactions. To prepare a working solution, the Bio-Rad reagent (Bio-Rad, Hemel Hempstead, UK) is diluted (1:5) and the protein standard bovine serum albumin (0.2mg/ml stock; 1 – 20 µg/ml) was prepared in a final volume of 1ml in the Bio-Rad working solution. Protein samples (2µl) were added to working solution

(998µl) in test tubes, vortexed briefly and transferred to cuvettes. Standards and samples were read at 595nm in a spectrophotometer, previously blanked with Bio-Rad working solution

4.7.3 Radio-labelling of the NF-κB consensus oligonucleotide

The NF-κB consensus oligonucleotide (Promega, Southampton, UK) containing the binding sequence for the transcription factor NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3', 3'-TCA ACT CCC CTG AAA GGG TCC G-5') was radio-labelled with [γ-^{32}P]-ATP by incubating 2µl of oligonucleotide (1.75pmol/µl) with 1µl of 10X T4 polynucleotide kinase buffer (700mM Tris-HCl, pH 7.6; 100mM $MgCl_2$, 50mM DTT), 1µl [γ-^{32}P]-ATP (3000 Ci/mmol in 10 mCi/ml), 5µl of deionised water and 1µl T4 polynucleotide kinase (5U/µl) at 37°C for 30min. The reaction was terminated by addition of 1µl of 0.5M EDTA and 89µl of TE buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA) was then added to give a 1X stock solution of labelled NF-κB oligonucleotide in a final volume of 100µl.

4.7.4 Analysis of NF-κB DNA binding activity by EMSA

10µg of nuclear protein sample was incubated for 20min at room temperature with 5X binding buffer (20% glycerol, 5mM MgCl2, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl, 50mM Tris-HCl pH7.5, 0.25 mg/ml poly (dI-dC) (Promega, Southampton, UK) and [γ-^{32}P]-labelled NF-κB consensus oligonucleotide. To confirm the specificity of the binding reaction, a cold competitor consisting of 2µl (2.5pmol) of unlabelled NF-κB and a non-competitor of 2µl (2.5pmol) of unlabelled activator protein-1 (AP-1) oligonucleotide were included in two incubations. The excess of NF-κB should compete out the retarded bands of interest whereas the excess of AP-1 should have no effect on the binding reaction. The reaction mixture was loaded onto a 6% non-denaturing polyacrylamide gel prior to electrophoresis at 100 volts. The gel was dried for 1h onto Whatman filter paper using a vacuum drier (Bio-Rad, Hemel Hempstead, UK). NF-κB DNA binding was assessed by autoradiography and quantitative analysis performed using densitometry with the UVP Grab and Gelplate program (UltaViolet Products Limited, Cambridge, UK). For selected experiments, analysis was also performed by phosphimager (Molecular Dynamics, Santa Cruz, USA).

4.8 STATISTICAL ANALYSIS

The data were expressed as mean ± SEM and were analysed for significance using analysis of variance (ANOVA), especially to asses the significance of trends in the data.

5 RESULTS

5.1 LTPSP CYTOTOXICITY IN A549 CELLS

A549 cells were treated with different particle concentrations to determine the cytotoxic concentrations using LDH release into the supernatants after 24h treatment as a marker of cytotoxicity. We were seeking to identify concentrations that would produce little or no LDH release from the A549 cells after treatment. These concentrations would be used for examining the more subtle endpoints in subsequent experiments. At the different particle concentrations tested (15, 31, 62, 125 and 250 μg/ml), the fine particles, carbon black (CB), titanium dioxide (TiO_2), barium sulphate ($BaSO_4$) and ultrafine TiO_2 did not produce any substantial cytotoxicity (<20%) (Figure 5.1).

In the case of ultrafine CB there was a trend towards an increase in cytotoxicity at low concentrations, namely 15, 31 and 62 μg/ml, compared to the higher concentrations. However, the apparent lack of effect at high concentrations was we believe due to absorption of LDH onto the high area of ultrafine CB surface (see Table 4.1). Preliminary experiments examining the effects of mixing ultrafine CB with LDH protein suggested the adsorption of LDH protein onto the surface of the ultrafine carbon black (Appendix 1; Figure 11.1).

DQ12 quartz was tested at particle concentrations of 48, 96, 192 and 384 μg/ml. The 48 μg/ml produced negligible LDH release. LDH release rose almost linearly with dose of DQ12 reaching significant cytotoxicity at 192 and 384 μg/ml, with toxicity being at 27 and 54%, respectively (Figure 5.1).

5.2 THE EFFECTS OF LTPSP ON IL-8 GENE EXPRESSION IN A549 CELLS

We examined the effects of all the particles on IL-8 gene expression in A549 cells after treatment for 6h and 24h. We measured IL-8 mRNA using RT-PCR. The PCR products were electrophoresed on agarose gels and bands for IL-8 and GAPDH (Figure 5.2A) were quantitated by densitometry. The ratio of IL-8/GAPDH was used to determine the level of IL-8 after treatments.

The 6h treatment with ultrafine particles and DQ12 caused a significant increase in IL-8 mRNA expression (Figure 5.2C, D and B, respectively). There was a significant trend for the DQ12 quartz and the LTPSP except $BaSO_4$ and fine CB; the IL-8 mRNA increased with concentration.

Following a 24h treatment, only DQ12 quartz showed a sustained increase in IL-8 mRNA (Figure 5.2E). Thus while LTPSP activated IL-8 transiently (at 6h), DQ12 quartz activation of IL-8 persisted, suggesting a greater reactivity.

5.3 THE EFFECTS OF LTPSP ON IL-8 PROTEIN EXPRESSION IN A549 CELLS

Following treatment of A549 cells with particles, fine and ultrafine particles had minimal effect on IL-8 protein release at 6h. However, significant IL-8 release was observed with DQ12 quartz at this time-point (Figure 5.3A).

Following a 24h treatment, fine particles did not produce a significant increase in IL-8 protein release (Figure 5.3B). Nevertheless, there was an indication of a trend as there appears to be a rise in IL-8 protein at the top concentration. The ultrafine particles induced a significant increase in IL-8 protein release from A549 cells, mirroring their effect on IL-8 mRNA at 6h

(Figure 5.3C). In the case of ultrafine CB, at high concentrations, 125 and 250 μg/ml, the measured amount of released IL-8 decreased. As these concentrations of ultrafine CB were non cytotoxic, cytotoxicity cannot explain the decrease in the amount of IL-8 protein release. (Under cytotoxic conditions, cells lift off the culture dish leaving fewer cells on the dish to produce IL-8.) However, supplementary experiments showed that IL-8 protein may adsorb on to the surface of the ultrafine CB (Appendix 2; Figure 11.2) and suggests that high concentrations of CB suppressed the IL-8 protein signal measurement. DQ12 quartz induced a higher release of IL-8 protein compared to the ultrafine particles, probably due to its extra surface reactivity (Figure 5.3D).

5.4 THE EFFECTS OF LTPSP ON TOTAL GLUTATHIONE (GSH and GSSG) LEVELS IN A549 CELLS

To assess the involvement of oxidative stress in particle-mediated inflammation, we measured the amount of GSH depletion after 4h treatment with the range of particles (Table 4.1). Glutathione, both oxidised (GSSG) and reduced (GSH), was isolated from the cells; GSSG was converted to GSH and total GSH was measured.

There was a significant depletion of GSH with the fine particles at 250μg/ml for $BaSO_4$, 125μg/ml and 250μg/ml for CB and at all concentrations for TiO_2 (Figure 5.4A and B). The ultrafine particles (Figure 5.4A and C), DQ12 quartz (Figure 5.4A and D) and ultrafine metals, Ni (Figure 5.4E) and Co (Figure 5.4F), produced significant decreases in GSH at each of the three particle concentrations tested.

5.5 THE EFFECTS OF LTPSP ON NF-κB ACTIVATION IN A549 CELLS

The present studies attempted to measure the activation of NF-κB in A549 cells exposed to LTPSP and DQ12 quartz by analysis of NF-κB DNA binding activity using a consensus oligonucleotide sequence for NF-κB in an EMSA. There were inherent problems in this assay (see Discussion, Chapter 7) and we were not able to produce reliable data for this end-point.

5.6 THE EFFECT OF LTPSP SURFACE AREA ON IL-8 mRNA, IL-8 PROTEIN RELEASE AND TOTAL GLUTTHIONE LEVELS IN A549 CELLS

The dependence on particle surface area was examined by replotting all of the data obtained from the experiments described above. IL-8 mRNA, IL-8 protein release and GSH levels following particle exposure in A549 cells were plotted against surface area of the different particle types.

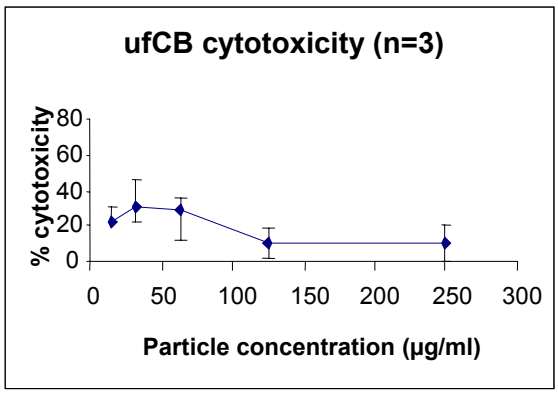
In the case of IL-8 mRNA, there are several clear trends between particle surface area and IL-8 mRNA expression with a clear dose-threshold observed at a dose of about 1 cm^2/cm^2 (particle surface area/area of culture dish [confluent layer of cells in dish]) (Figure 5.5). We express particulate surface area dose as "dose per surface area of culture dish" as the dish is covered with a confluent layer of cells; thus this is the dose per unit area of cells. Above this dose of about 1 cm^2/cm^2, three different lines are observed. The steepest is for DQ12, followed by ultrafine TiO_2, and finally a flatter line relating to ultrafine CB. Interestingly, fine TiO_2 showed an increase in IL-8 mRNA that was not mirrored by IL-8 protein release.

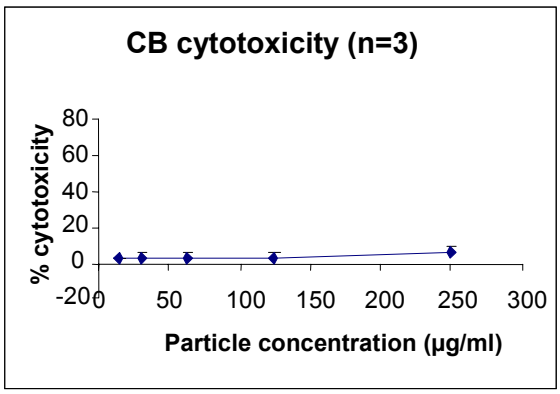
With regard to IL-8 protein release, concentrations of LTPSP below about 10 cm^2/cm^2 (particle surface area/surface area culture dish) did not have any effect on the release of this chemokine from A549 cells. There appears to be a dose-threshold (between about 1 to 10 cm^2/cm^2), because above this dose the release of IL-8 protein increases with surface area of LTPSP (Figure 5.6). The exception to this relationship was ultrafine CB at high

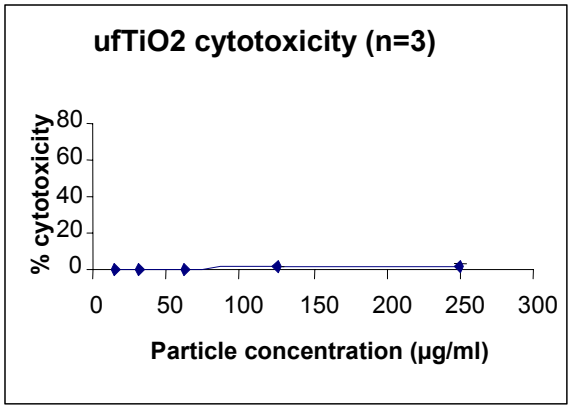
concentrations. DQ12 quartz follows a steeper line than the LTPSP and seems to have a much lower threshold. However, where the mRNA data had four doses for quartz, only the two lower doses were used for IL-8 protein release.

