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Research Report

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The Soufrière Hills volcano on the Caribbean island of Montserrat has been erupting intermittently since July 1995 producing widespread deposition of volcanic ash on the island, and leading to concern as to the health effects of the inhaled ash.

This study examined the toxicity of a sample of the ash using a variety of methods. The most important test involved the exposure of rats by inhalation to clouds of the ash, in parallel with similar exposure of another group of rats to a benchmark low toxicity control dust, titanium dioxide. The inhalation exposures were for 6 hours per day, 5 days per week, and for periods of 2, 4, 6 and 8 weeks at concentrations calculated to produce similar lung burdens for the two dusts and high enough to produce inflammation with a low toxicity dust. This comparison showed that the ash produced more inflammation in the lungs than the low toxicity dust. The ash also produced the earliest signs of fibrosis. Comparison with results from previous inhalation studies on quartz (a very toxic dust) and dust from two coalmines showed that the volcanic ash was much less toxic than the quartz, and was close in toxicity to the coalmine dusts.

The study also included other toxicity tests, using either cells in bench top tests, or dust injected into the lungs of rats. These tests confirmed that the ash was less toxic than quartz dusts. They also showed some variation in toxicity between samples of ash. However, they produced only limited and less reliable indications of the difference between the effects of the ash those of the low toxicity control dusts.

The report recommends that existing data sets on the effects of coalmine dust in miners would be valuable in assessing the risks to the people exposed to the Montserrat volcanic ash.

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SUMMARY

Introduction

The Soufrière Hills volcano on the Caribbean island of Montserrat, a British dependency, has been erupting intermittently since July 1995 leading to widespread deposition of volcanic ash on the island. The fine particle size of the ash and its high content of the toxic silica mineral, cristobalite, suggested that it could be potentially hazardous to human health. *In vitro* toxicity tests carried out at the Institute of Occupational Medicine (IOM) and at Napier University showed that the volcanic ash was toxic in some tests. In vivo experiments, with ash instilled into rat lungs, conducted by Richards and colleagues at Cardiff, had indicated that the ash might be similar in toxicity to low toxicity (nuisance) dusts. Subsequently, the UK Department for International Development (DfID) commissioned the Institute of Occupational Medicine to conduct further toxicological tests. These included an inhalation experiment conducted in rats by IOM and *in vitro* and lung instillation experiments conducted by Napier University.

Aims

The overall aim of the study was to better determine whether there is a significant possibility that volcanic ash is likely to be hazardous to the pulmonary health of the population of Montserrat. The specific objectives of the proposed study were as follows.

Inhalation experiment

The inhalation experiment was designed to test the null hypothesis that, dose for dose, inhaled volcanic ash causes no greater pulmonary inflammation than a benchmark, low toxicity, control dust. The experimental design also sought to estimate the extent of any difference from the control dust.

In vitro and instillation experiments

The aim of the *in vitro* experiments was to compare various ash samples, thereby linking the sample tested by inhalation with that tested by instillation (at Cardiff) and the samples used in previous *in vitro* tests.

An additional instillation experiment (not part of the contract) was conducted. The aim of this test was to compare the inflammatory response of ash samples with those of toxic and low toxicity control dusts.

Methods

Inhalation experiment

Wind-blown volcanic ash was collected from inside two houses in Corkhill, Montserrat. Before use, the ash was sieved, mixed, and sterilised by heating at 250°C for 1 hour. Rats were exposed by nose-only inhalation to an aerosol of Montserrat volcanic ash, five days per week for eight weeks. A control group of rats were exposed to a heat-treated sample of the low toxicity dust, titanium dioxide (TiO_2). The aerosol generated from the Montserrat volcanic ash was passed through a size selector to reduce the amount of coarse dust delivered to the rats. The TiO_2 was dispersed initially without a size selector, but later this was added to achieve a more stable aerosol.

After measurement of the aerodynamic size distributions of test dust clouds, target exposure concentrations were calculated to achieve the deposition in the lungs of similar mass amounts (burdens) of both dusts at levels that would ensure overload of the dust clearance mechanism of the lungs and consequent lung inflammation.

Animals were sacrificed 14, 28, 42, and 56 days from the start of exposure and their lungs lavaged with saline. The lavage fluid was examined for the presence of markers of inflammation, namely increased numbers of cells, especially polymorphonuclear neutrophils (PMN) and increased levels of the enzyme lactate dehydrogenase which leaks from damaged cells. Following lavage, lungs were stored frozen and then digested in bleach to recover deposited dust. The ash and TiO_2 in the lungs were quantified using X-ray diffraction (XRD) techniques.

Additional groups of six animals were sacrificed at the 8-week time point for histopathological examination of the lungs.

In vitro experiments

Two *in vitro* tests were used: (i) the haemolysis of sheep red blood cells as an indicator of surface reactivity and potential cytotoxicity; (ii) release of lactate dehydrogenase (LDH) from cells of the human type II epithelial cell line, A549, as an indicator of the potency of dusts to damage the membrane. A number of volcanic ash samples were compared to TiO_2 (low toxicity control) and samples of the toxic dusts, DQ12 quartz and cristobalite (positive controls). Three of the ash samples (M1, M2, M3) had been studied in previous *in vitro* experiments at IOM and Napier University, the fourth sample (M4) was that used in the inhalation experiment. The fifth ash sample (RMVA), provided by Prof. Richards, Cardiff University, had been used in their rat instillation study.

Lung instillation experiment

Two ash samples (M4 and RMVA), as used in the inhalation experiment and the instillation experiment at Cardiff University, were instilled into rat lungs, and the inflammatory profile in the bronchoalveolar lavage (BAL) determined approximately 24 hours later. Results were compared to those obtained using TiO_2 and DQ12 quartz instillation. The ash M4 and TiO_2 samples were instilled at similar doses expressed as particle surface area. The ash RMVA was used at the same mass dose as ash M4.

Results

Inhalation experiment

The lung burden measurements confirmed that the calculated exposure concentrations had produced lung burdens in the intended range. At the first time point (14 days), the TiO_2 mean lung burden was substantially lower than for the Ash, but by the final two time points, the mean TiO_2 lung burdens were higher than the corresponding means for the ash.

Pulmonary inflammation, as number of PMN recovered by lavage, and cellular damage, as released LDH in the lavage fluid, were greater for ash-exposed rats than the corresponding values for TiO_2-exposed rats at all time points. PMN numbers, or LDH plotted against mass of dust in the lung, showed that the volcanic ash produced a markedly greater response relative to mass lung burden. The TiO_2 lung burdens rose to nearly twice the final mean level for the ash, but still produced much less inflammation. In previous studies where the TiO_2 was compared to a coarser nuisance dust (barium sulphate), we showed that if the inflammation (number of PMN) was plotted against surface area of the particulate lung burden, then the

data collapsed onto a single trend. As the TiO_2 is a finer dust than the ash, differences in particulate surface area do not explain the results. It is clear that the volcanic ash is markedly more toxic than nuisance dust particles of similar size.

Histopathological examination after the 8-week exposure showed relatively minor pathology changes consistent with exposure to dust. In general, the changes were more marked in the ash-treated lungs and it was thought that continued exposure could have eventually led to fibrosis. Alternatively, if the animals had been allowed to survive for a sufficient period without further exposure, it is likely that the pulmonary lesions observed would have resolved.

In vitro experiments

Haemolysis was determined following exposure to the test dusts at 5, 10, and 20 $mg.ml^{-1}$. The relatively large amounts of dust required for this assay meant that only M1 (used in previous in vitro experiments) and M4 (used in the inhalation experiment) could be tested for haemolysis. DQ12 induced significantly greater haemolysis than TiO_2 or the two ash samples. M1, but not M4, was statistically more active than TiO_2 at the highest dose of 20 mg/ml ($p<0.01$), but the difference was only small and probably not biologically meaningful.

In the LDH assay, the test dusts were used at 50, 100, 200, and 400 $\mu g.ml^{-1}$. Only cristobalite at 100 and 200 $\mu g/ml$ was significantly more active than the low toxicity control dust, TiO_2. For the middle concentrations, some of the ash samples, especially RMVA used in the Cardiff instillation study, appeared to be more active than TiO_2, but the differences were not statistically significant.

Instillation experiment

Twenty-four hours following instillation, the two ash samples, M4 and RMVA, and TiO_2 had induced similar levels of inflammatory cells as the saline control. Only DQ12 quartz produced significantly greater inflammation than saline ($p<0.01$).

Discussion

Results from the *in vitro* and lung instillation experiments showed that the ash was less toxic than cristobalite and, in some experiments, less toxic than a standard quartz sample DQ12. These experiments showed only minor, and statistically insignificant, indications of a difference among ash samples or between ash samples and the low toxicity control TiO_2.

The inhalation experiment showed that the Montserrat volcanic ash sample produced more pulmonary inflammation than the low toxicity control dust. This result was in keeping with published studies on the toxicity of volcanic ash from the Mount Saint Helens eruption in 1980. Further comparisons of the Montserrat inhalation results with those from other dust studies showed that the volcanic ash was much less active than quartz, again confirming findings from Mt. St. Helens studies and also our *in vitro* and instillation studies. However, the volcanic ash was seen to be more comparable to coal mine dust in terms of the inflammation induced for a given mass deposited in the lung. Because of differences in protocols between studies, the comparisons with quartz and coal mine dust required some assumptions and extrapolations to be made. Nevertheless, the comparisons are sufficient to indicate that coal mine dust provides an excellent benchmark against which to compare the toxicity of volcanic ashes and, together with the wealth of exposure-response data available from coal miners, to aid risk assessment for those exposed on Montserrat.

Conclusions

From the inhalation experiment, Montserrat volcanic ash is clearly more toxic than a benchmark low toxicity (nuisance) dust, titanium dioxide.

The data from the instillation experiment, most of the data from the *in vitro* tests, and the comparisons made between the inhalation results and those from inhalation studies with other dusts, showed that Montserrat ash is considerably less active than quartz, a dust that can cause disabling lung fibrosis and cancer.

Comparisons with other inhalation studies indicate that Montserrat volcanic ash has similar lung toxicity to that seen with coal mine dusts.

Recommendations

1. The oxidative activity of the ash sample used in the experiments reported here, should be determined using the techniques employed by Horwell and colleagues (2001).

2. Given that the Montserrat volcanic ash appears to have similar toxicity to at least two samples of coal mine dusts, data on the risks from coal mine dust could be used as a benchmark to estimate the risks from ash exposure. Mathematical modelling would probably be needed to take account of differences in exposure scenarios, and to allow for the differences in the age and health of the exposed population.

3. The comparison between the toxicity of volcanic ash and coal mine dusts should be explored further experimentally using sensitive *in vitro* techniques to measure toxicity and cell function. The IOM has appropriate samples of coal mine dusts in storage.

4. The inhalation experiment used only one sample of ash. The *in vitro* assays indicated that the effects seen in the inhalation experiment are likely to be reasonably representative of all Montserrat ash samples. However, recent understanding of the molecular basis of the inflammatory response means that sensitive *in vitro* assays, in addition to those used in the present study, could be used to strengthen the comparison of the toxic potential of several different ash samples. Differences in the *in vitro* cellular responses to different ash samples could be of practical importance in predicting the outcome of chronic exposure to ash with different physico-chemical characteristics, as seen with ash from explosive plumes versus that from pyroclastic flows.

1. INTRODUCTION

1.1 BACKGROUND

1.1.1 Volcanic eruptions on Montserrat

Montserrat, a British dependency, is an island in the Lesser Antilles group in the Caribbean. In July 1995 the Soufrière Hills volcano in the south of the island began erupting and later that year a lava dome started to build up with episodes of explosive eruptions and the generation of numerous pyroclastic flows (mixtures of volcanic ash and gases that hug the ground and flow down valleys) leading to the production of substantial falls of volcanic ash, especially over the southern part of the island. Following a major eruption in June 1997 when 19 people died, the southern and central areas of the island were evacuated. Today, about 4,500 of the original 12,000 population remain on the island, although the volcano remains active. Exposure of humans to the ash occurs through continuing volcanic emissions and pyroclastic flows and by the disturbance of ash by wind and human activity.

The ash has a substantial respirable component (particles small enough to be deposited in the deep lung) and contains unexpectedly large amounts of the toxic silica mineral, cristobalite (Baxter *et al*., 1999). The fine particle size of the ash and its high content of cristobalite suggest that it may be potentially hazardous to human health.

1.1.2 Toxicity of ash from Mt. St. Helens volcano

Following the eruptions of Mount Saint Helens in the USA in 1980, a flurry of studies assessed the toxicity of the resulting ash. Those studies showed that, although the ash was considerably less toxic than quartz, it could cause inflammation and pathological changes in animal lungs, particularly at high doses (Martin *et al*., 1986). The toxic effect of the ash was possibly due to its crystalline silica content (3-7% by weight) (Dollberg *et al*. 1986; Martin *et al*. 1986).

1.1.3 Previous studies on toxicity of Montserrat volcanic ash

There have been three previous studies on the toxicity of ash samples from the Montserrat eruption, two *in vitro* studies (Cullen and Searl, 1998; Wilson *et al*., 2000) and one lung instillation study in rats (Housley *et al*., 2002). The *in vitro* findings varied, depending on the assays used. In the Cullen and Searl study, three samples of Montserrat ash were more toxic than a standard quartz sample (DQ12) as measured by the release of lactate dehydrogenase from cell of the epithelial line A549. But in another type of assay, MTT metabolism in alveolar macrophages, the samples were less toxic than quartz, but still more toxic than a control "nuisance dust", TiO_2. The study reported by Wilson *et al*. (2000) used the same three ash samples, but with different *in vitro* assays and also found that the ash samples were more active than TiO_2. The *in vivo* study carried out by Professor Richards' group at Cardiff indicated that the Montserrat ash was of low toxicity (personal communication; Housley *et al*., 2002). Since this was the only *in vivo* data available when planning the present study, it was agreed that the inhalation experiment should test the null hypothesis that the ash would produce a response similar to that produced by a low toxicity dust (see Section 1.4).

1.2   QUARTZ AND CRISTOBALITE AS INHALATION HAZARDS

Silica forms a major part of the earth's crust and occurs in three main crystalline forms: quartz (the most abundant), tridymite, and cristobalite. Silica may exist in both crystalline and amorphous forms with the crystalline form being more toxic *in vivo*.

Inhalation of quartz, a crystalline form of silicon dioxide, can lead to the fibrotic lung disease, silicosis. Exposure to quartz alone is rare and most exposures occur through the presence of quartz in other dusts produced by activities such as mining, tunnelling, quarrying, stone grinding, and sandblasting. Thus silicosis is often seen with mixed dust pneumoconiosis Morgan and Seaton, 1984; Pilkington *et al.*, 1996). The International Agency for Research on Cancer (IARC) has classified crystalline silica as a human carcinogen (IARC 1997).

The majority of the crystalline silica in Montserrat volcanic ash is in the form of cristobalite which is generally regarded as being more toxic than quartz in *in vitro* and *in vivo* experiments (Kozin *et al.*, 1982; Pratt 1983; Nolan *et al.*, 1987; Hemenway *et al.*, 1986, 1990).

1.3   DEPOSITION AND CLEARANCE OF PARTICLES IN THE LUNG

The deposition of particles in the lower respiratory tract is mainly influenced by the breathing pattern, the branched morphology of the airways, and by the particle shape and size. In inhalation toxicology particle size is expressed in terms of the aerodynamic diameter, d_{ae}, which is the diameter of a spherical particle of unit density (1 $g.cm^{-3}$) having the same gravitational settling velocity as the particle studied. In rats, the alveolar deposition fraction (i.e., the fraction of inhaled particles which penetrate to the alveolar region and deposit there) is at a maximum ($\approx$ 10 percent) for particles of $d_{ae} \approx$ 1 μm, and decreases rapidly for increasing aerodynamic diameter (Jones, 1993; Ménache *et al.* 1996).

Particles that deposit on the surface of the main airways are mostly trapped in the mucus covering the airway surface. Ciliated cells move the mucus with trapped particles up to the throat where it is eliminated by swallowing or expectoration. This mechanism is known as the muco-ciliary escalator. Particles that penetrate to the non-ciliated alveolar, gas-exchange, region of the lung are mostly cleared by phagocytic alveolar macrophages, which have a primary role in the defence of this lung region against microorganisms and other deposited material. Material, such as insoluble mineral dust, that is not digested by the macrophages, may be cleared through the migration of macrophages to the muco-ciliary escalator (Lehnert, 1992). Some particles may reach the interstitium, either carried by macrophages or through direct penetration of alveolar epithelium; and hence particles reach the lymph nodes draining the deep lung.

This macrophage-mediated removal process can be impaired when the alveolar macrophage phagocytoses cytotoxic particles such as quartz, or fibrous particles such as asbestos. However, it is also known that dust clearance is affected when individual macrophages become overloaded with a high burden of relatively inert particles such as titanium dioxide (Bolton *et al.* 1983, Morrow 1988, 1992). Morrow (1988) argued that overload was initiated when the particle volume exceeded ~ 60 μm^3 *per* macrophage and was complete, with a virtual cessation of clearance, when the particle volume exceeded ~ 600 μm^3 *per* macrophage.

Injury to macrophages and epithelial cells by toxic particles, or overload of low toxicity particles, can precipitate inflammation, characterised by the recruitment into the lung of other cellular components of the host defence system, principally a class of phagocytic cells called polymorphonuclear neutrophils (PMN). These events are associated by a build up of dust in

the lung and greater carriage of dust to the lymph nodes (Bolton *et al*. 1983; Vincent and Donaldson 1990; Tran *et al*. 1997). A number of recent studies have demonstrated that the surface area of deposited particles provides a better correlate to lung inflammation than particle mass or volume (Driscoll (1996); Lison *et al*., 1997; Cullen *et al*., 2000; Tran *et al*., 2000). Persistent inflammation may play a key role in both the development of fibrosis and the promotion of carcinogenesis, even with relatively inert particles (Lee *et al*. 1985, 1986; NTP 1993; Dungworth 1994; Driscoll, 1996).

1.4   INHALATION MODEL TO STUDY PULMONARY EFFECTS OF DUSTS

This study exploits our prior knowledge of lung overload and resulting inflammation in rats arising from the inhalation of titanium dioxide to use the low toxicity dust as a benchmark against which to assess the pulmonary toxicity of Montserrat volcanic ash. We decided to conduct an 8-week inhalation experiment that would allow comparison at various delivered doses of ash, including those at which we would expect inflammation with a low toxicity dust. This study thus provides both negative and positive responses with the same control dust. The experimental design used for the inhalation study is described in Chapter 2.

1.5   EXPERIMENTAL PROGRAMME

In addition to the 8-week inhalation experiment conducted by the IOM, the study programme included both lung instillation and *in-vitro* assays carried out at Napier University. The inflammatory response of the rat lung was measured one day after instillation of small amounts of ash or control dusts. The differences in toxicity of various samples of Montserrat volcanic ash towards red blood cells and a human epithelial cell line (A549) were investigated *in vitro*. These assays measure the potential of dusts to cause cell injury and/or reduce cell viability. The *in vitro* assays provide a comparison of the current sample of ash with those used in the previous *in vitro* experiments (Cullen and Searl 1998; Wilson *et al*., 2000), and with the sample used by Professor Richards in a long-term instillation study at Cardiff University.

1.6   LAYOUT OF THIS REPORT

The inhalation experiment design, materials and methods and results are presented in chapters 2, 3, and 4 respectively. Chapter 5 provides a summary of the *in vitro* and instillation experiments conducted at Napier University, which are fully described in the Appendix. Chapter 6 provides discussion of the experimental results as a whole and Chapter 7 provides conclusions and recommendations.

2. INHALATION STUDY: AIMS /EXPERIMENTAL DESIGN

2.1 AIMS

The overall aim of the study was to better determine whether there is a significant possibility that volcanic ash is likely to be hazardous to the pulmonary health of the population of Montserrat. The specific objectives of the proposed study were to undertake short term experiments with rats to:

(i) determine the *in vivo* inflammatory and pathological effects of inhaled ash in rat lungs;

(ii) compare the *in vivo* response to volcanic ash to that for a relatively inert dust (titanium dioxide);

(iii) to estimate the critical burden of the ash in the lung that is associated with a significant increase in numbers of polymorphonuclear neutrophil cells (PMN), a marker of inflammation.

2.2 HYPOTHESIS TO BE TESTED

The inhalation experiment was designed to test the null hypothesis that, dose for dose, inhaled volcanic ash causes no greater pulmonary inflammation than the low toxicity control dust.

2.3 AEROSOLS AND DOSE

The effects of volcanic ash were compared to those of the known low toxicity (nuisance) dust, titanium dioxide, at similar delivered mass doses. Note that mass is important for dust control purposes in the workplace. If the particle aerodynamic size distributions had been very similar for the ash and the control dust, then equal aerosol mass concentrations would have produced similar mean lung burdens for the two dusts. However, the aerodynamic size distributions were significantly different. Alternatively, if the rat and human respiratory systems produced identical aerodynamic size selection of particles that would deposit in the lung, then again the study would have used the same target aerosol concentration for the two dusts with different size distributions. However, the aerodynamic size selection for rats is similar but not identical to that for humans. Therefore the study design sought to compare the toxicity relative to the achieved lung burden in rats.

2.4 DESIGN OF THE INHALATION EXPERIMENT

The experimental protocol comprised nose-only inhalation for 8 weeks (6 hours per day, 5 days per week) to the two dusts in parallel. The target exposure concentrations were selected to deliver similar mass burdens for both dust types. Exposure concentrations were measured daily by gravimetric sampling.

Based upon our previous experience (Cullen *et al*. 2000) the target aerosol concentration of TiO_2 was selected to ensure that, by approximately four weeks, there would be sufficient dust burden to initiate lung inflammation. The aerosol concentration of the ash was then selected to provide similar mass lung burdens to those animals exposed to TiO_2.

At 14, 28, 42, and 56 days from the start of inhalation, groups of rats were sacrificed and the following end points determined.

- Cell types and cell numbers recovered by bronchoalveolar lavage

- LDH concentrations in lavage fluid

- Lung wet weight

- Lung dust burden

- Lymph node dust burden (nodes draining the lung)

- Histopathology of lungs at the end of inhalation (56 days only)

As in previous studies of inflammation by our group (Cullen *et al*. 2000), the animals were sacrificed on the day after the last exposure day for that test group. A group of untreated, sentinel, animals were used to observe background levels of lung inflammation and dust at each of the sacrifice days (2 rats per day). In addition, the lungs from six of these control animals were examined by histopathology at the end of the study.

Table 2.1 shows the numbers of animals assigned to the various treatment groups.

Table 2.1 Numbers of rats sacrificed per time point

Test dust	Time point (from start of exposure)				Histopath 56 days	Total rats
	14 days	28 days	42 days	56 days		
Ash	10	10	10	10	6	46
TiO$_2$	10	10	10	10	6	46
Sentinel	2	2	2	2	6	14

3. INHALATION STUDY: MATERIALS AND METHODS

3.1 TEST DUSTS

3.1.1 Montserrat ash sample

Collection of ash

Ash was collected during September 1998 from internal ledges and floors of two undamaged buildings in Cork Hill. The ash was windblown and had penetrated the buildings through window louvres. The ash was dry when collected and would not have been exposed to rain or standing water while inside the buildings. The volcanic history prior to collection indicated that the ash was likely to be predominantly from pyroclastic flows. The ash was stored in sealed plastic bags until required for analysis and experimentation.

Preparation of ash

Ash from the collection bags was sieved through a 1 mm sieve to remove very coarse material and pooled in a 30 litre plastic tub. The ash was mixed first by hand and then by rotating the tub vertically and horizontally.

Following mixing, aliquots of the mixed ash were treated with dry heat at 250 $^{\circ}$C for 60 minutes to kill any contaminating microorganisms and to inactivate bioactive molecules such as endotoxin. Confirmation that endotoxin was not present in the heat-treated ash was obtained by mixing the ash in water and testing the resulting eluate for endotoxin in the Limulus Amoebcyte Lysate (LAL) assay ("Coatest" kit; Chromogenix AB, Molndal, Sweden) as described previously (McGorum *et al*. 1999). Heat-treated ash was stored in sterile plastic containers until required for experimentation.