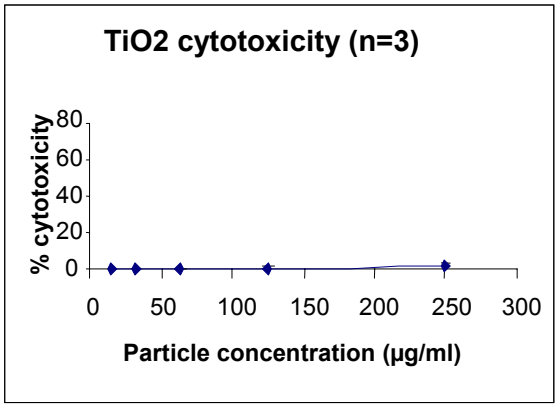
When we normalised the IL-8 protein data by dividing the amount of IL-8 released by the corresponding particulate surface area dose, so that the response is expressed as IL-8 per cm^2 of particle surface per cm^2 of culture dish. All the normalised results were then relative to a normalised dose of 1, as in Figure 5.7. The normalised results clearly distinguished the two categories of particles. The normalised release of IL-8 protein is very low for the LTPSP and they fall into one group. In contrast the DQ12 produced high normalised IL-8 release, approximately 13-fold greater than the LTPSP.

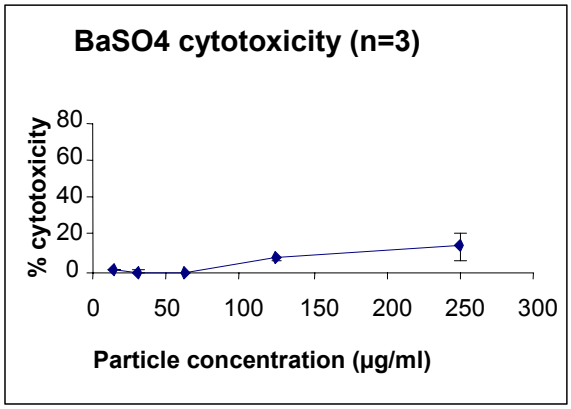
The levels of GSH depletion following a 4h treatment with particles, expressed relative to surface area dose (particle surface area/surface area of culture dish), showed that as the surface area dose increases all the LTPSP and DQ12 deplete intracellular GSH. All dusts showed a significant dose-response trend. There appear to be three groups, DQ12, then the LTPSP and ultrafine metals, and ultrafine CB on its own (Figure 5.8A and B). Figure 5.8 shows plots for geometric and arithmetic means; only for ultrafine carbon black does the choice of mean make a noteworthy difference, and this is due the greater variation between replicate experiments for the ultrafine CB as compared to the other particles.











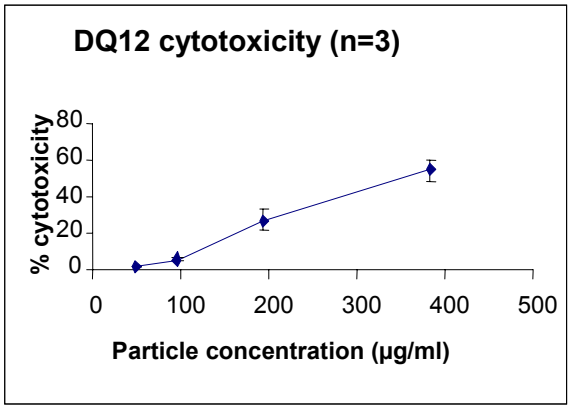


Figure 5.1

Effect of LTPSP and DQ12 quartz on cytotoxicity in A549 cells as measured by release of lactate dehydrogenase (LDH)

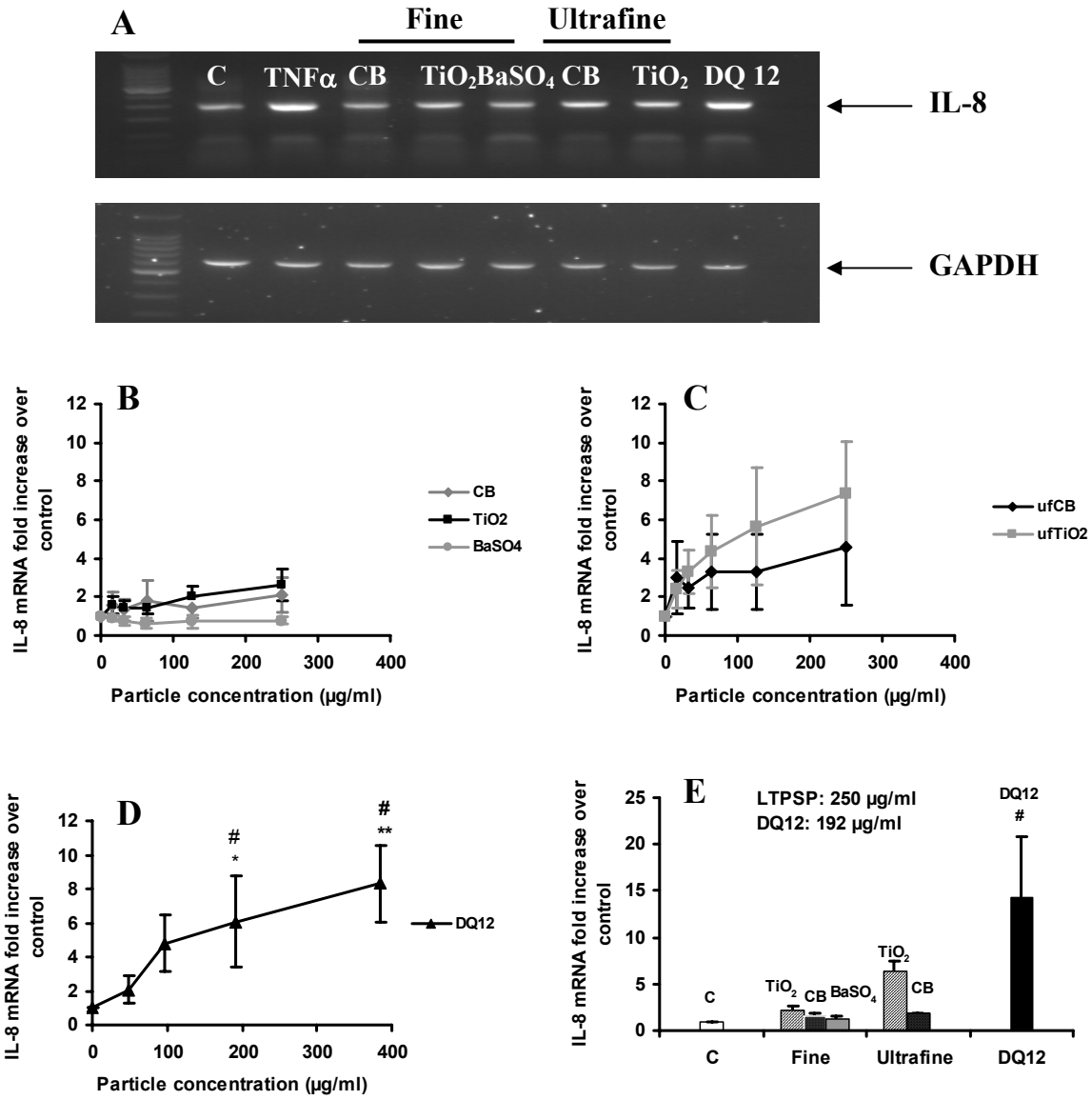


Figure 5.2

RT-PCR analyses of IL-8 mRNA expression in A549 cells.

(A) Agarose gel with IL-8 and GAPDH mRNA bands from a representative experiment. The ratio of the band intensities gives the data in the graphs which show means and standard error of four different experiments.
(B) fine particles (C) ultrafine particles, all at 15, 31, 63, 125 and 250 µg/ml
(D) DQ12 at 48, 96, 192 and 384 µg/ml.
(E) Cells treated for 24h at 250 µg/ml for fine and ultrafine particles, and at 192 µg/ml for DQ12.

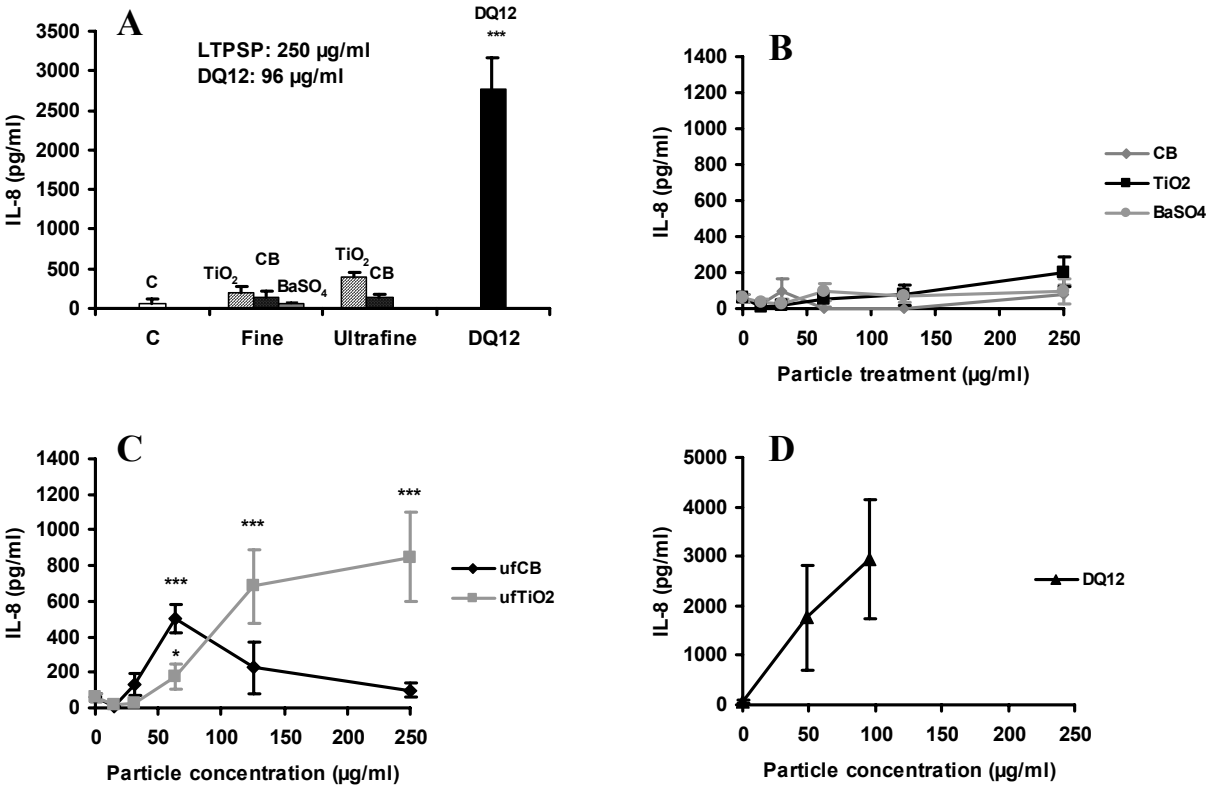


Figure 5.3

IL-8 protein release from A549 cells upon 6h and 24h treatment with fine particles, ultrafine particles and DQ12. Mean and standard errors from four replicate experiments

(A) Cells treated for 6h, at 250 µg/ml for fine (CB, TiO_2 and $BaSO_4$), ultrafine (CB and TiO_2) and at 96 µg/ml for DQ12.

(B and C) Cells treated for 24h with fine particles (B), ultrafine particles (C) at doses of 15, 31, 63, 125 and 250 µg/ml.

(D) Cells treated for 24h with DQ12 at doses of 48, 96, 192 and 384 µg/ml.