Cristobalite content of ash

The cristobalite content of the Montserrat ash was measured by X-ray diffraction (XRD) against bulk mixtures containing standard reference material, 1879 Respirable Cristobalite Quantitative XRD Standard. Measurements were made at the main cristobalite peak of $22.0^{\circ}2\theta$ with no correction made for plagioclase interference. Plagioclase was present as a substantial component of the ash but a full mineralogical assessment was not carried out.

3.1.2 Titanium dioxide (control dust)

Titanium dioxide (rutile; TiO_2) was obtained from Tioxide Europe Ltd, Cleveland, UK. A 1.5 kg sample of this TiO_2 was mixed thoroughly by hand and aliquots heat-treated as described above for volcanic ash.

3.2 AEROSOL GENERATION

An aerosol of Montserrat volcanic ash was generated using a Wright dust feed (Wright 1950). The principal of the Wright feed is that the test dust is pre-compressed into a metal cylinder and is cut away by a slowly rotating blade to be carried into the exposure chamber by a stream of compressed air. Before reaching the exposure chamber, the aerosol was passed through a size selector to reduce the amount of coarse dust delivered to the rats. The aerosol was diluted with a separate airstream just before entry to the particle separator.

The TiO$_2$ aerosol was generated using a rotating brush generator (Model RBG 1000; Palas GmBh, Karlsruhe, Germany). The principal of operation is similar to that for the Wright feed except that the dust compressed into the supply cylinder is carried into the airstream by a rotating nylon brush. The TiO$_2$ was dispersed initially without a size selector, but later this was added to achieve a more stable aerosol (as described in Chapter 4).

Control of the concentrations of dust entering the exposure chambers was achieved for both dusts by adjusting the cylinder advance rate and by varying the compressed air flows to the aerosol generators and size selectors.

3.3   AEROSOL CONTROL

For each dust, total and respirable gravimetric samples were taken from each nose-only chamber approximately every 30 minutes throughout each exposure day. To ensure comparability from day to day, samples were always taken from the same ports. Running averages of these measurements were recorded and, from time to time, appropriate changes made to the aerosol generation systems to bring the average concentrations closer to the target concentration. Total samples were measured using Gelman filter holders fitted with a Whatman 47 mm glass fibre filter and sampling at a flow rate of 0.5 l.min^{-1}. The respirable dust concentration was measured using Simpeds cyclone samplers fitted with a 25 mm Whatman glass fibre filter and sampling at a flow rate of 2.2 l.min^{-1}. Note that this sampler is used to measure the respirable dust concentration for humans in workplaces.

3.4   AEROSOL CHARACTERISATION

In aerosol optimisation trials, the size distribution of the Montserrat dust was evaluated using the real time measuring instrument, the TSI Aerodynamic Particle Sizer (APS) model 3320. This device provides real time information on the aerodynamic diameter of aerosol particles in the size range 0.5 to 20 μm. Volcanic ash aerosols were generated in a test chamber and the size distribution measured. Using this distribution and the known deposition in rat lungs of particles of differing aerodynamic diameters, we were able to estimate the deposition profile of respirable ash particles in the rat lung.

The size distribution of TiO$_2$ particles in our sample had been measured previously (all particles respirable) and no new measurements were made with the TSI machine (Cullen *et al*., 1999).

Mean aerodynamic diameters were also measured for both test dusts using two types of cascade impactor, the Marple and the PIDS. In addition to measurements during the aerosol optimisation phase, cascade impactor measurements were conducted weekly during the inhalation exposure to confirm consistency of the aerosols.

3.5   ANIMALS

Specific-pathogen-free male Wistar rats (approximately 225 g) were obtained from Charles River (UK) Limited, Margate, England.

3.5.1   Acclimatisation

The animals were allowed to acclimatise to the cage accommodation for at least 7 days before the commencement of dosing. During this period, animals were also acclimatised to the restraining tubes used in the nose-only exposure chambers.

3.5.2   Allocation to treatment group and identification

Animals were randomly assigned to treatment groups and individual animals were identified by a unique earmark. A colour coding system was used to identify cages and exposure systems for particular treatments.

3.5.3   Husbandry

When not being exposed, the rats were housed 5 per cage (of the same exposure group) in polypropylene cages (58 x 40 x 18 cm) with stainless steel mesh tops and bottoms. The cages were suspended over trays containing absorbent paper.

There was automatic control of temperature and humidity in the ranges 20°C± 2°C and 55%±15% respectively, with a minimum of 15 air changes per hour. Temperature and humidity ranges were monitored and recorded daily.

There was automatic control of the light cycle with light hours normally from 0700-1900.

3.5.4   Body weights

Individual body weights were recorded to the nearest 0.1g before the start of the first day's exposure, and on the day of sacrifice.

3.5.5   Clinical examination

All animals were checked daily for illness or injury. During the 6-hour exposure period animals were checked at least once every hour.

3.6   INHALATION EXPOSURE TO TIO$_2$ AND VOLCANIC ASH

Rats were exposed by nose-only inhalation for 6 hours per day, 5 days per week. Exposure continued for 8 weeks, with groups being sacrificed for assays after 14, 28, 42 and 56 days.

The exposure to the test dusts was performed using an appropriately sized modular nose only flow past system (see Figure 3.1). This exposure technique allows a continuous supply of test material to be delivered to each animal; the biased flow ensures that there is no re-breathing of the test atmosphere.

A vacuum pump system was used to continuously exhaust the test atmosphere. Each exposure chamber was located in an extract booth to prevent any cross-group contamination and for the protection of the personnel undertaking the animal inhalation exposure procedures.
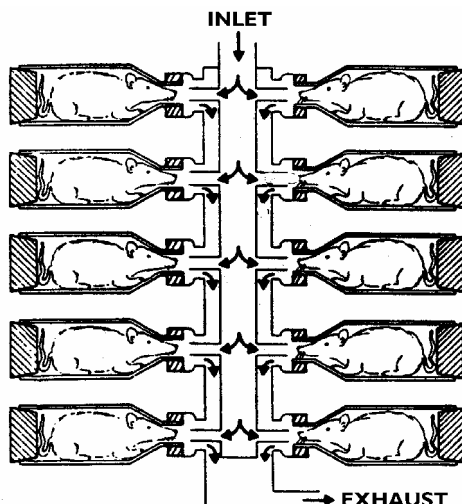
Figure 3.1 Schematic of the nose-only exposure system

Each exposure chamber was operated to sustain a dynamic airflow with an evenly distributed exposure atmosphere. The chamber airflow rate was in the range 20-55 litres.min^{-1}. Flow rates were monitored continuously using calibrated flow meters. Chamber airflow rates were monitored and recorded at appropriate intervals during each exposure period.

For inhalation exposure, the rats were restrained in clear, tapered, polycarbonate tubes with an adjustable back-stop to prevent the animals from turning in the tubes. The animals' snouts protruded through the anterior end of the restraint tubes which were connected to the exposure chamber by way of a push-fit through rubber 'o' rings in the aerosol delivery port. This exposure technique minimised concurrent exposure by the oral and dermal routes.

3.7   BRONCHOALVEOLAR LAVAGE (BAL)

At each time point, animals were sacrificed approximately 18 hours after the end of the previous day's exposure by intraperitoneal injection of an overdose of sodium pentobarbitone (approximately 70 mg per Kg body weight). Following sacrifice, the thoracic cavity was opened and the trachea cannulated with a blunt 19-gauge needle tied in place with suture. The bronchoalveolar space was then lavaged with 4 x 8 ml volumes of warm (37 ^{o}C) saline (no calcium or magnesium salts present). A sample of the first lavageate from each lung was used for the measurement of protein and LDH levels (see below).

Cells were recovered from lavage fluid by centrifugation, pooled from the four aliquots, and resuspended in RPMI-1640 medium (Sigma Aldrich, Poole, UK) containing 0.2% bovine serum albumin (Sigma Aldrich, Poole, Dorset). Total cell counts were made, and cytocentrifuge smears were prepared and stained with Diffquick (Merz Dade, Switzerland) to obtain differential cell counts. At least 400 cells were categorised for the differential count for each animal. The cells were kept on crushed ice until the counting procedures were completed. The cells remaining after counting were centrifuged and the cell pellets retained for dust analysis (see section 3.10.2).

Two age-matched untreated (sentinel) animals were sacrificed and lavaged at each time point to provide confirmation that the study animals were free of any non-treatment-related pulmonary inflammation.

3.8   MEASUREMENT OF LDH IN LAVAGE

A sample of the fluid from the first lavage from each lung was used to determine the concentrations of lactate dehydrogenase (LDH). The assay was performed using a Hitachi 717 automated clinical chemistry analyser using an optimised Roche LDH method (Weißhaar *et al*. 1975).

3.9   HISTOPATHOLOGY

Histopathology was restricted to examining the lungs of six animals per dust at the end of the 8-week inhalation period. They were sacrificed on the day following the final exposure day. Lungs were inflation-fixed with 10% formalin in phosphate-buffered saline at a pressure of 30 cm water. The left lobe from each lung was later embedded in paraffin. Sections were cut and stained with haematoxylin and eosin for light microscopy. Additional sections were stained with Masson's trichrome to highlight collagen.

Using light microscopy, an experienced pathologist examined the stained lung sections for the presence of particles and evidence of tissue responses to particles.

3.10   MEASUREMENT OF LUNG AND LYMPH NODE BURDENS

3.10.1 Collection of lungs and lymph nodes

The lung and lymph node contents of test dusts were measured on the same animals used for lavage. Following lavage, lung lobes from each animal were separated from the main bronchi and placed in a universal container. In addition, the lung-draining lymph nodes (bronchial/thoracic) from each animal were separated from connective tissue and placed in a small tube. Lung lobes and lymph nodes were stored frozen until required for processing.

3.10.2 Recovery of dust from lavage

To complete the lung burden total, dust was recovered from lavage as follows. Following total and differential counting, the cell fractions were centrifuged at 2000 rpm for 20 minutes and the supernatant discarded. The cell pellets, plus associated dust, were combined with any material recovered by further centrifugation of the cell-free lavage supernatants and stored frozen until required for dust analysis.

3.10.3 Dust measurements

The wet weights of each lung and each set of lymph nodes were recorded. The analysis of lung burden for both dusts was performed using X-ray diffraction (XRD). All minerals have unique XRD traces consisting of a series of peaks each of which represents a characteristic spacing of lattice planes within the mineral. The intensity of the peaks is related to the mass of the mineral in the sample.

The lungs, lymph nodes, and cell pellets were digested in bleach and aqueous suspensions were prepared from samples of each digest. Measured aliquots of the digests were deposited on filters. Calibration filters were prepared from aqueous suspensions of each of the pure test dusts. In pilot experiments, the bleach treatment was shown not to affect the XRD analysis of the ash. The amount of test dust on each filter was determined by measuring the intensity of the primary X-ray diffraction peak and calculating mass from the previously determined calibration factors. The amount of dust present in each lung was then calculated from the mass on the filter. The detection limit for this method was 0.05 mg of mineral per filter.

3.11   STATISTICAL ANALYSES FOR INHALATION EXPERIMENT

The statistical analyses were aimed at testing the null hypothesis that inhaled volcanic ash in the lung is no more inflammatory than the low toxicity control dust TiO_2. Variables analysed as markers of inflammation were lung wet weight, total cells, total PMN, and LDH.

All analyses were carried out by the technique of Analysis of Variance (ANOVA). The bronchoalveolar lavage variables analysed have been noted in the past to have distributions in which the variation, expressed as a standard deviation, increases with the mean value. To adjust for this, analyses were carried out on the scale of the logarithms of the data. There were a few zero values, and the logarithm of zero is undefined. We adopted the standard practice of replacing zeroes with a small value, here using one half of the smallest non-zero value recorded. Percentage PMN was analysed using logistic regression, which yields an Analysis of Deviance analogous to the ANOVA.

The results of the ANOVA analyses are summarised in Chapter 4 in tables of means, with 95% confidence intervals calculated assuming normality of the random variation on the logarithmic scale. These values have then been exponentiated, to give geometric means and their associated confidence intervals, on the scale of the original measurements.