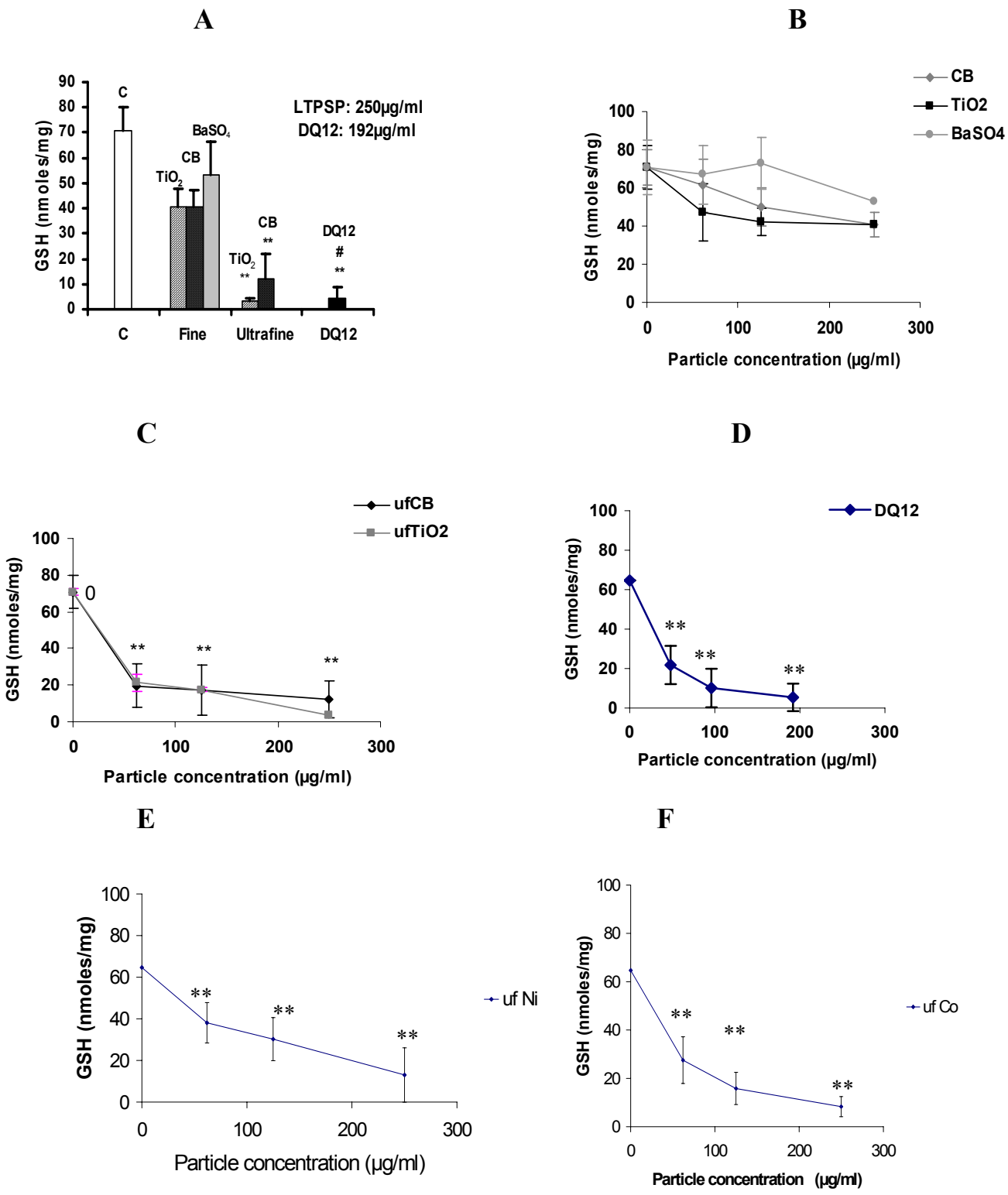


Figure 5.4
Total intracellular glutathione (GSH) in A549 cells following a 4h treatment. (A) fine and ultrafine particles and DQ12. Dose-response for (B) fine, (C) ultrafine particles, (D) DQ12, (E) ultrafine Ni, and (F) ultrafine Co. Means and standard errors of three experiments. # indicates cytotoxicity, ** significant decrease at this dose relative to control.

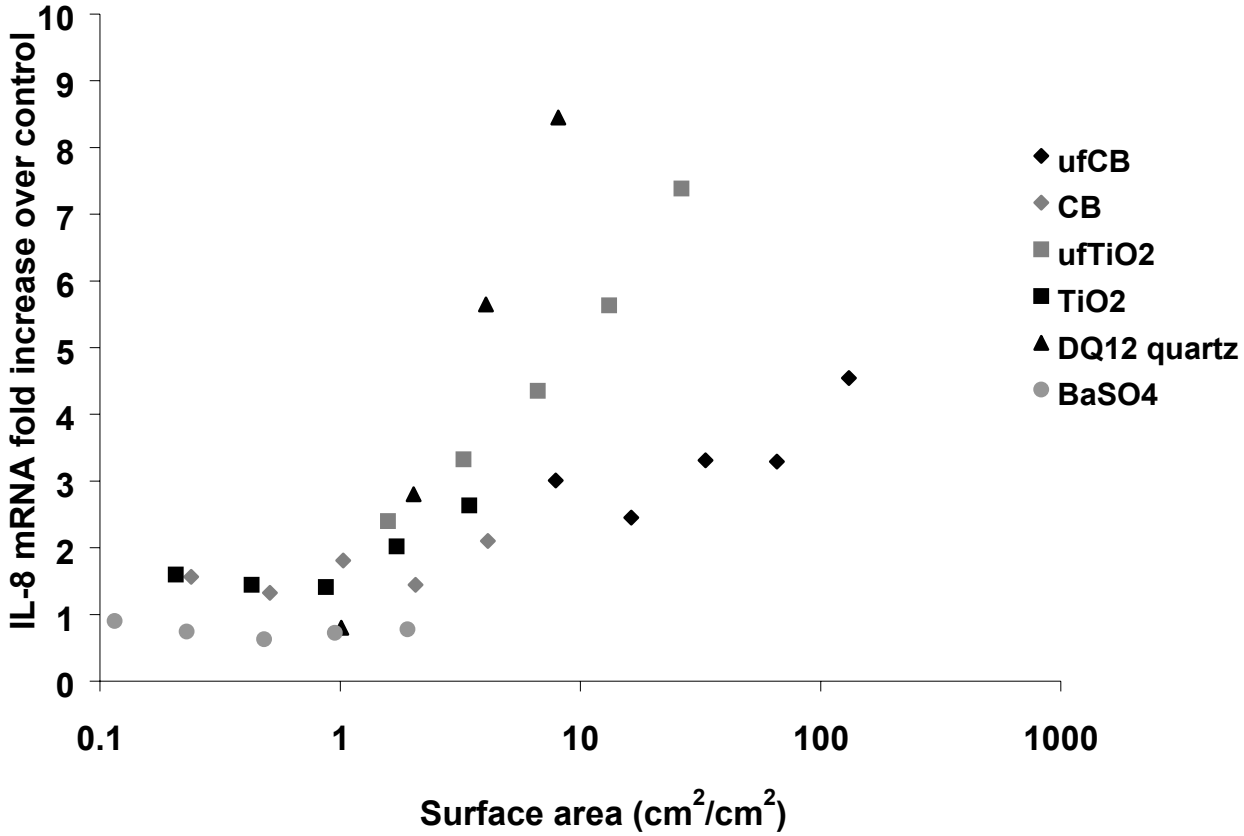


Figure 5.5

Effect of surface area dose on IL-8 mRNA expression after exposure of A549 cells to LTPSP and DQ12 quartz for 6h

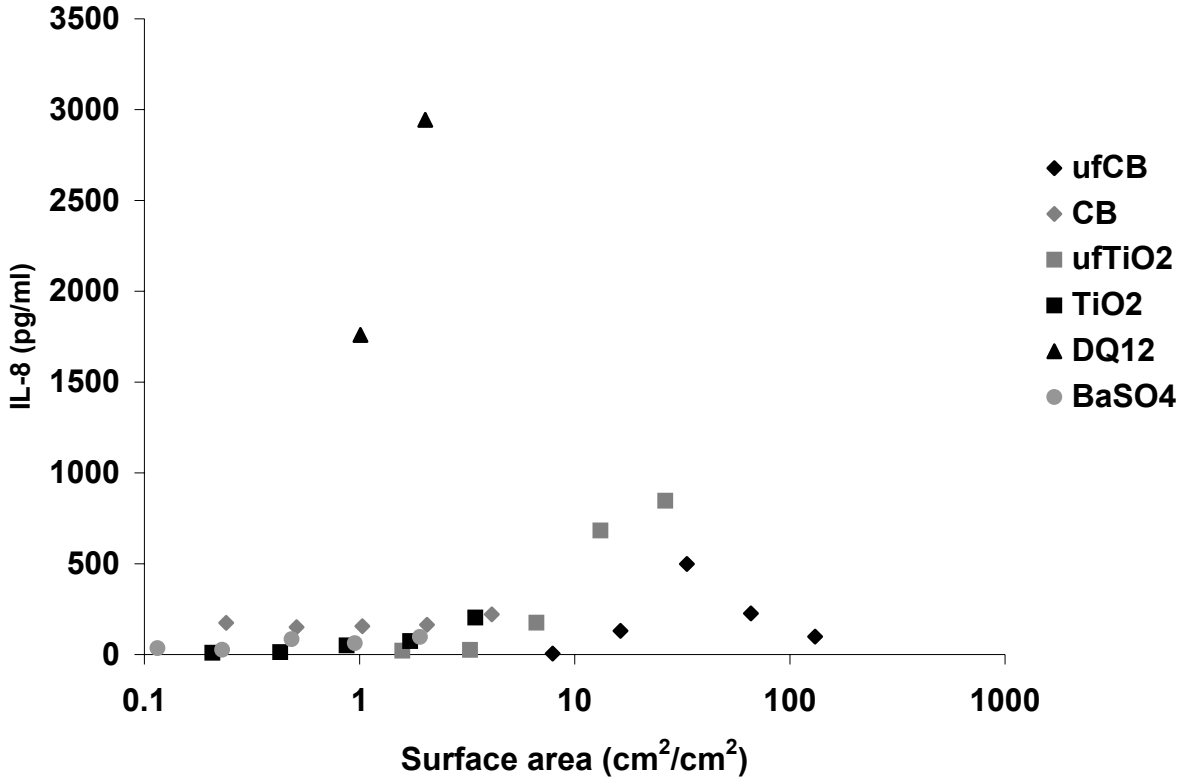


Figure 5.6

Effect of surface area on IL-8 protein release following a 24h exposure of A549 cells to LTPSP and DQ12 quartz

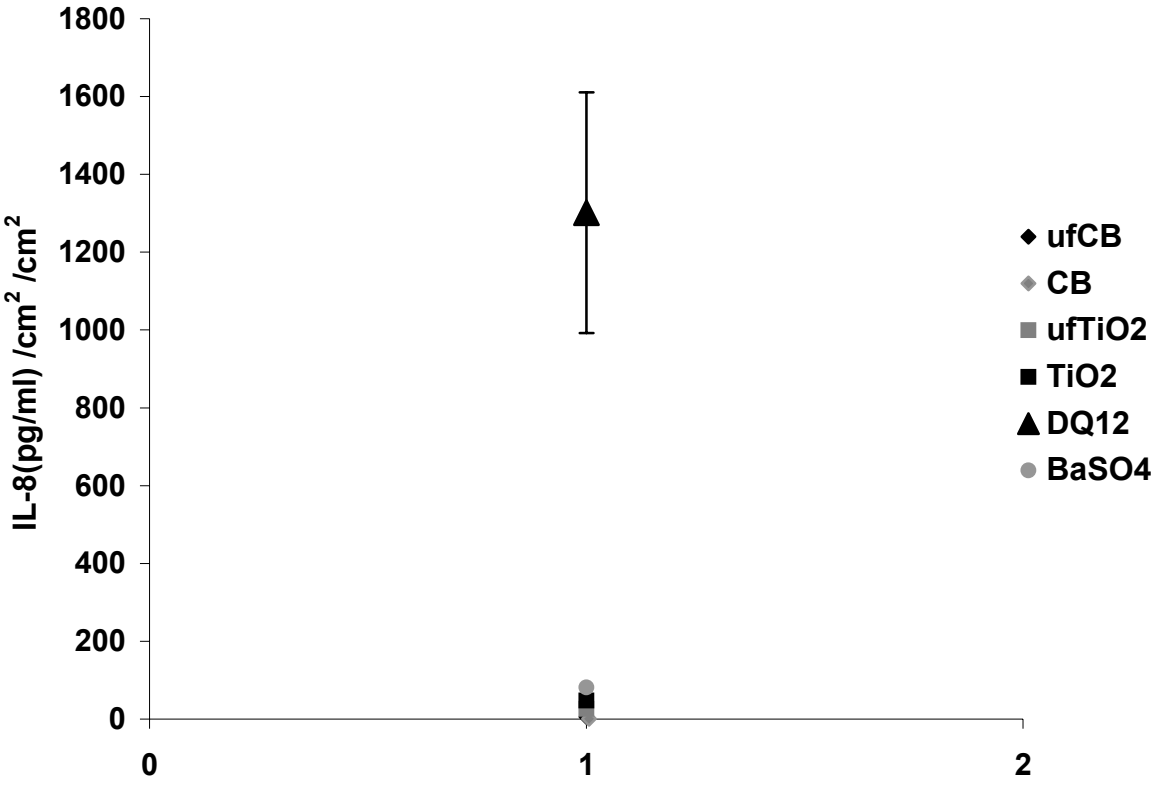


Figure 5.7

Normalised IL-8 protein release in A549 cells exposed for 24h to LTPSP and DQ12 quartz. The normalised response is in pg/ml of IL-8 protein per cm^2 of particulate surface area dose per cm^2 of cell culture dish.