4.   INHALATION STUDY: RESULTS

4.1   SIZE DISTRIBUTION OF BULK ASH

Following sieving and blending, the size distribution of the collected volcanic ash was determined. The size distribution in terms of mass is shown in Figure 4.1. Using this distribution and the known deposition in rat lungs of particles of differing aerodynamic diameters, we were able to estimate the deposition profile of respirable ash particles in the rat lung (Figure 4.2). It was clear from this exercise that the small respirable fraction in the ash would have to be increased to avoid having to use very high aerosol concentrations in the inhalation experiments. This was achieved by using a particle separator (elutriator) that trapped the larger particles before entry of the aerosol to the exposure chambers. Preliminary trials and previous experience with the control dust, TiO_2, showed that a particle separator would not be required for this dust (but see Section 4.3.2 below).

4.2   CHARACTERISATION OF MINERAL CONTENT OF ASH

The cristobalite content of the elutriated ash was 17.7 % (SD 2.1), based upon five measurements. Plagioclase was present in the ash but a full mineralogical assessment was not carried out. However, it would be expected that the composition would be broadly as described by Baxter *et al*. (1999).

Horwell *et al*. (2001) have measured radical activity in several samples of Montserrat ash and found that this activity was not associated with cristobalite particles but mainly with surface iron ions on other types of particle. It is likely that the ash sample used in our inhalation experiment would have a similar radical profile.

4.3   AEROSOL CONCENTRATIONS AND SIZE DISTRIBUTION

4.3.1   Aerodynamic size distributions

Measurements of the size distribution of the aerosolised dusts were obtained from preliminary test aerosols, with the ash elutriator in place, to enable calculation of the fraction likely to deposit in the rat lungs and hence calculation of initial target aerosol concentrations that would produce lung burdens in the same range for the two dusts.

Aerosol size distributions were also monitored during the inhalation experiment, and the calculations and target concentrations revised accordingly. During the inhalation phase, the mass median aerodynamic diameter (MMAD) of the ash was 5.3 µm (gsd 2.4), and the TiO_2 was 1.2 µm (gsd 2.2). This MMAD is for the latter period of exposure when the size distribution appeared to be stable, and when the deposition was much more effective for the TiO_2 (see next section).
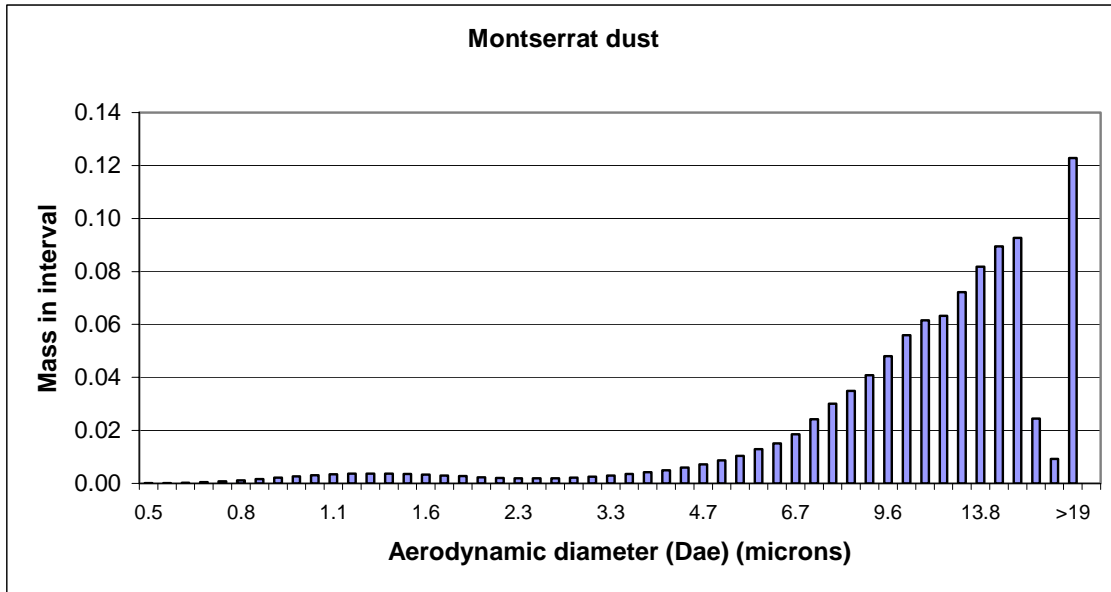
Figure 4.1 Aerodynamic size distribution of the bulk, non-elutriated
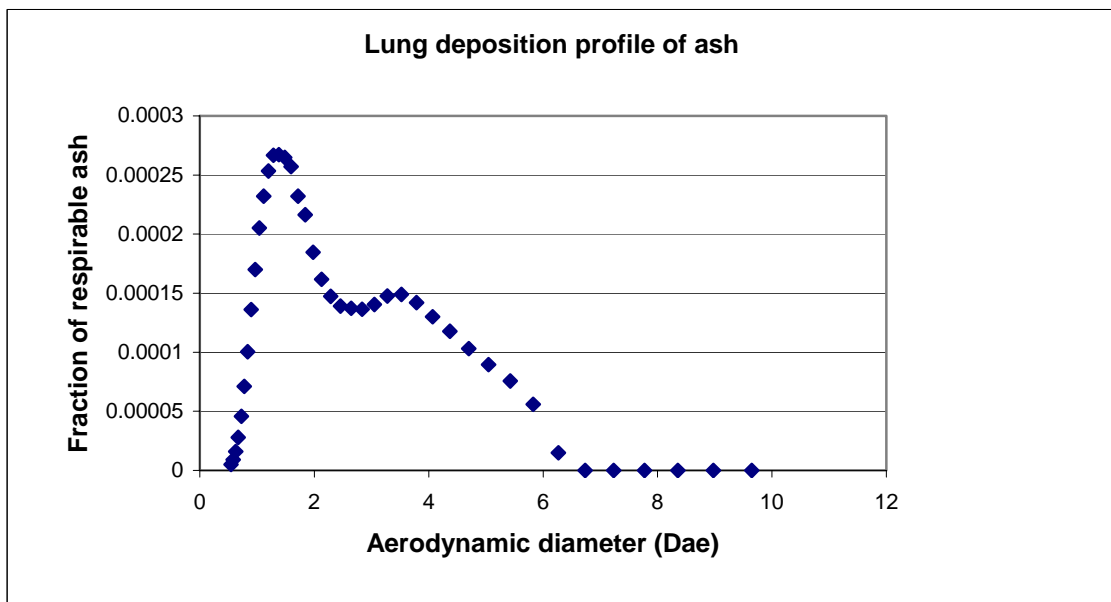Montserrat ash sample (The instrument measures in the range 0.5 to 20 μm)

Figure 4.2 Estimated deposition of non-elutriated ash in rat lungs

4.3.2 Aerosol concentrations

Rats were exposed by nose-only inhalation for 6 hours per day, 5 days per week over an 8-week period. Total and respirable samples were taken from spare ports on the exposure chambers to monitor the aerosol concentrations. Because of the differences in the aerodynamic size distributions of the two dusts, the total volcanic ash concentration had to be approximately twice that of the TiO_2 control dust in order to achieve equal mass deposition in the rat lungs (see Chapter 2, Aims and Experimental Design). Thus, for the volcanic ash, the mean total dust concentration for the eight weeks was 253 mg.m^{-3}, with the daily concentrations fluctuating about this level with a standard deviation of 137 mg.m^{-3}. The overall mean daily total dust concentration for the TiO_2 was 140 mg.m^{-3}, with a standard deviation of 43 mg.m^{-3}.

Initially, the TiO_2 was dispersed without using a size selector. In pilot trials, this produced an aerosol with mass median diameter of about 1 μm and it was thought that monitoring of the total dust would be sufficient. However, the TiO_2 aerosol generation system was changed to include a size selector (as used for the ash) after the first fortnight, as the TiO_2 aerosol was found to be less well dispersed than in the pilot trials. At this time we started to monitor the respirable fraction of the TiO_2 aerosol. Possible reasons why the size distribution of the TiO_2 aerosol had become coarser, compared to the pilot trials, may include longer residence of dust in the brush generator feeder tube leading to greater aggregation of particles, and/or re-suspension of TiO_2 aggregates from the internal surfaces of the aerosol generation system and the exposure chamber.

The mean daily respirable concentrations for the ash and TiO_2 aerosols are shown in Figure 4.3. Following the realisation that the size distribution of the TiO_2 had changed from the pre-exposure trials, the respirable exposure concentration was increased in order to compensate for the expected lower lung deposition. The mean respirable concentration for TiO_2 (based on data from the final 6 weeks) was 120 mg.m^{-3}, with a standard deviation of 40 mg.m^{-3} and for the ash it was 68 mg.m^{-3}, with a standard deviation of 26 mg.m^{-3} (based on data from the whole exposure period). The standard deviations reflect the variation in concentration from day to day (Figure 4.3).
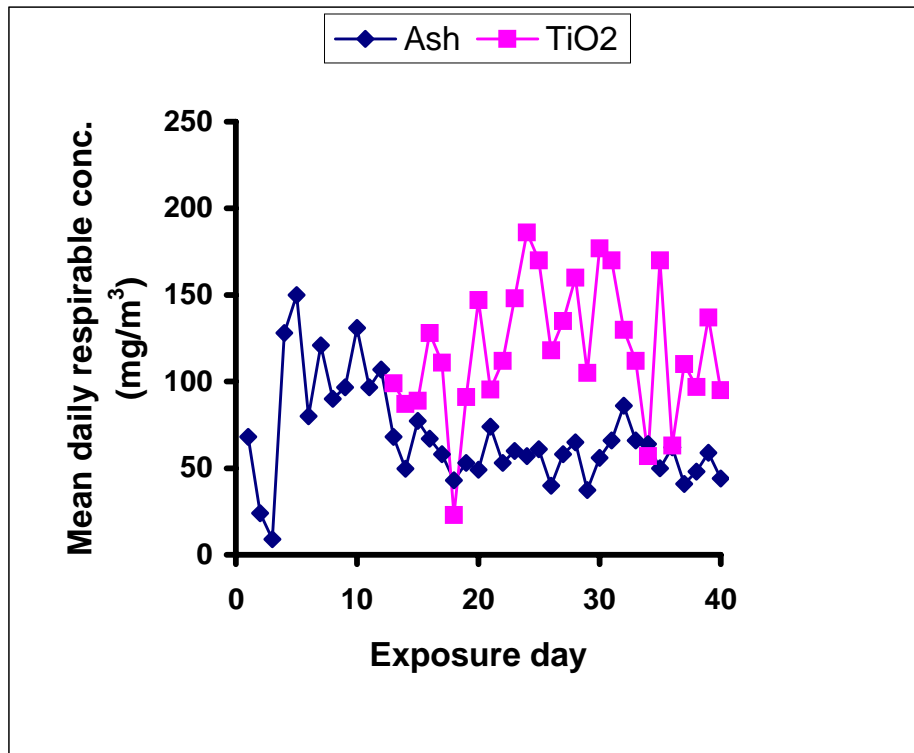
Figure 4.3 Daily mean respirable concentration of ash and TiO_2 aerosols sampled from the exposure chambers ($mg.m^{-3}$). Note that respirable measurements for TiO_2 were started at day 12.

4.4   DUST CONTENT OF LUNGS

4.4.1   Mass burden

The changes in the mass lung burdens of the test dusts with length of exposure, are shown in Figure 4.4a. Lung dust masses for the untreated sentinel controls were almost all zero, as would be expected, and are not shown here, and were not included in the ANOVA. There were no zero burdens in the treated animals. The pattern of TiO_2 lung deposition, lower than ash at the start of exposure and then higher than ash for the later time points, reflects the changes in TiO_2 aerosol concentration described in Section 4.1 above. These divergent patterns were reflected in the results from the ANOVA showing highly statistically significant differences across the dust types and lengths of exposure, and a significant dust×exposure interaction.