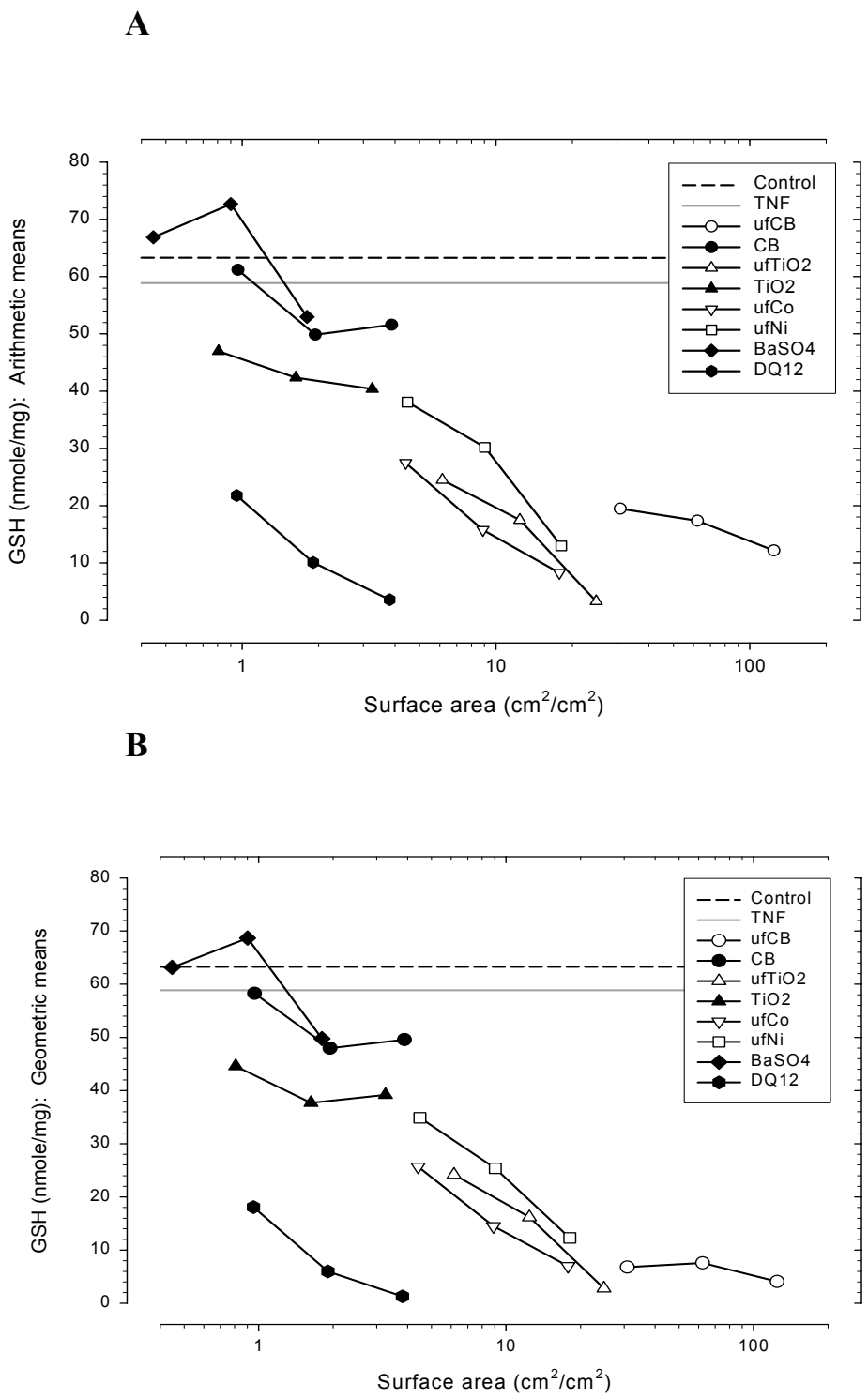


Figure 5.8

Effect of particle surface area on GSH depletion in A549 cells exposed for 4h to LTPSP and DQ12 quartz. The data are expressed as the (A) Arithmetic and (B) Geometric means.

6. RELATING *IN VITRO* AND *IN VIVO* RESULTS

6.1 DEPOSITION OF INHALED PARTICLES

Inhaled particles, depending on their size, may deposit in the upper airways, the tracheo-bronchial region or the alveolar region of the lung. If they deposit in the upper airways or the ciliated airways of the bronchi then clearance of inhaled particles is relatively rapid, with most of the particles being removed within 24 hours. If the particles deposit in the alveolar region, then clearance is much slower, with half times of the order of 65 days for rats and much slower for humans. So the particles that deposit in the alveolar region are the major concern for chronic health effects caused by poorly soluble particles. A few studies have examined the exact sites of deposition in the alveolar region, and have shown that most of these particles tend to be deposited in the centri-acinar region (Brody *et al*, 1981). The centri-acinar region is defined as the terminal bronchiole and the first 400 to 600 microns beyond the bronchiole-alveolar duct junction (or proximal alveolar region - PAR), that is the transitional zone between the airways and the alveoli, the gas exchange regions of the lungs. The cells in this region are mainly type II epithelial cells.

For many purposes, it is sufficient to use the term alveolar deposition without elaborating on the exact location of the deposited particles. For example, alveolar deposition efficiency (i.e. the fraction of the total mass inhaled) is sufficient to calculate the mass dose (lung burden), accumulating in the lung (Tran *et al*, 2000). However, a more precise description of the site of deposition in the alveolar region is needed for a proper understanding of the dose-response relationship for inhaled poorly soluble particles (PSP).

The present study has examined the mechanisms of particulate toxicity in the lungs using an *in vitro* system. Specifically, we have examined the effects that directly impact on human alveolar type II epithelial cells, as represented by A549 cells. Since epithelial cells are the first cells in the respiratory tract to come into contact with inhaled particles, we hypothesise that damage to these cells can serve as a direct and highly sensitive measure of particle toxicity. Epithelial cells lining the central acinus play a key role in the initiation and progression of particle-induced pulmonary injury. We hypothesise that the interactions between particles and epithelial cells initiate a cascade of events that underlie the adverse effects associated with inhaled particles. We also hypothesise that particle toxicity begins with the oxidation of cellular glutathione (GSH & GSSG) in epithelial cells, culminating in inflammation (Rahman and MacNee, 2000). Oxidation of GSH unbalances the redox equilibrium inside the cell, which depends on reduced GSH, pulling it towards oxidation. The oxidised GSH is excreted by the cell, producing GSH depletion. The oxidative stress-responsive transcription factors (NF-κB and AP-1) are then activated, leading to activation of many stree-responsive and pro-inflammatory genes (Rahman and MacNee, 2000) including IL-8, which leads to inflammation and γGCS, which is activated to restore the GSH levels and thereby the redox balance. Thus there is a clear a clear sequence of events leading from GSH depletion to the activation of NF-κB and, subsequently, the transcription of genes for chemokines such as IL-8, that attract neutrophils (PMN) and alveolar macrophages (AM) to the site of particle deposition to produce inflammation.

In vitro results are known to be difficult to relate to their *in vivo* counterparts, because of a lack of understanding of the correspondence between the dosimetries of *in vitro* and *in vivo* systems. There is also the difficulty that some types of response cannot be produced in both *in vivo* and *in vitro* systems; for example, the number of PMNs in the bronchoalveolar lavage (BAL) fluid, which represents the extent of pulmonary inflammation *in vivo*, cannot be replicated *in vitro*.

6.2 LTPSP *IN VIVO* RESULTS

Tran *et al* (2000) have established a dose-response for two PSP with low toxicity, TiO_2 and $BaSO_4$. Figure 6.1 shows the dose-response pattern. The vertical axis represents the level of pulmonary inflammation, as quantified by the number of PMNs in the broncho-alveolar lavage (BAL) fluid; the horizontal axis represents the alveolar dose (lung burden), described in surface area units (cm^2). The figure clearly indicates a threshold dose at approximately 200-300 cm^2. However, for comparison with *in vitro* data, it is more informative to express the dose in terms of surface area dose per unit of epithelial surface area affected.

As explained above, the affected area, where particles are deposited, is the centri-acinar region i.e. the terminal bronchiole region plus the PAR region of the alveolar lung. Table 6.1 summarises the values of the parameters needed to calculate the affected surface area. Each terminal bronchiole was taken as being approximately cylindrical, with length l_{TB} and diameter d_{TB}. The total surface area of N_{TB} terminal bronchioles was then estimated as:

$$TB\ surface\ area = N_{TB} \times \pi \times l_{TB} \times d_{TB}$$

The volume of the PAR region of the rat is approximately 5 percent of the total volume of the lung (Pinkerton, personal communication). The surface area to volume ratio for the rat lung parenchyma is approximately 500 to 600 cm^2/ml (Mercer and Crapo, 1987).

$$PAR\ surface\ area = VOL \times pc \times cf$$

where *VOL* is the parenchymal lung volume of the rat,
pc is the PAR volume as a percentage of the parenchymal lung volume and
cf is the surface area to volume ratio for the rat lung parenchyma.

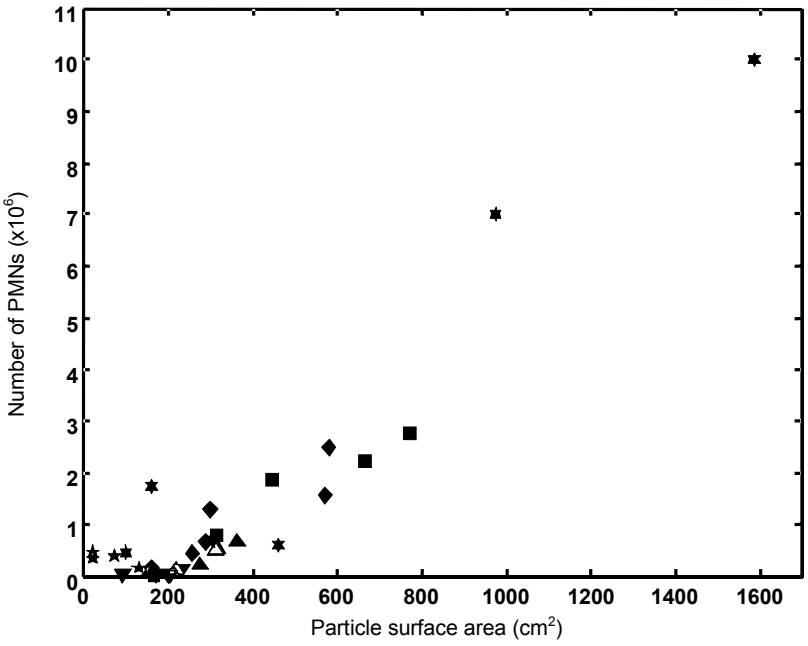


Figure 6.1. The dose-response from *in vivo* data.
Inflammation in the rat lung as a function of lung burden expressed as particle surface area for TiO_2 at two exposure concentrations (□ rectangle and diamond), $BaSO_4$ at two exposure concentration (triangles), and data from Oberdörster for TiO_2 (fine and ultrafine, stars).

From the values in Table 6.1, the centri-acinar surface area, defined as the terminal bronchiole surface area plus PAR area, is calculated as 300 cm^2 approximately.

Parameters	Value	Units	Reference
N_a	2500		Pinkerton *et al* 1997
l_a	450 (range 400 to 500)	μm	
d_a	180	μm	
VOL	10.9	*ml*	Gehr *et al.* (1981)
pc	5	percent	Pinkerton, personal communication
cf	550 (500-600)	cm^2/ml	Mercer and Crapo (1987)

Table 6.1. The parameter values used to estimate the centri-acinar surface area. For l_a and *cf* we use the mid-point value; the range of values is given in brackets.