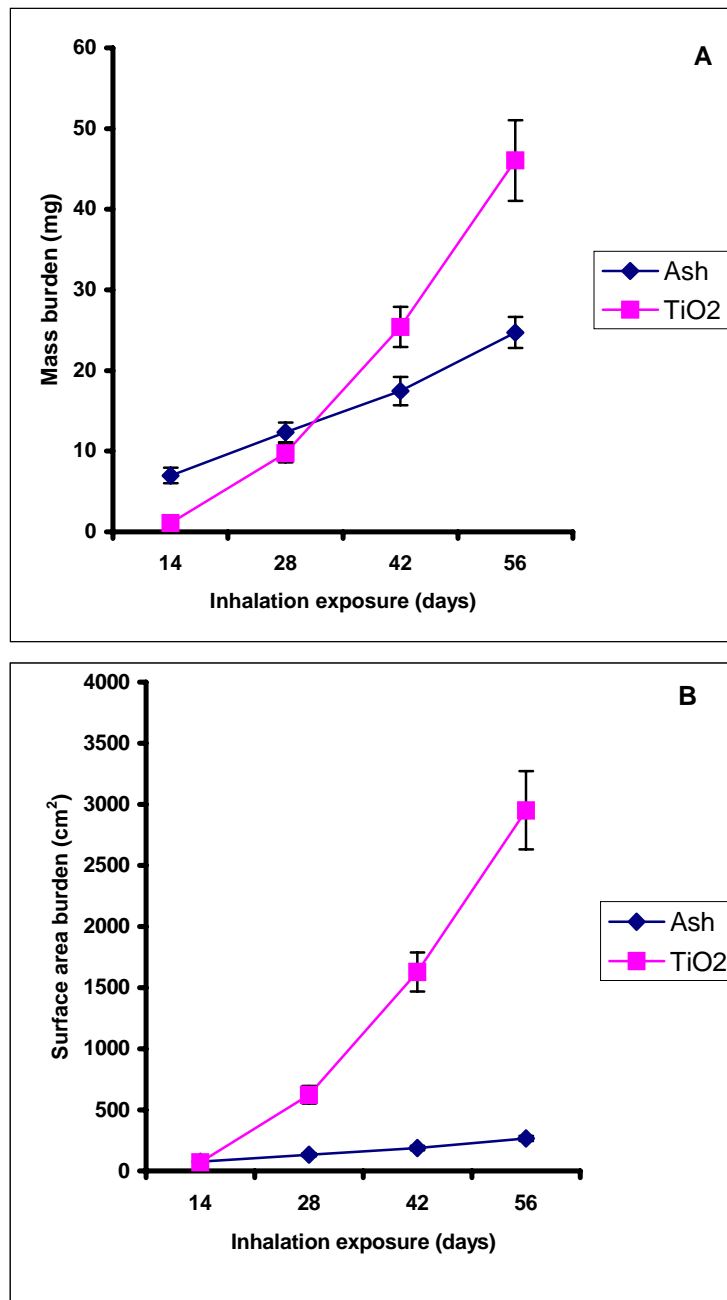**Figure 4.4** Lung burden as mass (A) or particle surface area (B) plotted against period of exposure. Data points are the arithmetic means and the error bars represent 95% confidence intervals.

Table 4.1 shows the pattern of the differences and of the interaction. Burdens of ash increased almost exactly linearly with duration of exposure as would be expected from aerosol concentration history (Section 4.3).

Table 4.1 Lung dust mass burden (mg):
geometric means with *95% Confidence Intervals (CI)*

Treatment	Duration of exposure (days)							
	14		**28**		**42**		**56**	
	Mean	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
Montserrat Ash	6.8	*(6.1, 7.7)*	12.2	*(10.9, 13.7)*	17.3	*(15.4, 19.4)*	24.5	*(21.9, 27.5)*
TiO_2	1.1	*(1.0, 1.2)*	9.6	*(8.5, 10.8)*	25.1	*(22.4, 28.2)*	45.4	*(40.4, 51.0)*

4.4.2 Surface area burden

Assuming that clearance or retention is equivalent at all particle size classes, lung mass burdens can be converted to surface area burdens. Specific surface area measurements on elutriated samples of the dusts from the aerosol generators were made using the nitrogen absorption (BET) method (Brunauer *et al.*, 1938). These values were 10.8 and 64.1 $cm^2.mg^{-1}$ for ash and TiO_2 respectively. The estimated particle surface area burdens are summarised in Table 4.2 and illustrated in Figure 4.4b. Interestingly, the ash and TiO_2 surface area burdens were very similar at 14 days; at the later time points they show much greater divergence than the mass burden data.

Table 4.2 Inferred lung dust surface area burden (cm^2):
geometric means with *95% Confidence Intervals (CI)*

Treatment	Duration of exposure (days)							
	14		**28**		**42**		**56**	
	Mean	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
Montserrat Ash	74	*(66, 83)*	132	*(117, 148)*	186	*(166, 209)*	265	*(236, 297)*
TiO_2	69	*(61, 77)*	614	*(546, 689)*	1608	*(1433, 1805)*	2910	*(2593, 3267)*

4.4.3 Corrections to the lung burden measurement

In previous studies, it has been our normal practice to measure inflammation by bronchoalveolar lavage (BAL) on the day after inhalation exposure, and to measure lung burdens in rats killed three days after exposure. The three-day interval would ensure that any dust deposited in the bronchi and lower trachea would clear, leaving only the alveolar lung burden. However, in the present study, we used the same animals for both purposes (dust and BAL) and kept to the usual time point for BAL, i.e. the day after the final inhalation exposure. Thus there may have been incomplete clearance of dust deposited in the upper airways, which would have been present in the measured lung burden. Furthermore, the fraction of dust depositing in the upper airways would have been different for the two dusts as the ash and the

TiO$_2$ had different aerodynamic size distributions. Therefore, we calculated an estimate of how much dust would have been expected to deposit in the upper airways. The estimate showed that the correction was negligibly small for these two dusts after these exposure periods of 2 to 8 weeks.

4.5   LYMPH NODE BURDEN

Lung-draining lymph nodes were digested and their mass burden of dust determined. Results are summarised in Table 4.3 as the arithmetic mean burdens at each of the time points. The amounts of dust at the first two time points for TiO$_2$ and at the first three points for the ash were below the limit of detection (0.05 mg). At the final point, 56 days, both treatments gave similar mean lymph node burdens of about 1 mg, despite the lung mass burden for the ash being approximately half that of the TiO$_2$ lungs.

Table 4.3 Mass (mg) of ash and TiO2 recovered from lung-draining lymph nodes: arithmetic means with 95% confidence intervals (CI)

| Treatment | Duration of exposure (days) | | | | | | | |
| | 14 | | 28 | | 42 | | 56 | |
	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
Montserrat Ash	<0.05		<0.05		<0.05		1.05	(0.73, 1.37)
TiO$_2$	<0.05		<0.05		0.11	(0.05, 0.17)	0.93	(0.50, 1.36)

4.6   BRONCHOALVEOLAR LAVAGE

4.6.1 Total Cells

The numbers and types of cells recovered by bronchoalveolar lavage (BAL) were recorded from the same lungs used for dust burden. Total cell numbers are shown in Figure 4.5 as the arithmetic means with 95% confidence intervals. There was a clear upward trend in cell numbers with duration of exposure for the ash. For TiO$_2$, the total numbers did not exceed those for sentinel controls at the first two time points, and then increased at the two later time points. This pattern was probably due to the low TiO$_2$ exposure during the first two weeks. There was no trend with time in the untreated sentinel controls.

ANOVA of the log-transformed data confirmed that differences between types and lengths of exposure were highly significant, with volcanic ash showing a greater response than TiO$_2$. Table 4.4 shows the geometric means and 95% confidence intervals for the total cell data. There was a significant type×exposure interaction, confirming that the treatments did not show the same pattern with time.
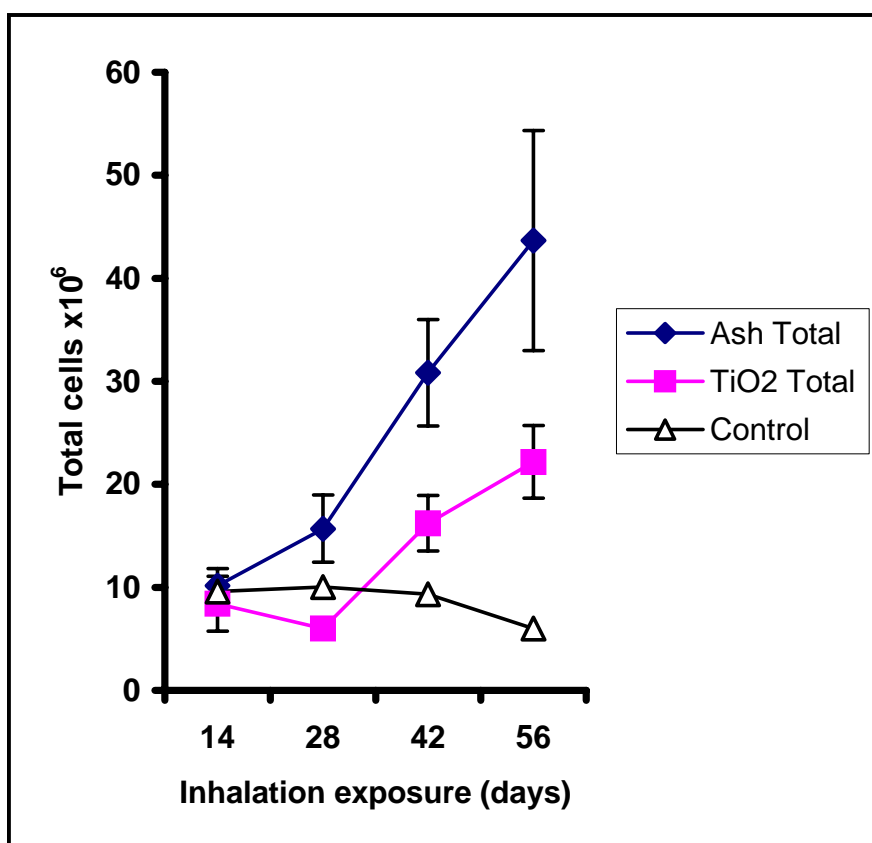
Figure 4.5 Total cells (in millions) recovered by bronchoalveolar lavage.
Data points are the arithmetic means and the error bars represent 95% confidence
intervals.

Table 4.4 Total cells recovered by BAL (millions):
geometric means with *95% Confidence Intervals (CI)*

Treatment	Duration of exposure (days)							
	14		**28**		**42**		**56**	
	Mean	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
Montserrat Ash	9.9	*(8.1, 12.0)*	14.8	*(12.2, 18.0)*	29.8	*(24.6, 36.3)*	41.0	*(33.7, 49.8)*
TiO$_2$	7.6	*(6.3, 9.3)*	5.8	*(4.8, 7.1)*	15.8	*(13.0, 19.1)*	21.5	*(17.7, 26.2)*
Sentinel control	9.5	*(6.1, 14.6)*	10.0	*(6.5, 15.4)*	9.3	*(6.0, 14.3)*	6.0	*(3.9, 9.3)*

4.6.2 Macrophages and Lymphocytes

Numbers of individual cell types (macrophages, lymphocytes and PMN) were derived from the total cell number in BAL and the percentage of the various cell types determined by differential counting of a sample of stained cells.