6.3 RELATING *IN VIVO* AND *IN VITRO* RESULTS

6.3.1 Relating the dose

Figure 6.2 shows the *in vivo* data from Figure 6.1 but with the horizontal axis values expressed as particulate surface area dose divided by the calculated centri-acinar surface area, i.e. dose as cm^2/cm^2. In Chapter 5, we showed the *in vitro* data for a range of LTPSP; now, for directness of comparability, Figure 6.3 shows the *in vitro* results for IL-8 mRNA with the same particles (TiO_2, fine and ultrafine, and $BaSO_4$). In the *in vitro* data, a threshold dose can be identified at around 1 cm^2/cm^2.

With a normalised unit implemented for the *in vivo* data, the dose ranges in the two datasets (Figures 6.2 and 6.3) are very similar. Most importantly, the threshold dose identified in each dataset appears to be approximately the same dose of particulate surface area per unit surface area of epithelial cells (1 cm^2/ cm^2). In both cases the particle dose is expressed relative to surface area of the cells exposed; in the former case for cells in a layer in the lung, in the latter case for the epithelial cell line in a test cell

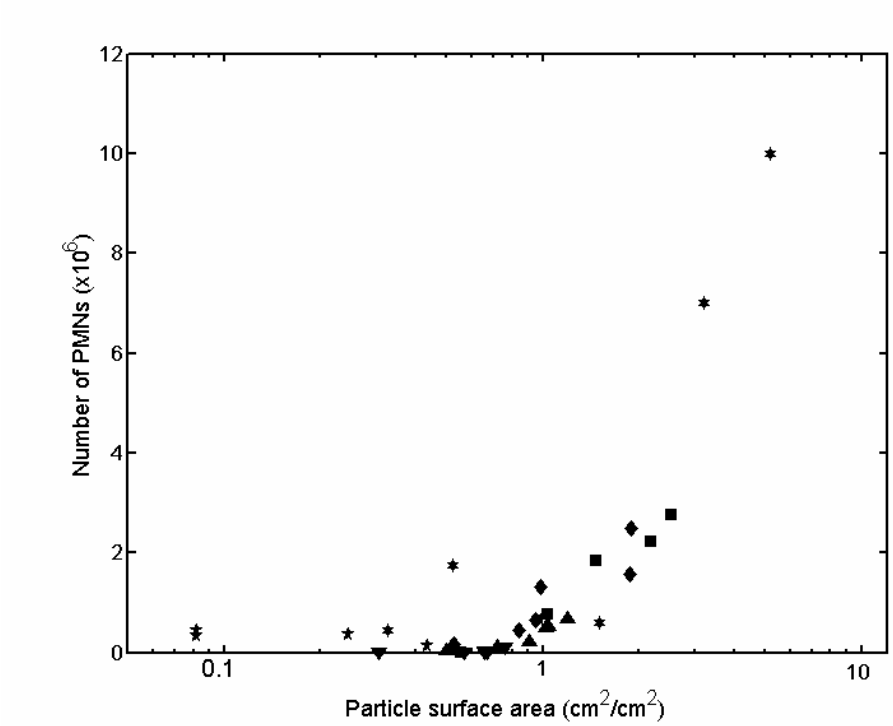


Figure 6.2. The dose-response *in vivo* using normalised dose (cm^2/cm^2).

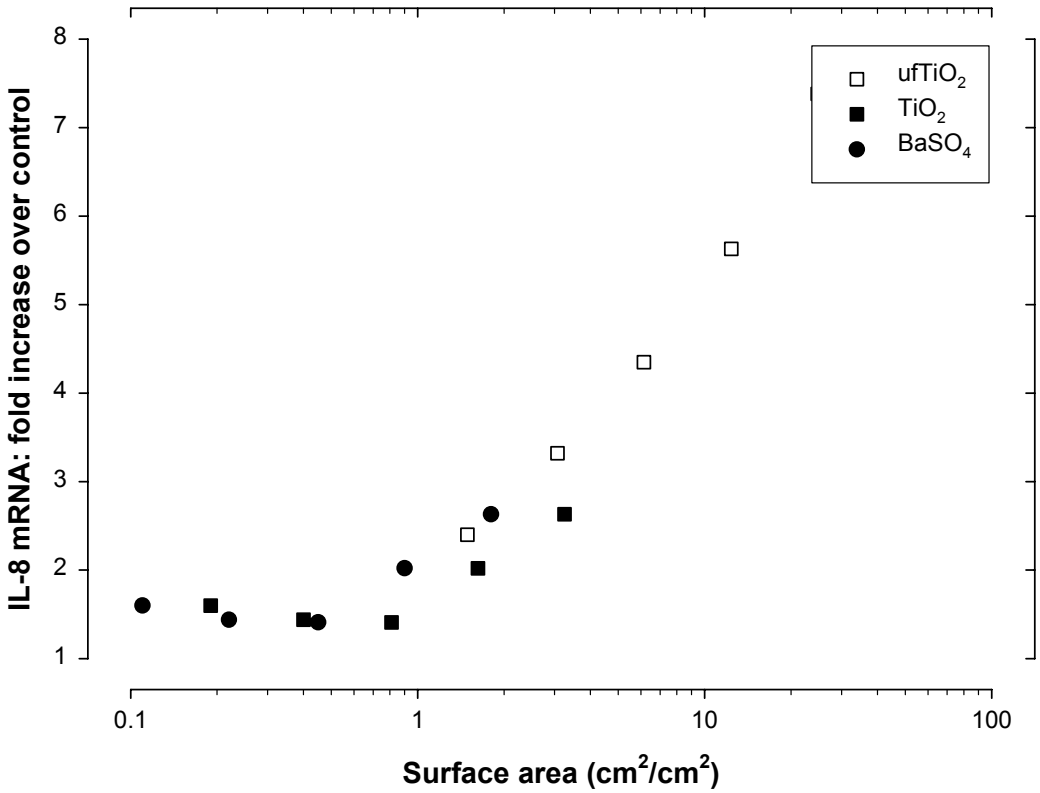


Figure 6.3. The dose-response *in vitro* using normalised dose (cm^2/cm^2).

6.3.2. Relating the response

In this comparison of *in vivo* and *in vitro* dose and response, the IL-8 mRNA observed *in vitro* has been compared to the number of PMNs measured in the BAL fluid. While IL-8 is one of the pro-inflammatory chemokines responsible for the recruitment of PMN to the site of the lesions, the actual relationship between the two parameters within the same system remains to be investigated. Since the comparison is based on two related but different response parameters, the slopes of the dose-response relationship *in vivo* (Figure 6.2) and *in vitro* (Figure 6.3) are not expected to be the same. This study has demonstrated an approach to relating the doses in the two experimental systems, and opens the ways to using one system (e.g. *in vitro* tests) to predict the outcome from the other. However, the comparisons are based on the two different parameters, with one measured in inhalation experiments lasting several weeks whereas the other was measured in *in vitro* tests where cells were exposed to particles for 6 or 24 hours. Therefore, to substantiate the comparability and relationship between the responses in these systems, one would need the same response to be measured in both systems (e.g. IL-8, or the rat equivalent MIP2). Currently, such data are not available. In the future, when such measurements become available, a complete, quantitative mapping of the dose-response between experimental systems can be completed.

7. DISCUSSION

7.1 BACKGROUND

There is increasing interest and debate about the basis for regulating "nuisance dust" or "particulate not otherwise regulated" in the occupational environment. These particles have three main characteristics. Firstly they are of respirable size, secondly they are poorly soluble and thirdly they have very little intrinsic toxicity associated with their surface. Accordingly, they have been grouped together as low toxicity poorly soluble particles (LTPSP) and are controlled in occupational settings in terms of their mass concentrations in the air. Occupational exposure standards (OES) have been set for these LTPSP as 8h time-weighted average concentrations of 10 mg/m^3 and 4 mg/m^3 for the inhalable and respirable dust fractions, respectively (HSE, 1997). Similarly in the US, the limits are 10 mg/m^3 and 5mg/m^3 for inhalable and respirable fractions, respectively.

Experimental data have emerged over the past 10 years to reveal that volumetric overload is not the complete explanation for the impairment of clearance of these low toxicity particles. Exposure of rats to ultrafine particles (with large surface area per unit mass) showed features of overload at a lower mass and volume lung burden compared to larger fine particles with the same mineral and chemical properties (Oberdorster *et al.,* 1994).

Recent studies have emphasised the role of surface area in the initiation of overload. Tran *et al.* (2000) compared the inflammatory response of two LTPSP types with different particle sizes. At equal lung mass burden, the smaller particles produced more inflammation but the two LTPSP types were equally inflammogenic when the lung burden was expressed as surface area (Tran *et al.,* 2000). These findings imply an important change in our understanding of the LTPSP driven inflammation and suggest that surface area dose rather than mass or volumetric overload may be a better monitor in assessing the increased toxicity of ultrafine particles over their fine counterparts (Donaldson *et al.,* 2000). This has a direct relevance to workplace exposures since the current regulations are set on the basis of mass concentrations (mass per unit volume of air (mg/m^3)) for any type of particle and any size.

The initiation of inflammation *in vivo* is characterised by the generation of ROS causing an oxidant/antioxidant imbalance, leading to oxidative stress and causing the activation of transcription factors, such as NF-κB. Transcription factors, such as NF-κB control the expression of many pro-inflammatory genes (Rahman and MacNee, 1998). Among these NF-κB-controlled genes is IL-8, a potent chemoattractant and activator of neutrophils, an event that is crucial in the initiation of the inflammatory response (Kelley, 1990; Kunkel *et al.,* 1991; Kunch and Rosen, 1993). The recruitment of neutrophils cannot be modelled *in vitro*, but it is possible to measure IL-8 gene expression and subsequent release of IL-8 protein, an event crucial for neutrophil recruitment *in vivo.*

7.2 HYPOTHESIS

We hypothesised that surface area of LTPSP drives the ability of these particles to cause oxidative stress in A549 cells and subsequent induction of the oxidative stress-sensitive transcription factor NF-κB, and thereby induce IL-8 protein release. In addition, we expected that DQ12 quartz would be more active per unit surface area because of its greater surface reactivity.

7.3 AIMS

The purpose of the present study was to investigate the role of particle size and surface reactivity in initiating pro-inflammatory effects in a human alveolar type II epithelial cell line, A549, *in vitro*. This *in vitro* model was used to determine if particle surface area was a better descriptor of the ability of LTPSP to stimulate pro-inflammatory end-points rather than mass. In addition, the development of this approach provides a means of screening particles *in vitro* and hence a cost effective and ethical way of testing new particles.

7.4 MEASURES OF CELLULAR TOXICITY, OXIDATIVE STESS AND PRO-INFLAMMATORY EFFECTS *IN VITRO*

7.4.1 Cellular toxicity by measurement of LDH

To select particle concentrations that were non-toxic for the assays to assess pro-inflammatory markers and oxidative stress. we first looked at LDH leakage from the A549 cells following treatment with a range of particle concentrations. Measurement of LDH leakage from cells is a reliable way of assessing cellular toxicity *in vitro.* DQ12 at the highest concentrations used (192 and 384 μg/ml) produced gross toxicity, whereas the LTPSP were non-toxic for all tested concentrations (up to 250 μg/ml). This is in accord with previous findings with DQ12 (Duffin *et al.,* unpublished observations) and ultrafine particles (Stone *et al.,* 2000).

7.4.2 Depletion of GSH as a marker of oxidative stress

We tested the battery of particles shown in Table 4.1 for their ability to deplete intracellular GSH levels following a 4h exposure. GSH is a reliable measure of oxidative stress in A549 cells exposed to particulates (Stone *et al.,* 2000).

Recent studies from a number of laboratories have demonstrated a role for ROS in promoting the cellular effects of ultrafine particles by the induction of oxidative stress (Stone *et al.,* 2000). The plasmid DNA scission assay showed that ultrafine CB has greater free radical activity than the same mass of fine CB (Stone *et al.,* 2000). The same was true for TiO_2 (Gilmour *et al.,* 1997). In cases of oxidative stress, the most abundant antioxidant in the lung GSH is depleted and a rapid induction of intracellular GSH synthesis occurs (Shi *et al.,* 1994; Rahman *et al.,* 1999). Previous studies have shown that ultrafine CB was more potent than its fine counterpart in depleting GSH levels in A549 cells (Stone *et al.,* 1998, 2000). The present results support the hypothesis that the production of ROS and the subsequent oxidative stress exerted on A549 cells is a mechanism whereby ultrafine particles may exert their effects. Furthermore, these results are in accord with those previous observations of Stone *et al.* However, the present studies have used a larger panel of particles.

When GSH levels were plotted as a function of surface area dose, then the LTPSP data fell within a single broad band of results with the GSH levels being inversely proportional to the log of the surface area dose.

7.4.3 Assessment of the oxidative stress sensitive transcription factor NF-κB

NF-κB is a transcription factor that is activated under conditions of oxidative stress and it is involved in the expression of pro-inflammatory and proliferative genes such as IL-8 (Rahman and MacNee, 1998). To assess the activation of NF-κB we have used EMSA. This is an assay with several stages involving incubating nuclear extracts with a radio-labelled oligonucleotide prior to polyacrylamide gel electrophoresis. Unfortunately we did not get any increases in

NF-κB DNA binding in our studies and this may have been due to the annealing of the double-stranded oligonucleotide or the binding conditions used in the assay. Our negative results with ultrafine CB and DQ12 suggest problems with the assay as increases in NF-κB had been observed with these particulates in previous investigations (Schins *et al.,* 2000). However, from previous knowledge of the relationship between NF-κB and IL-8 we can assume that there was activation of this transcription factor prior to IL-8 gene expression (Kunsch and Rosen, 1993).

7.4.4 Induction of IL-8 mRNA and IL-8 protein release as pro-inflammatory markers *in vitro*

Gene expression occurs in two steps. Firstly transcription of the information encoded in DNA into mRNA and then secondly, translation of the information encoded in the nucleotides of mRNA into a defined sequence of amino acids in a protein. The processes of transcription and translation are separated both spatially and in time. Transcription of mRNA occurs in the nucleus and translation of mRNA into proteins occurs in the cytoplasm.

To assess the pro-inflammatory effects of DQ12 and LTPSP in A549 cells we measured IL-8 mRNA by RTPCR and IL-8 protein release by ELISA at 6h and 24h. These times were chosen in the light of GSH depletion having been observed at 4h, because the oxidative stress precedes the changes in IL-8 mRNA and IL-8 protein release.

We demonstrated that IL-8 mRNA levels in A549 cells treated with LTPSP had risen after 6h and that only at surface area dose greater than $1cm^2/cm^2$ (surface area particles/surface area dish) was IL-8 mRNA levels dose-dependently increased with LTPSP. These levels of IL-8 mRNA expression then fell to background levels at 24h. In comparison, DQ12 quartz increased IL-8 mRNA at both 6h and 24h at low surface area dose. In line with these findings, several studies have reported persistent upregulation of NF-κB-controlled genes in response to mineral particles (Janssen *et al.,* 1995; Simenova and Luster, 1996; Driscoll *et al.,* 1997).

After a 6h treatment, there was a significant increase in IL-8 protein release from A549 cells with DQ12 quartz and tumour necrosis factor-α (TNF-α), but not with ultrafine or fine particles. However, after 24h, ultrafine particles elicited a modest dose-dependent increase in IL-8 protein release.

There were problems in the measurement of IL-8 protein in the case of ultrafine CB. In the present studies, A549 cells showed an apparent decrease in IL-8 protein production at high concentrations that were non-cytotoxic and which did not mirror the increase in mRNA levels of IL-8 at these concentrations. Other studies have encountered a problem of the 'disappearance' of protein in the supernatants in cells treated with ultrafine CB (Stone *et al.,* 2000) and with ultrafine polystyrene beads (Brown *et al.,* 2001). We have interpreted the effects as an *in vitro* artefact due to the large surface area of the ultrafine particles. Some supplementary experiments have confirmed that IL-8 protein is adsorbed to ultrafine CB that may explain the decrease at higher concentrations of this particle.

The IL-8 protein assay proved problematic at some dose levels for several of the particles, with relatively large variation between replicate experiments performed on the same day. Therefore we would recommend that the other three assays (LDH, GSH, IL-8 mRNA) are the more reliable for testing new dusts.

In the present studies, it is interesting to note that IL-8 mRNA levels present in cells exposed to LTPSP and DQ12 were similar relative to mass dose (Figure 5.2) and this may be explained by a difference in speed of reactivity. The DQ12 may have produced an earlier induction of IL-8 mRNA. This earlier induction has been observed with other end-points using DQ12 (Schins *et al.,* 2000). This explanation is also supported by the release of IL-8 protein at 6h by DQ12 and not by LTPSP; the latter only produced this chemokine at 24h. In addition, at 24h DQ12 produced much more IL-8 protein than the LTPSP. The effect of the DQ12 was not only more rapid but it was a persistent effect. (This difference could be investigated further by performing time-course experiments with DQ12 in A549 cells to determine how rapidly the DQ12 switches on IL-8 mRNA expression and protein release.)

In the present studies we have shown oxidative stress in the form of GSH depletion and IL-8 gene induction. Oxidative stress has been implicated in the induction of IL-8 (Deforge *et al.,* 1993). Recent studies showed that the antioxidant nacystelyn, a thiol donor to cells, inhibited IL-8 gene expression following treatment with the model oxidant H_2O_2 (Antonicelli *et al.,* 2002). (Further studies with antioxidants could help to further substantiate the role of oxidative stress in the induction of IL-8 mRNA and protein release.)

Following elaboration of our hypothesis given in Chapter 2, we decided to concentrate on the effects of the LTPSP in alveolar epithelial cells. We believe that it is the epithelial cell that is responsible for the failed clearance of the particles by macrophages. In the light of this, and after reviewing previous results from within our laboratory, the project team made a decision in the present studies not to perform experiments to look at the effect of LTPSP on macrophage chemotaxis. The previous studies by Donaldson *et al.* (1988) had shown that recruited neutrophils release substances that inhibit macrophage movement. Following inhalation of quartz or TiO_2, there was a slowing of the movement of macrophages lavaged from the lungs.

7.5 SURFACE AREA AS A UNIFYING FACTOR DRIVING THE PRO-INFLAMMATORY EFFECTS

In order to determine if the surface area of particles is a unifying factor driving the inflammatory response *in vitro*, we re-plotted the depletion of GSH, IL-8 mRNA induction and IL-8 protein release with dose expressed as surface area.

7.5.1 GSH depletion and effect of surface area

In the present studies, we have demonstrated that the surface area of the particles is the important factor for the oxidative stress in the form of the depletion of intracellular GSH in A549 cells and that this drives the pro-inflammatory effects of these particles. We have shown that the dusts in the present studies fall into three groups. The line with DQ12 is the steepest, probably due to the reactive surface of DQ12. Then the next line is all the LTPSP, with the exception of ultrafine CB that produced less response relative to its surface area. These results emphasise the importance of surface reactivity in the ability of any particle to induce inflammation (Donaldson *et al.,* 2000). We have reported extensively on the increased oxidative activity (relative to mass dose) of ultrafine particle surfaces in cell free systems (Brown *et al.,* 2001), in cells *in vitro* (Stone *et al.,* 2000) and *in vivo,* where antioxidants ameliorated the inflammatory effects of these particles (Donaldson *et al.,* 2000). In this respect, ultrafines with their greater surface area available for free radical chemistry would favour surface reactions and production of ROS or other free radical species, such as reactive nitrogen species.

It was interesting to note that in our present studies investigating GSH depletion with the ultrafine metals, Ni and Co, we have shown that they behave like the LTPSP. In previous

studies *in vivo* by Duffin *et al.* (2002), the ultrafine metals were more reactive in producing inflammation, in the form of neutrophil influx, than the LTPSP. In particular, the ultrafine Co lay in between DQ12 and the LTPSP, with the ultrafine Ni slightly more reactive than the LTPSP. The results of the present studies were unexpected and we have concluded that the ultrafine metals may have been chemically modified in some way (Cotton and Wilkinson, 1972).

Ni and CO are well-documented lung sensitisers. This study did not attempt to address the sensitising effects of Ni and Co but used these agents as exemplar surface- reactive ultrafines hypothesised to have greater pro-inflammatory effects than LTPSP.Respiratory sensitisation is an inappropriate and excessive immune response, and probably involves passage of the sensitiser through the epithelium to produce a hapten-driven immune response in the lung tissue. Therefore the inflammatory and epithelial-injuring effects shown here might well have adjuvant effects and increase trans-epithelial passage of the metal and so contribute to, or exacerbate, the process of respiratory sensitisation.

7.5.2 Effect of surface area on IL-8 mRNA induction and IL-8 protein release

Expressing the data in terms of surface area demonstrated that there was a clear threshold concentration for IL-8 mRNA expression at around 1 cm^2/cm^2 and for IL-8 protein release between 1 and 10 cm^2/cm^2 *in vitro*. It is interesting to note that above these concentrations all of the LTPSP show approximately linear increase with dose. For IL-8 mRNA there appears to be three lines, the steepest being for DQ12 followed by ultrafine TiO_2 and then ultrafine CB. In the case of IL-8 protein release, the line for DQ12 is much steeper than for LTPSP and the prolonged release (at 24h) of IL-8 protein was only found with quartz. This may be a determining factor in its chronic inflammatory effects (Duffin *et al.,* 2001).

All the assays measured in these studies showed a clear distinction between the LTPSP and DQ12 confirming previous observations with ultrafine CB and DQ12 (Stone *et al.,* 1998; Schins *et al.,* 2000). However, in the present studies we used a larger panel of LTPSP. Expressing the amount of IL-8 protein released per cm^2 of particle surface clearly distinguished the two categories of particles, with the LTPSP showing a low value for this index and DQ12 quartz produced a much greater effect for the same unit of surface area. This finding from the present studies confirms the extra factor that surface chemistry plays in the ability of certain particles to elicit inflammation.

DQ12 quartz has been well documented to cause much more inflammation than LTPSP at similar mass lung burdens confirming its greater reactivity per unit particle surface area (Donaldson *et al.,* 1988). Coating the surface with aluminium lactate effectively converts DQ12 to a LTPSP (Duffin *et al.,* 2001).

In summary, the effects of ultrafine particles relate to the large surface area dose that they deliver to epithelial cells. This surface area dependence supports results from studies reported for neutrophil recruitment *in vivo* after inhalation (Tran *et al.,* 2000) and instillation (Duffin *et al.,* 2002). The present study has also shown a pro-inflammatory effect of a low surface area of a particle with high surface reactivity, such as DQ12 quartz.

7.6 CORRELATION OF PRESENT *IN VITRO* STUDIES WITH EXISTING *IN VIVO* INFORMATION

7.6.1 Consistency with other studies

The present *in vitro* findings are strikingly in accord with previous *in vivo* instillation studies from our laboratory (Duffin *et al.,* 2002). In those studies, particle surface area was shown to be the driving factor in the inflammatory response as measured by neutrophil influx and release of macrophage inflammatory protein-2 (MIP-2), the rat equivalent of human IL-8, into bronchoalveolar lavage (BAL) fluid of rats exposed to the LTPSP used in our *in vitro* studies (Duffin *et al.,* 2002). Recent studies by Brown *et al.* (2001) have demonstrated a similar surface area-dose response relationship when instilling polystyrene beads of different particle sizes.

Previous studies have emphasised the role of surface area in the *in vitro* and *in vivo* harmful effects of particles of various types, including CB (Murphy *et al.,* 1999), manganese dioxide (Lison *et al.,* 1997), beryllium metal (Finch *et al.,* 1991) and TiO_2 (Hohr *et al.,* 2002). We contend that the present study is the first to describe the role of surface area in the pro-inflammatory effects of particles across a range of different types of LTPSP *in vitro.*

7.6.2 Comparability of *in vitro* and *in vivo* dose response

In the present studies we have compared the *in vivo* and *in vitro* data with respect to PMN and IL-8 mRNA measurements, respectively for three LTPSP namely ultrafine TiO_2, fine TiO_2 and $BaSO_4$. There is a difficulty in making these comparisons as the inhalation experiments last several weeks whereas the *in vitro* experiments are over 6h exposure. Nevertheless, the present study has demonstrated a feasible approach for relating these two parameters. The dose-response curves are remarkably similar in slope. Furthermore, in each case there is a dose-threshold at about 1 cm^2/cm^2 (particulate surface area per unit surface area of epithelial cells). This comparison uses particle dose expressed as dose per unit surface area of cells exposed, where *in vivo* that is relative to epithelial cells in the lung and *in vitro* epithelial cells in a culture dish.