Macrophage numbers and lymphocyte numbers in BAL fluid followed similar trends to those seen with total numbers of cells and the data are summarised as the arithmetic means in Figures 4.6 and 4.7 respectively. The main difference is that at the first time point, the total cell numbers are similar for ash, TiO_2 and controls, but these cells comprise, for ash, more lymphocytes and PMN with relatively fewer macrophages.
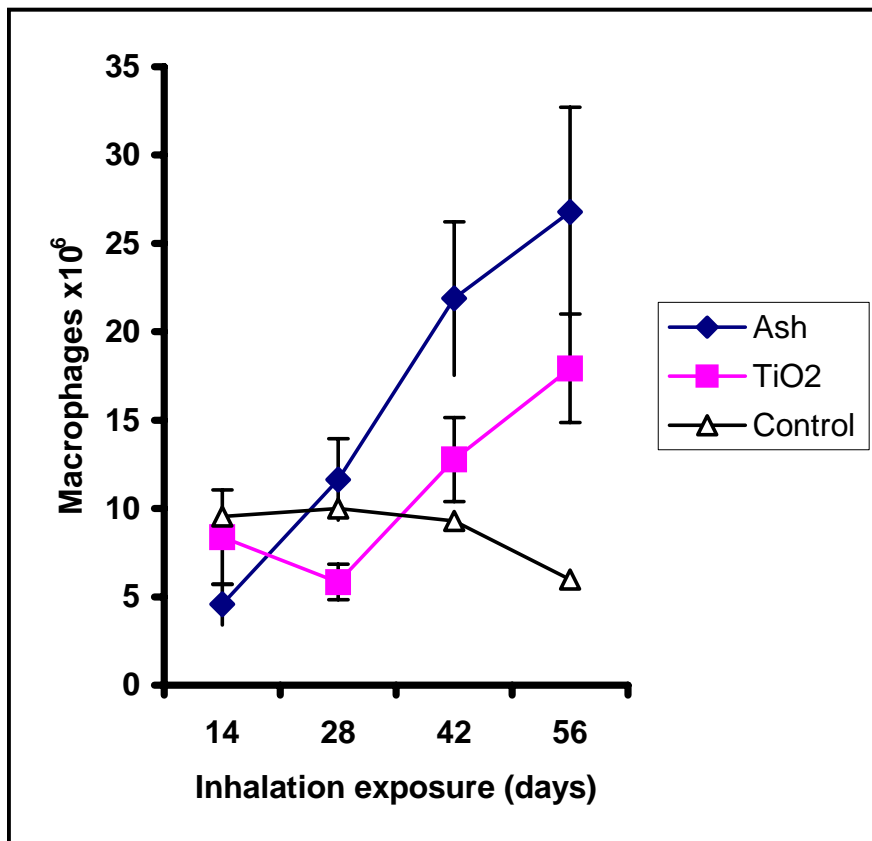

Figure 4.6 Macrophage numbers (millions) recovered by bronchoalveolar lavage. Data points are the arithmetic means and the error bars represent 95% confidence intervals.

Figure 4.7 Lymphocyte numbers (millions) recovered by bronchoalveolar lavage. Data points are the arithmetic means and the error bars represent 95% confidence intervals.

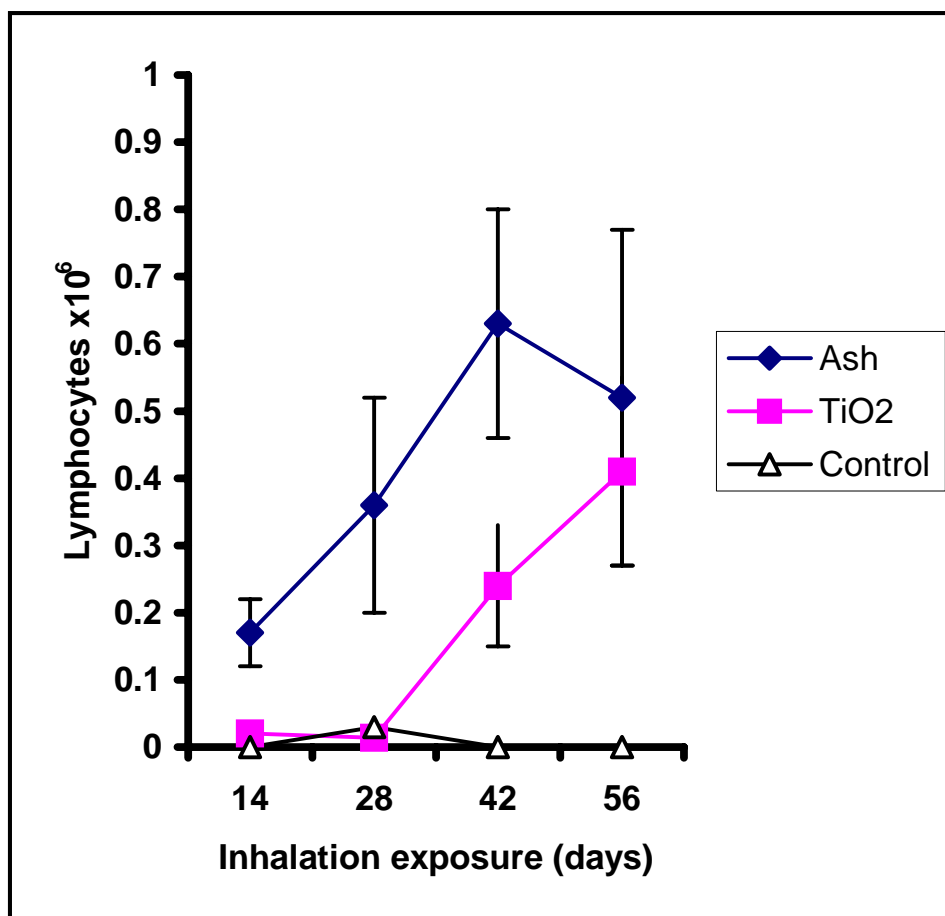### 4.6.3 PMN

The presence of marked numbers of polymorphonuclear neutrophils (PMN) within lavage demonstrate that an inflammatory response has occurred within the lung. PMN numbers are shown in Figure 4.8 as the arithmetic means with 95% confidence intervals. The ash shows a much greater recruitment of PMN than the TiO_2 for all exposures and the dependence on exposure time appears rather different for the two dusts. For the TiO_2-treated animals, inflammation begins to appear between day 28 and day 42 which is what was predicted for this dust. The sentinel animals, as expected, had very low levels of PMN and no trend with time.

Table 4.5 summarises the geometric means and 95% confidence intervals. Results of the ANOVA showed highly statistically significant differences between the types and the lengths of exposure, and a highly significant type×exposure interaction, confirming that the treatments did not follow the same pattern with time.
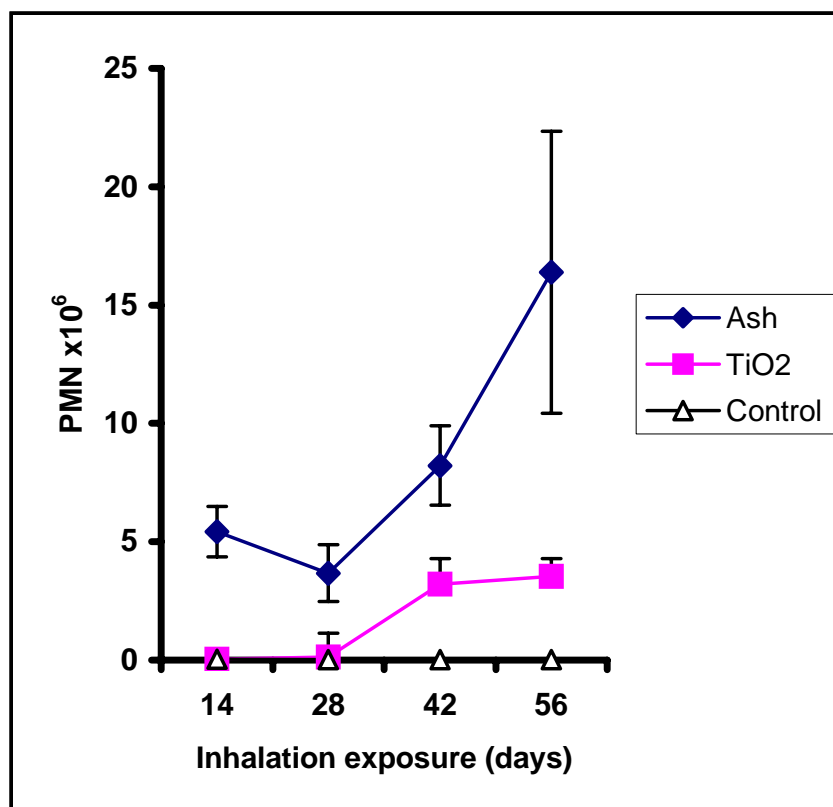
Figure 4.8 Numbers of inflammatory PMN cells (millions) recovered by
bronchoalveolar lavage.
Data points are the arithmetic means and the error bars represent 95% confidence
intervals.

Table 4.5 Total PMN (millions):
geometric means with *95% Confidence Intervals (CI)*

Treatment	Duration of exposure (days)							
	14		28		42		56	
	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
Montserrat Ash	5.20	*(3.21, 8.42)*	2.84	*(1.75, 4.60)*	7.82	*(4.83, 12.67)*	14.43	*(8.91, 23.38)*
TiO$_2$	0.02	*(0.01, 0.02)*	0.08	*(0.05, 0.13)*	2.57	*(1.59, 4.17)*	3.55	*(2.19, 5.76)*
Sentinel control	0.03	*(0.01, 0.09)*	0.01	*(0.00, 0.04)*	0.02	*(0.01, 0.06)*	0.02	*(0.01, 0.05)*

4.6.4   Percentage PMN

PMN numbers in BAL fluid provide a measure of inflammation in the lung. The relative
numbers of PMN to other cell types, such as macrophages, may also be important in defining

the inflammatory response. Accordingly, PMN numbers expressed as percentages of the total cell numbers, are shown in Figure 4.7; most of the other cells in lavage were macrophages. The ash data show the highest mean value (>50%) at the 14-day point, with a fall at day 28 and an upward trend for the final two points. The pattern was different for the TiO_2 treatment group where there was an upward trend through the first three time points and possibly a levelling off by day 56. There was no trend with time for the sentinel animals.
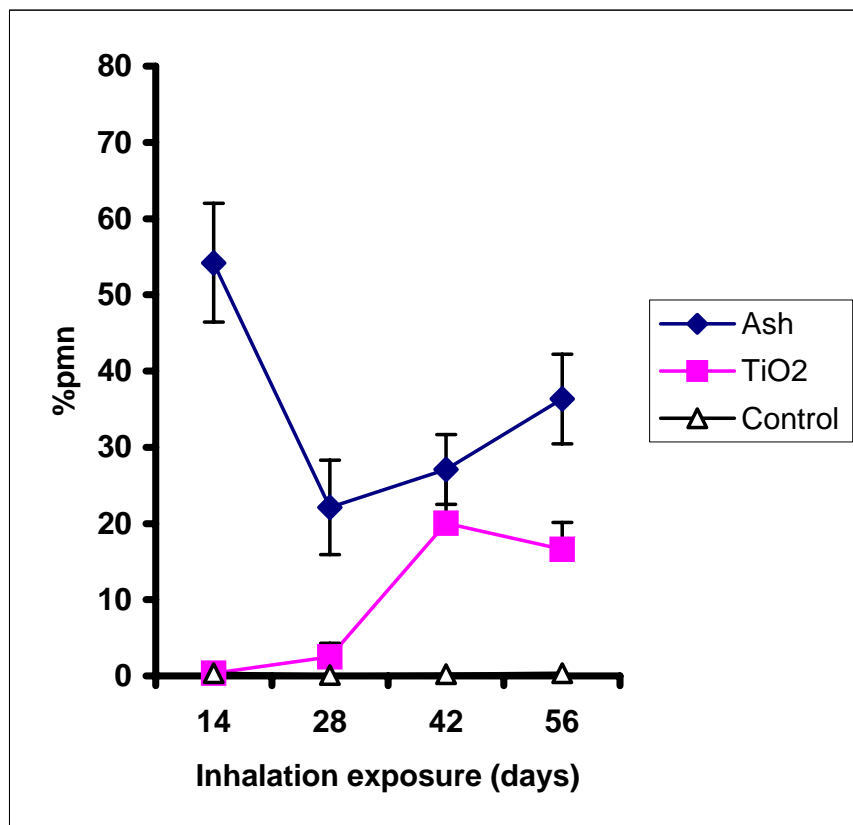

Figure 4.9 Percentage PMN recovered by bronchoalveolar lavage.
Data points are the arithmetic means and the error bars represent 95% confidence intervals.

The variable %PMN was analysed using logistic regression, with the count of PMN as the numerator and the total cells counted as the denominator of the proportion. The residual deviance was 16.3 where binomial theory predicts exactly 1.0, so that there was evidence of considerable extra-binomial variation, and this has been taken into account in the calculation of standard errors and confidence intervals. This is most likely due to inter-animal variation in response. Predicted means and confidence intervals are given in Table 4.6.

Results of the analysis showed, as for total PMN numbers, highly significant differences, and evidence that the response was not parallel in the treatment groups. As can be seen from the predicted means in Table 4.6 and Figure 4.7, %PMN did not show such a clear exposure-response relationship with increasing ash nor with increasing TiO_2. There was no response in the sentinel controls.