To substantiate the comparability in the responses in the two systems, further measurements are required. The comparability should be supported by the same measurement in the two systems, e.g. IL-8 *in vitro* and the rat equivalent MIP-2 *in vivo*. Currently such data on MIP-2 are not available for the LTPSP. Nevertheless, the similarity between the data sets on PMN levels and IL-8 *in vivo* and *in vitro* respectively, is so clear that we would expect a positive result from further comparison. When the MIP-2 data become available in the future, we will be able to make a direct comparison of the dose-response relationship between the two experimental systems. The approach we have used demonstrating the similarity between the two systems opens the way for using just one system to test particles in the future, namely the *in vitro* system.

7.7 CONCLUDING REMARKS

These findings on the role of surface area of LTPSP in driving the inflammatory response are of special importance with regard to the regulation of workplace exposures, which are currently based on airborne mass concentrations. Since the inflammatory potential is driven by surface area rather than mass, then a given airborne respirable dust mass concentration of a material in the form of very small particles would be much more inflammogenic than the same airborne mass concentration of the same material in the form of larger particles. The data produced for the ultrafine TiO_2 could help in the setting of an occupational exposure limit (OEL) for ultrafine LTPSP.

In the LDH and IL-8 protein assays we had interference problems with the ultrafine CB, but not with the ultrafine TiO_2. Supplementary experiments showed that this appeared to be due to CB, at high concentration, binding to LDH and IL-8 protein. The fractal structure of the ultrafine CB with very high surface area per unit mass (Table 4.1) is one potential explanation of its binding of LDH and IL-8 protein to cause interference. The possibility that some aspect of a particle's characteristics might interfere with an assay will always be a consideration in testing other particles, so we recommend that this risk be minimised by using a range of tests rather than rely on a single type of assay.

The development of an approach to comparing dose response relationship that produced such good similarity between *in vitro* and *in vivo* results has great potential for enabling *in vitro* assays of inhalation hazards.

The present studies have shown that LTPSP surface area is a better dose metric than mass for relating dose to IL-8 mRNA induction, IL-8 protein release and GSH depletion in A549 cells *in vitro* for a range of different particle types. These signals, observed *in vitro*, would when produced *in vivo* stimulate inflammation. Thus this *in vitro* cellular approach has confirmed our hypothesis that surface area of LTPSP is a better descriptor of the ability to stimulate inflammation than mass.

The surface area was measured by the BET gas adsorption method, using nitrogen. For particles with complex surface, the technique of measurement will influence the value obtained for surface area. For example, with a porous particle (such as carbon black), the surface area measured by gas adsorption may include some internal pore surface, and the gas molecule size may affect the penetration of gas molecules into the pores. Measurement by adsorption of other gas or fluid molecules might be more appropriate for obtaining the surface area that would be available to biological molecules (proteins etc) in the lung milieu. Nevertheless, the present surface area measurements produced some approximately common thresholds, both *in vitro* and *in vivo*, and helped to collapse the data for dose response relationships.

DQ12 quartz with a highly reactive surface has a much greater activity per unit surface area when compared to LTPSP. However, the concomitant depletion of GSH also suggests the involvement of oxidative stress in the toxicity of high surface area, low surface reactivity LTPSP and low surface area, high surface reactivity DQ12 quartz. These results would suggest similar mechanisms for DQ12 and LTPSP in that the same effects are observed. However, the DQ12 clearly produces a more rapid and more persistent response consistent with its known greater surface reactivity.

We believe that this *in vitro* approach could form the basis of a screening test that could be used with particles of unknown toxicity. We would recommend that the testing scheme would incorporate the LDH assay to assess gross toxicity, measurement of GSH depletion to assess evidence of oxidative stress and measurement of IL-8 mRNA to determine pro-inflammatory effects in this *in vitro* model. The approach developed in the present studies could be used to discriminate particles that can be accepted as LTPSP from those with greater toxicity due to an element of surface reactivity. An *in vitro* predictive assay such as this represents a less expensive, more ethical approach to testing that might replace or reduce testing in animals.

8 CONCLUSIONS AND RECOMMENDATIONS

There are five main conclusions that can be drawn from the present studies:

1. For a given dust type, we have observed differences in effect related to particle size with finer particles (with greater specific surface area) having greater biological effects.

2. For LTPSP, surface area is a better predictor than mass or particle number of pro-inflammatory effects in A549 cells *in vitro.* The observed effects include changes in IL-8 mRNA, IL-8 protein release and GSH depletion. Therefore, from these findings, we would predict that exposure to an airborne respirable dust mass concentration of very small particles will cause more inflammation than the same mass concentration of larger particles of the same chemical composition.

3. DQ12 quartz with a low surface area has greater reactivity per unit surface area than the LTPSP with a much larger surface area.

4. The paradigm developed here represents a cost-effective *in vitro* system that can be used to test new particles less expensively, much faster and more ethically than in animal experiments. We would recommend that the *in vitro* testing scheme would incorporate the LDH assay to assess gross toxicity, measurement of GSH depletion to assess oxidative stress, and measurement of IL-8 mRNA to determine pro-inflammatory effects.

5. Dose response relationships observed in *in vitro* assays appear to be directly comparable to dose response relationships *in vivo* when dose is expressed relative to the surface area of the exposed cells.

There are four recommendations from the present studies.

1. Since the dependence on surface area is potentially highly important, it should be substantiated by testing a wider range of dusts with this *in vitro* test system.

2. Further investigations are needed into the surface reactivity of the particles to determine if there is a difference in the ROS or reactive nitrogen species that are being produced by DQ12 quartz and LTPSP.

3. More *in vivo* data on GSH, NF-κB and MIP-2 mRNA and MIP-2 protein release into BAL fluid of rats exposed to LTPSP are needed to substantiate the apparent correlation between dose response *in vivo* and *in vitro*. At the present time we are only able to compare PMN levels *in vivo* with IL-8 *in vitro.* Directly comparing the above parameters *in vivo* with the same parameters *in vitro* would substantiate the comparability of the responses in the two different systems. This could be achieved without further animal exposures as lung and BAL fluid samples are available from previous TiO_2 inhalation exposed animals. In these animals, samples are available from animals with exposure that gave lung burdens below and above overload (i.e. at the relevant levels for comparison). They represent a valuable resource that would allow testing and confirmation of the hypothesised comparability of dose response. If confirmed, it would have major implications for extrapolating *in vitro* results.

4 Where airborne dust concentrations continue to be monitored in terms of mass, then it may be important to characterise the dust in terms of specific surface area, just as some dusts are commonly analysed for composition relevant to surface reactivity (e.g. quartz content).

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10 REFERENCES

Antonicelli F, Parmentier M, Drost EM, Hirani N, Rahman I, Donaldson K, MacNee W (2002). Nacystelyn inhibits oxidant-mediated interleukin-8 expression and NF-κB nuclear binding in alveolar epithelial cells. Free Radicals in Biology and Medicine 32: 492-502.

Borm PJA, Driscoll KE (1997). Particles, inflammation and respiratory tract carcinogenesis. Toxicology Letters 88: 109-113.

Brown DM, Wilson MR, MacNee W, Stone V, Donaldson, K. (2001). Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines. Toxicology and Applied Pharmacology 175: 191-199.

Brunaeur S, Emmett PH, Teller E. (1938). Adsorption of gases in multi-molecular layers, Journal of the American Chemical Society. 60: 309-319.

Deforge LE, Preston A, Takeuchi E, Kenney J, Boxer LA, Remick DG (1993). Regulation of interleukin-8 gene expression by oxidant stress. Journal of Biological Chemistry 268: 25568-25576.

Donaldson K, Borm PA (1998). The quartz hazard: a variable entity. Annals of Occupational Hygiene 42: 287-294.

Donaldson K, Bolton RE, Jones AD, Brown GM, Robertson MD, Slight J, Cowie H, Davis JMG (1998). Kinetics of the bronchoalveolar leukocyte response in rats during exposure to equal airborne mass concentration of quartz, chrysotile asbestos and titanium dioxide. Thorax 43: 525-533.

Donaldson K, Brown DM, Robertson MD, Slight J, Cowie H, Jones AD, Bolton RE, Davis J MG (1990). Contrasting bronchoalveolar leukocyte responses in rats inhaling coal mine dust, quartz, and titanium dioxide: Effects of coal rank airborne mass concentration, and cessation of exposure. Environmental Research 52: 62-76.

Donaldson K, Stone V, Gilmour PS, Brown DM, MacNee W (2000). Ultrafine particles: mechanisms of lung injury. Philosophical Transactions of the Royal Society of London 358: 2741-2749.

Driscoll KE, Lindenschmidt RC, Maurer JK, Higgins JM, Ridder G. (1990). Pulmonary response to silica or titanium dioxide: Inflammatory cells, alveolar macrophages-derived cytokines, and histopathology. American Journal of Respiratory Cell and Molecular Biology 2: 381-390.

Driscoll KE, Carter JM, Howard BW, Hassenbein DG, Pepelko W, Baggs RB, Oberdorster G (1996) Pulmonary inflammatory, cytokine, and mutagenic responses in rats after subchronic inhalation of carbon black. Toxicology and Applied Pharmacology 136: 372-380.

Driscoll KE (1996) Role of inflammatory in the development of rat lung tumours in response to chronic particle exposure. Inhalation Toxicology 8: 139-153.

Driscoll KE, Baggs RB, Carter JM, Howard BW, Hassenbein D, Pepelko W, Baggs RB, Oberdörster G (1996). Pulmonary inflammatory chemokine and mutagenic responses in rats

after subchronic inhalation of carbon black. Toxicology and Applied Pharmacology 136: 372-380.

Driscoll KE, Carter JM, Hassenbein DG, Howard BW (1997). Cytokines and particles-induced inflammatory cell recruitment. Environmental. Health Perspectives 105: 1159-1162.

Duffin R, Gilmour PS, Schins RP, Clouter A, Guy K, Brown DM, MacNee W, Borm PJ, Donaldson K, Stone V (2001). Aluminium lactate treatment of DQ12 quartz inhibits its ability to cause inflammation, chemokine expression, and nuclear factor-kappaB activation. Toxicology and Applied Pharmacology 176: 10-17.

Duffin R, Tran CL, Clouter A, Brown DM, MacNee W, Stone V, Donaldson K (2002). The importance of surface area and specific reactivity in the acute pulmonary inflammatory response to particles. Annals of Occupational Hygiene 46: 242-245.

Finch GL, Lowther WT, Hoover MD, Brooks AL (1991). Effects of beryllium metal particles on the viability and function of cultured rat alveolar macrophages. Journal of Toxicology and Environmental Health 34: 103-114.

Gilmour PS., Brown DM., Beswick PH, Benton E, MacNee W, Donaldson K (1997). Surface free radical activity of PM_{10} and ultrafine titanium dioxide: a unifying factor in their toxicity? Annals of Occupational Hygiene 41: 32-38.

Huaux F, Arras M, Vink A, Renauld J-C, Lison D (1999). Soluble TNF receptors and interleukin-10 (IL-10) down-regulate tumor necrosis factor alpha (TNF-α) activity during the lung response to silica particles in NMRI mice. American Journal of Respiratory Cell Molecular Biology 21: 137-145.

Hetland RB, Schwarze PE, Johansen BV, Myran T, Uthus N, Refsnes M (2001). Silica-induced cytokine release from A549 cells: importance of surface area versus size. Human and Experimental Toxicology 20: 46-55.

Hohr, D., Steinfartz, Y., Schins, R. P., Knaapen, A. M., Martra, G., Fubini, B., and Borm, P. J. (2002). The surface area rather than the surface coating determines the acute inflammatory response after instillation of fine and ultrafine TiO_2 in the rat. International Journal of Hygiene and Environmental Health 205: 239-244.

HSE (1997). Occupational Exposure Limits 1997 (EH 40/97), Sudbury, UK, HSE Books.

Ichinose T, Yajima Y, Nagashima M, Takenoshita S, Nagamachi Y, Sagai M (1997). Lung carcinigenesis and formation of 8-hydroxy-deoxyguanosine in mice by diesel exhaust particles. Carcinogenesis. 18: 185-192.

ILSI (2000). The relevance of the rat lung response to particle overload for human risk assessment: a workshop consensus report. Inhalation Toxicology 12: 1-17.

Janssen YMW, Barchowky A, Treadwell M, Driscoll KE, Mossman BT (1995). Asbestos induces nuclear factor κB (NF-κB) DNA-binding activity and NF-κB-dependent gene expression in tracheal epithelial cell. Proceedings of the National Academy of Sciences 92: 8458-8462.

Jimenez LA, Drost EM, Gilmour PS, Rahman I, Antonicelli F, Ritchie H, MacNee W, Donaldson K (2002). PM_{10}-exposed macrophages stimulate a pro-inflammatory response in lung epithelial cells via TNFα. American Journal of Physiology 282: L237-L248.

Katsnelson, B.A. Konysheva, L.K. Privalova, L.I. Morosova, K.I. (1992). Development of a multicompartmental model of the kinetics of quartz dust in the pulmonary region of the lung during chronic inhalation exposure in rats. British Journal of Industrial Medicine 49: 172-181.

Kelley J (1990). Cytokines of the lung. American Reviews in Respiratory disease 141: 765-788.

Kunkel SL, Standiford T, Kashara K, Strieter RM (1991). Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung. Experimental Lung Research 17: 17-23.

Kunsch C, Rosen CA (1993). NF-kappa B subunit-specific regulation of interleukin–8 promoter. Molecular and Cell Biology 13: 6137-6146.

Morrow PE (1988). Possible mechanisms to explain dust overloading of the lungs. Fundamental and Applied Toxicology 10: 369-384.

Lison D, Lardot C, Huaux F, Zanetti G, Fubini B (1997). Influence of particle surface area on the toxicity of insoluble manganese dioxide dusts. Archives of Toxicology 71: 725-729.

McClellan RO (1996) Lung cancer in rats from prolonged exposure to high concentrations of carbonaceous particles: implications for human risk assessment. Inhalation Toxicology 8: 193-226.

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. Journal of Immunology 136: 2348-2357.

Murphy S., BeruBe KA, and Richards RJ (1999). Bioreactivity of carbon black and diesel exhaust particles to primary Clara and type II epithelial cell cultures. Occupational and Environmental Medicine 56: 813-819.

Oberdörster G, Ferin J. Lenert BE (1994). Correlation between particle size, *in vivo* particle persistence, and lung injury. Environment Health Perspectives 102: 173-179.

Prahalad AK, Inmon J, Dailey LA, Madden MC, Ghio AJ, Gallagher JE (2001). Air pollution particles mediated oxidative DNA base damage in a cell free system and in human airway epithelial cells in relation to particulate metal content and bioreactivity. Chemical Research in Toxicology 14: 879-887.

Rahman I, MacNee W (1998). Role of transcription factors in inflammatory lung diseases. Thorax. 53: 601-612.

Rahman I, Antonicelli F, MacNee W. (1999). Molecular mechanism of the regulation of glutathione synthesis by tumour necrosis factor-α and dexamethasone in human alveolar epithelial cells. Journal of Biological Chemistry 274: 5088-5096.

Rahman, I. and W. MacNee. 2000. Regulation of redox glutathione levels and gene transcription in lung inflammation: therapeutic approaches. Free Radic.Biol.Med. 28:1405-1420.

Robock K (1973). Standard quartz DQ12 greater than 5 micro m for experimental pneumoconiosis research projects in the Federal Republic of Germany. Annals of Occupational Hygiene 16: 63-66.

Sacks M, Gordon J, Bylander J, Porter D, Shi XL, Castranova V, Kaczmarczyk W, Van Dyke K, Reasor MJ (1998). Silica-induced pulmonary inflammation in rats: activation of NF- φB and its suppression by dexamethasone. Biochemistry and Biophysics Research Communications 253: 181-184.

Schins RP, McAlinden A, MacNee W, Jimenez LA, Ross JA, Guy K, Faux SP, Donaldson K (2000). Persistent depletion of I kappa B alpha and interleukin-8 expression in human pulmonary epithelial cells exposed to quartz particles. Toxicology and Applied Pharmacology 167: 107-117.

Sempowski GD, Derdak S, Phipps RP (1996) Interleukin-4 and interferon-gamma discordantly regulate collagen biosynthesis by functionally distinct lung fibroblast subsets. Journal of Cell Physiology 167: 290-296.

Simeonova PP, Luster MI (1996). Asbestos induction of nuclear transcription factors and interleukin-8 gene regulation. American Journal of Respiratory Cell and Molecular Biology 15: 787-795.

Shi MM, Kugelman A, Takeo I, Tian L, Forman HJ (1994). Quinone-induced oxidative stress elevates glutathione and induces gamma-glutamylcysteine synthetase activity in rat lung epithelial cells. Journal of Biological Chemistry 269: 26512-26517.

Stöber W, Morrow PE, Koch W, Morawietz G (1994). Alveolar clearance and retention of inhaled insoluble particles in rats simulated by a model inferring macrophage particle load distributions. Journal of Aerosol Science 25: 975-1002.

Stone V, Shaw J, Brown DM, MacNee W, Faux SP, Donaldson K (1998). The role of oxidative stress in the prolonged inhibitory effect of ultrafine carbon black on epithelial cell function. Toxicology In Vitro 12: 649-659.

Stone V, Tuinman M, Vamvakopoulos JE, Shaw J, Brown DM, Pettersson S, Faux SP, Borm P, MacNee W, Michaelangeli F, Donaldson K (2000). Increased calcium influx in a monocytic cell line on exposure to ultrafine carbon black. European Respiratory Journal 15: 297-303.

Tieze F (1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidised glutathione: application to mammalian blood and other tissues. Annals of Biochemistry 27: 502-522.

Timbrell V, Ashcroft T, Goldstein B, Heyworth F, Meurman LO, Rendall REG, Reynolds JA, Shilkin KB, Whitaker D (1988). Relationships between retained amphiboles and fibrosis in human tissue specimens. Annals of Occupational Hygiene 32: 323-340.

Tran, CL, Jones, AD, Donaldson K. (1994) Development of a dosimetric model for assessing the health risk associated with inhaling coalmine dusts. Institute of Occupational Medicine (IOM report TM/94/01) 1-59.

Tran CL, Jones AD, Cullen RT, Donaldson K (1999a) Mathematical modelling of the retention and clearance of low-toxicity particles in the lung. Inhalation Toxicology 11: 1059-1076.

Tran CL, Jones AD, Cullen RT, Donaldson K. (1999b) Exploration of the mechanisms of retention and clearance of low-toxicity particles in the rat lung using a mathematical model. Inhalation Toxicology 11: 1077-1108.

Tran CL, Buchanan D, Miller BG, Jones AD, Donaldson K (2000a) Mathematical Modeling to Predict the Responses to Poorly Soluble Particles in Rat Lungs. Inhalation Toxicology 12: 403-409.

Tran CL, Buchanan D, Cullen RT, Searl A, Jones AD, Donaldson K (2000b). Inhalation of Poorly Soluble Particles II. Influence of Particle Surface Area on Inflammation and Clearance. Inhalation Toxicology 12: 1113-1126.

Vandeputte C, Guizon I, Genestie-Denis B, Vannier B, Lorenzon G (1994). A microtitre assay for glutathione and glutathione disulfide content in cultured/isolated cells: performance study of a new miniaturized protocol. Cell Biology and Toxicology 10: 415-421.

Wallace WA, Ramage EA, Lamb D, Howie SE (1995) A type 2 (Th2-like) pattern of immune response predominates in the pulmonary interstitium of patients with cryptogenic fibrosing alveolitis (CFA). Clinical and Experimental Immunology 101: 436-441.

Yu CP, Zhang L, Oberdorster G, Mast RW, Glass LR, Utell MJ (1994) Clearance of refractory ceramic fibres (RCF) from the rat lung: Development of a model. Environmental Research 65: 243-253.

11. APPENDICES

Appendix1

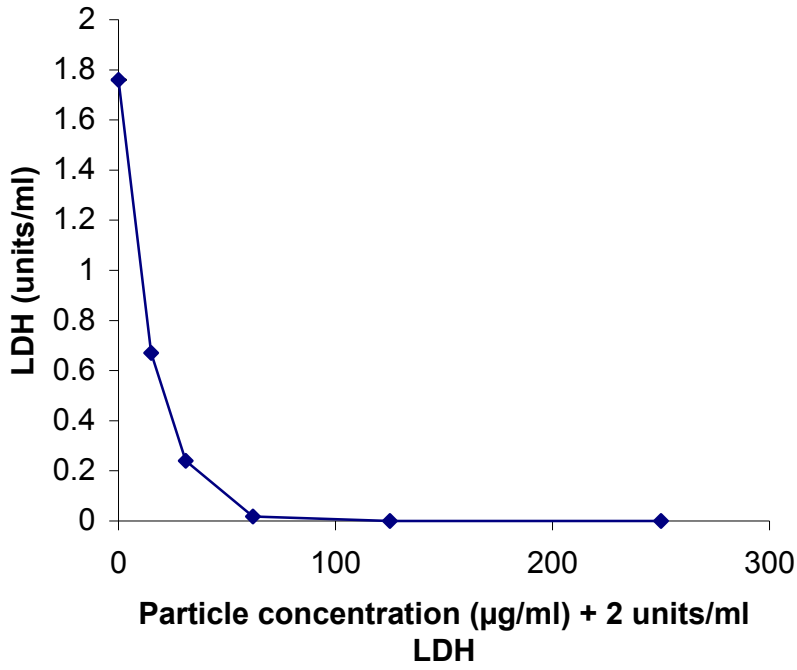

Figure 11.1

Effect of ultrafine CB on the measurement of LDH in a cell free system. Ultrafine CB was incubated with LDH and then the levels of LDH were measured as described in section 4.3. The data are the mean ±one standard error from three independent experiments.

Appendix 2

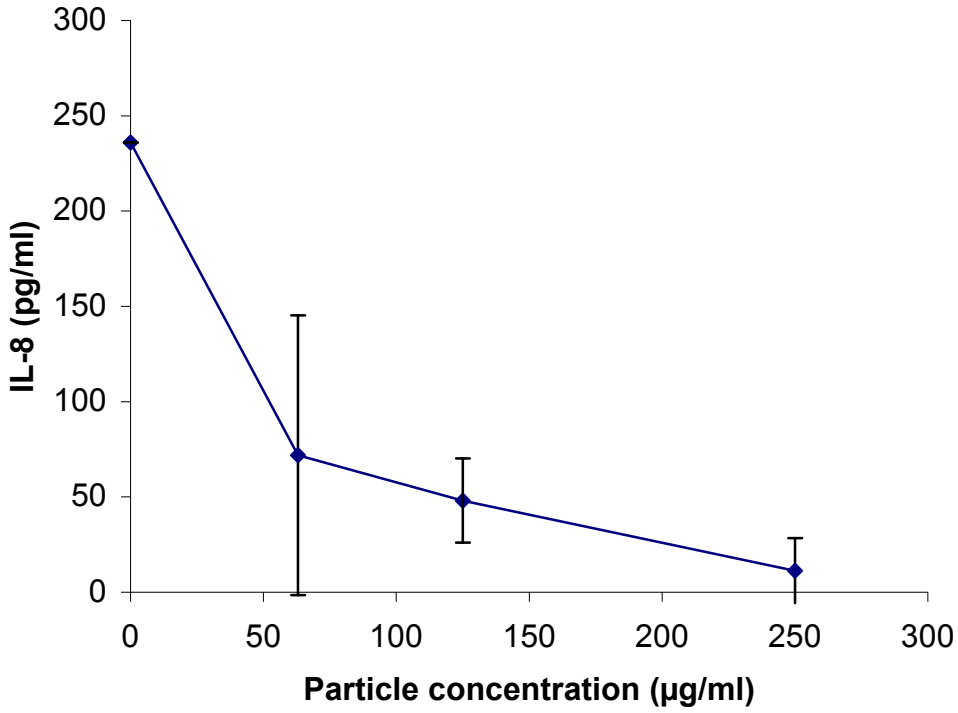

Figure 11.2

Effect of ultrafine CB on IL-8 protein measurement in a cell free system. Ultrafine CB was incubated with IL-8 (1000pg/ml) and IL-8 was measured as described in section 4.5. The data are the mean ± one standard error from three independent experiments.

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