Table 4.6 Percentage PMN amongst cells recovered by BAL:
predicted mean percentages and *approximate 95% confidence intervals (CI)*

Treatment	Duration of exposure (days)							
	14		28		42		56	
	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
Montserrat Ash	52.6	*(34.1, 81.2)*	19.2	*(12.4, 29.5)*	27.2	*(24.8, 29.7)*	36.5	*(33.7, 39.4)*
TiO$_2$	0.2	*(0.2, 0.35)*	1.43	*(0.93, 2.20)*	16.3	*(10.6, 25.2)*	16.5	*(10.7, 25.4)*
Sentinel control	0.3	*(0.13, 0.89)*	0.2	*(0.06, 0.45)*	0.2	*(0.09, 0.64)*	0.3	*(0.11, 0.73)*

4.6.5 Lactate dehydrogenase (LDH)

The presence of increased levels of the intracellular enzyme, LDH, in BAL fluid provides an indication of cell injury and death within the air spaces of the lung. Table 4.7 below shows LDH concentrations in lavage fluid as geometric means. There is a clear upward trend in LDH with duration of exposure for the TiO$_2$ and for ash, with the ash again showing much greater levels than the TiO$_2$ at all exposure times. There was no trend with time in the untreated sentinel controls. Results of the ANOVA showed highly statistically significant differences between the types of dust and the durations of exposure, and a significant type×exposure interaction, suggesting that the treatments did not show the same pattern with time.

Table 4.7 LDH (International Units) recovered by bronchoalveolar lavage:
geometric means with *95% Confidence Intervals (CI)*

Treatment	Duration of exposure (days)							
	14		28		42		56	
	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
Montserrat Ash	139	*(114, 169)*	312	*(256, 380)*	314	*(258, 383)*	560	*(460, 682)*
TiO$_2$	54	*(45, 66)*	63	*(51, 76)*	156	*(128, 190)*	282	*(231, 343)*
Sentinel control	45	*(29, 71)*	61	*(39, 95)*	39	*(25, 61)*	60	*(38, 93)*

4.7   COMPARISONS OF BURDEN WITH MARKERS OF INFLAMMATION

4.7.1   PMN numbers and mass burden

PMN numbers (Figure 4.10) plotted against mass of dust in the lung, show that the volcanic ash produced a markedly greater inflammatory response relative to mass lung burden. The TiO_2 lung burdens rose to nearly twice the final mean level for the ash, but still produced much less inflammation.
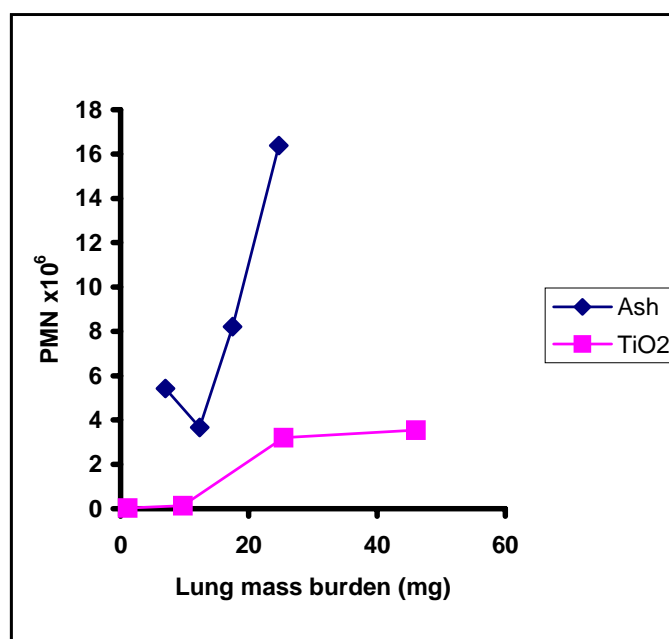

Figure 4.10 PMN numbers (millions) plotted against lung dust mass burden (mg)

Scatter plots of PMN number and mass burden for individual animals also indicate that the dependence of PMN number on mass is different for the two dusts (Figure 4.11). There is clear separation of the ash and TiO_2 data sets. This indicates that the specific toxic activity (per unit mass) of ash particles is greater than that for the TiO_2 particles in terms of inflammogenicity. The correlation coefficients for the trendlines were 0.623 and 0.730 for ash and TiO_2 respectively.
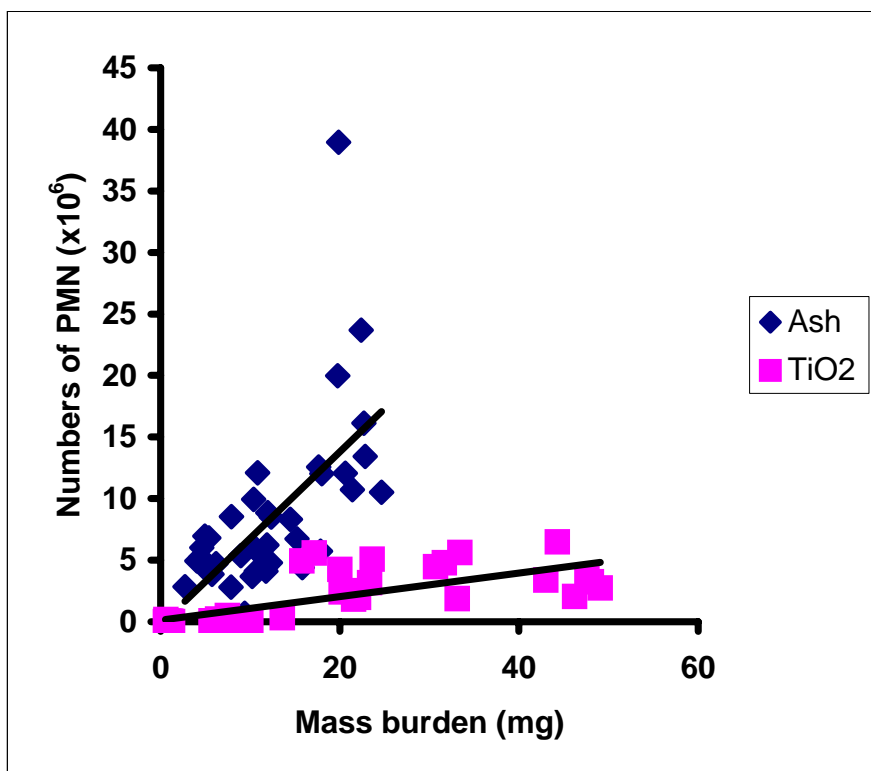
Figure 4.11 Scatter plots of PMN numbers and mass burden from individual animals exposed to volcanic ash or TiO_2

4.7.2   PMN numbers and surface area burden

In previous studies where TiO_2 was compared to a coarser nuisance dust, barium sulphate, we showed that if the inflammation, as PMN numbers, was plotted against surface area of the particulate lung burden, then the data collapsed onto a single trend (Tran *et al.*, 2000). So if the volcanic ash were behaving as a nuisance dust, then the data would have been expected to collapse after converting lung burdens to surface area. However, the conversion magnifies the difference between the two dusts (Figure 4.12). This comparison suggests that the ash is markedly more toxic than nuisance dust particles of similar size.

By day 28, inflammation in TiO_2-treated lungs was greater, on average, than that seen in untreated control lungs (Figure 4.8; Table 4.5). At this point the TiO_2 lung burden, expressed in terms of particulate surface area (Figure 4.12), was approximately 600 cm^2 a value that is consistent with our previous studies estimating a threshold for inflammation for this dust in the range 300 to 600 cm^2 (Tran *et al.* 2000). Derivation of a threshold value for the ash PMN data was not possible because of the high early response.
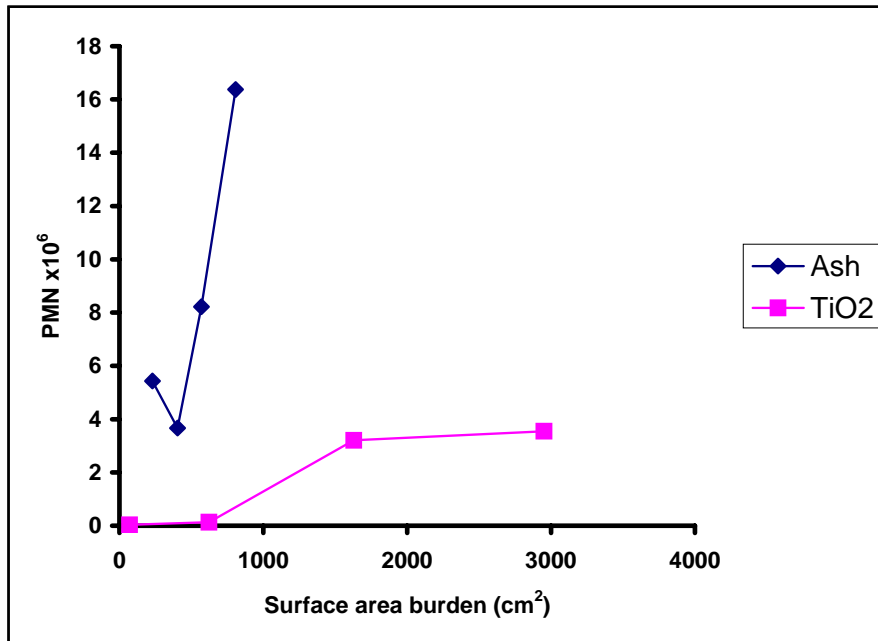
Figure 4.12 PMN numbers (millions) plotted against lung dust burden as particle surface area (cm2)

4.7.3 LDH and mass burden

The marker of cell injury, LDH, was also plotted against lung mass burden (Figure 4.13). As with PMN numbers, the ash results in a greater release of LDH than with TiO_2 for similar mass deposits.
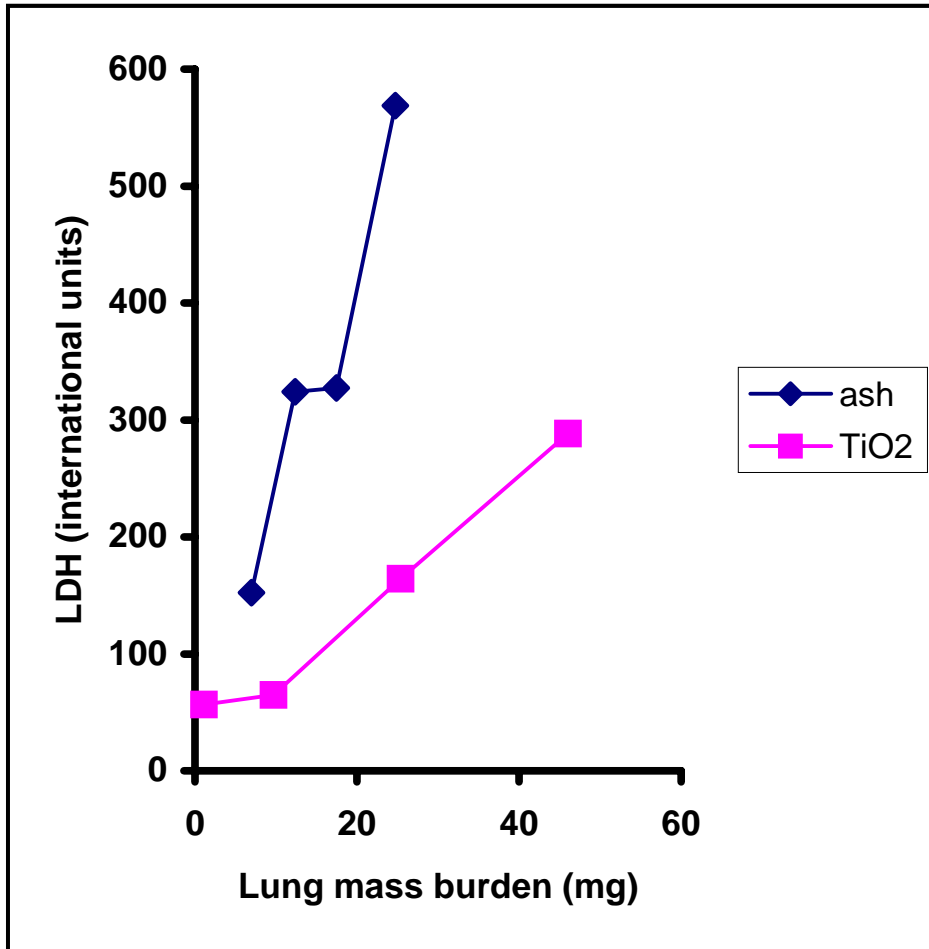
Figure 4.13 Lactate dehydrogenase (LDH) levels in lavage fluid plotted against lung mass burden (mg)

4.7.4 LDH compared to PMN

LDH and PMN are markers of pulmonary toxicity following inhalation of particles or other materials. In this study, LDH and PMN were both measured in each individual animal. Figure 4.14 shows PMN numbers plotted against their LDH equivalents for each animal. There is scatter in the data, which might be expected from inter-animal variation and uncertainties associated with determining PMN numbers and LDH from lavage, but it is clear that there is an upward trend. The data thus provide some evidence that LDH concentration in lavage is proportional to the numbers of PMN.
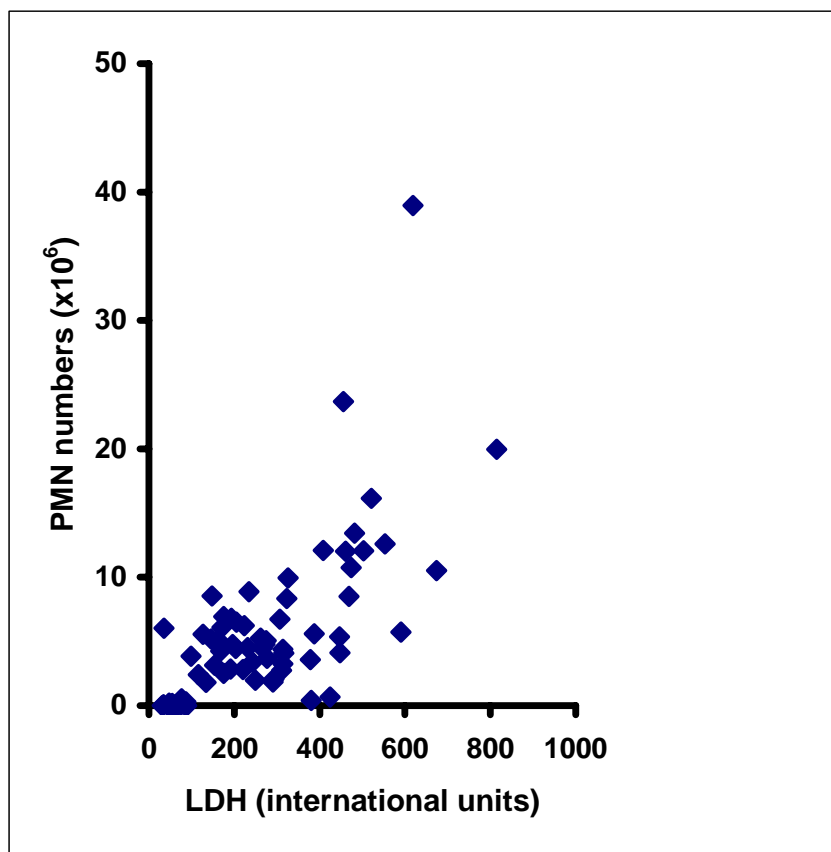
Figure 4.14 PMN numbers plotted against lactate dehydrogenase (LDH) levels in lavage fluid (pooled data from TiO$_2$ and ash treatment groups)

4.8   PATHOLOGY

The six slides from untreated control animals showed completely normal lung tissue. This would be expected from young rats from a healthy animal population. Rats treated with either titanium dioxide or volcanic ash for 56 days all showed a vigorous macrophage reaction. Large numbers of pulmonary macrophages were present around the bifurcations of the terminal and respiratory bronchioles and in the adjacent alveoli. Most macrophages contained dust particles but this was particularly marked in the titanium dioxide experiment where many macrophages were completely packed with this material which although white in reflected light is opaque and black in the transmitted light of the microscope. Even macrophages completely packed with titanium dioxide appeared close to normal size but macrophages from the volcanic ash experiment were frequently larger with a pale 'foamy' cytoplasm. Macrophages containing titanium dioxide had a particular tendency to aggregate together so that some alvioli were completely filled with these cells. In contrast macrophages with volcanic ash were more loosely distributed. At some of the bifurcation sites, particularly in animals tested with volcanic ash, macrophages with dust were present in the interstitial position where they were present with a few fibroblasts. At the same time epithelial cells were more rounded than normal and these bifurcation sites represented the earliest stages of 'microgranuloma' formation. The walls of the alvioli adjacent to the bifurcation sites were also thickened mainly due to the rounding of the epithelial cells. The use of Masson's trichrome stain demonstrated a slight positive reaction for collagen within some microgranulomas but

this was so slight that it may have been due to the separation and dispersion of collagen bundles already present at these sites.

5.   SUMMARY OF IN VITRO AND INSTILLATION EXPERIMENTS (NAPIER UNIVERSITY)

5.1   INTRODUCTION

As part of this study, Napier University conducted some *in vitro* assays to test the cytotoxicity of the Montserrat ash, and a short-term lung instillation experiment in rats to examine the inflammogenicity of the ash. This work is fully reported in the Appendix, but a summary is presented here.

The purpose of these tests was to assess the relative toxicity of various samples of Montserrat volcanic ash and to see whether *in vitro* tests could be used to indicate likely toxicity following *in vivo* exposure. Five samples of respirable Montserrat Volcanic Ash (MVA) were compared with three control dusts, titanium dioxide, DQ12 quartz, and a cristobalite. Three of the ash samples (M1, M2, M3) had been tested in previous *in vitro* studies (Cullen and Searl, 1998; Wilson *et al.* 2000) and M4 was derived from the sample collected for the inhalation study reported here. The fifth ash sample, RMVA, was kindly supplied by Professor Roy Richards and had been used for his DfID-funded lung instillation study (Richards, personal communication).

Three toxicological assays were carried out to determine biological reactivity:

1. the *in vitro* haemolysis of sheep red blood cells was used as an indicator of surface reactivity and potential cytotoxicity;

2. release of lactate dehydrogenase (LDH) from cells of the human type II epithelial cell line, A549, as an indicator of the potency of dusts to damage the membrane.

3. ash particles were instilled into rat lungs and bronchoalveolar lavage (BAL) used to determine inflammogenicity.

Each of the assays was repeated on three separate occasions using separate aliquots of particles, blood and cells. Three observations were made for each *in vitro* experiment and the mean calculated from these observations. The results represent the mean of the three experiments ± standard error of the mean (SEM). The LDH data were analysed using one way analysis of variance with Tukey's pairwise comparisons. All other data were analysed using a Students t-test assuming equal variance.

5.2   RESULTS

5.2.1   Haemolysis

This assay compared the effects of two samples of ash, M1 and M4, with the positive control dust, DQ12 quartz, and the negative control dust, titanium dioxide. There were insufficient amounts of the other volcanic ash samples available to enable use in this assay. DQ12 induced significantly greater haemolysis than TiO_2 at all doses tested (p<0.01). M1, a sample of Montserrat ash tested previously (Cullen and Searl, 1998; Wilson *et al.* 2000) exhibited a significantly greater haemolytic activity compared to TiO_2 at the highest dose of 20 mg/ml (p<0.01), but the difference was only small and probably not biologically significant. The haemolysis induced by M4, the ash used in the inhalation experiment, was not significantly different to that induced by TiO_2 at any of the doses tested.

5.2.2 LDH release from A549 cells

This assay compared the effects of five volcanic ash samples and the three control dusts. The only particle treatment that caused significant ($p < 0.01$) release of LDH compared to the TiO_2 (negative control) treatment, at any dose, was cristobalite at the doses of 100 and 200 µg/ml. However the impression gained from the data was that there was some activity in the ash samples, as the dose response curves do not cluster around the control but spread up towards the positive controls. It was especially notable that M4 and RMVA were at the extremes of the range of effects with M4 being close to TiO_2 and RMVA being closest to cristobalite. It is emphasised, however, that neither RMVA nor M4 differed significantly from the TiO_2 control.

5.2.3 Inflammatory effects of Montserrat volcanic ash

Rat lungs were lavaged approximately 24 hours following instillation of saline suspensions of the M4 and RMVA ash samples, TiO_2, or DQ12. Note that the ash and TiO_2 samples were instilled at similar doses expressed as particle surface area. The only significant ($p < 0.01$) differences from the saline control BAL cell profile were with DQ12 quartz and applied to total cells, macrophages and neutrophil numbers. There were no significant differences between TiO_2 and the ash samples.

5.3 CONCLUSIONS

Different assays gave different indications of the likely toxicity of volcanic ash.

- The *in vitro* haemolysis and *in vivo* short-term PMN recruitment assay following instillation into the rat lung indicate that the volcanic ashes have little activity compared to DQ12 quartz.

- The findings from the *in vitro* LDH assay of epithelial cell membranolysis were that:

  the ash samples were significantly less toxic than cristobalite;

  DQ12 quartz appeared to be less toxic than cristobalite at the lower concentrations;

  The results suggested that the volcanic ash samples were more toxic than TiO_2, although the differences were not statistically significant.

- On the basis of these *in vitro* assays, the volcanic ash samples have low toxicity.

- The failure of the instillation assay to demonstrate differences between TiO_2 and volcanic ash confirms the inability of the assay to discriminate between samples of different toxicity when the overall toxicity of the samples is low. The assay was, however, capable of discriminating between low and high (quartz) toxicity dust samples.

6.   DISCUSSION

6.1   INHALATION STUDY

6.1.1   Inflammation and burden

This inhalation study has shown that lung inflammation, as PMN recruitment, was greater for ash-exposed rats than the corresponding values for TiO_2-exposed rats at all time points. PMN numbers or LDH plotted against mass of dust in the lung, showed that the volcanic ash produced a markedly greater response relative to mass lung burden. The TiO_2 lung burdens rose to nearly twice the final mean mass for the ash, but still produced much less inflammation. Inflammation was not due to contaminating microorganisms or bioactive molecules such as endotoxin because the test dusts had been heat-treated (250 ^{o}C for 1 hour) before use in the inhalation experiment. By the end of the 8-week inhalation exposure, similar mass quantities of the ash and TiO_2 particles were recorded in the hilar lymph nodes, despite the approximately 2-fold greater mass lung burden in TiO_2-treated rats. As a more toxic dust would be expected to accumulate more quickly in the lymph nodes, this finding suggests that the volcanic ash was more toxic than the control dust.

In previous studies where the TiO_2 was compared to a coarser nuisance dust, barium sulphate, we showed that if the inflammation (as PMN numbers) was plotted against surface area of the particulate lung burden, then the data from the two dusts collapsed onto a single trend (Cullen *et al* 2000; Tran *et al*. 2000). So if the volcanic ash were behaving as a nuisance dust, then the data would have been expected to collapse after converting lung burdens to surface area. However, the conversion magnifies the difference between the two dusts. This comparison suggests that the ash is markedly more toxic than nuisance dust particles of similar surface area.

The trend for the rise in PMN with lung burden suggests that a plateau may be reached for the TiO_2 towards the end of the exposure. By contrast, PMN numbers in ash-treated animals go higher and show no sign of reaching a plateau. Extrapolation to other doses would need to take account of the apparently different dose dependence for ash and low toxicity dusts such as TiO_2.

The results for the TiO_2 showed inflammation occurring at a lung burden, expressed in terms of particulate surface area, of approximately 600 cm^2 and that value is consistent with our previous studies estimating a threshold in the range 300 to 600 cm^2 (Tran *et al*. 2000). The consistency supports the comparability with other data from previous IOM studies. It was not possible to determine a surface area threshold value for the ash because of the high early inflammatory response. If the ash had behaved as a low toxicity dust, then the experimental design would have enabled the estimation of a threshold, as seen with TiO_2.

6.1.2   Pathology

Additional groups of six animals were sacrificed at the 8-week time point for histopathological examination of the lungs. The widespread macrophage reaction present in the lungs of rats treated with either titanium dioxide or volcanic ash is consistent with a heavy exposure to respirable dust particles. The close aggregation of groups of macrophages packed with titanium dioxide has been reported before for other 'innocuous' dusts. In contrast the wider dispersion of foamy macrophages in animals treated with volcanic ash is seen with more toxic materials such as quartz. The observation of foamy macrophages is associated with a greater tendency for dusted macrophages to reach the interstitial position and form

microgranulomas. These lesions represent the earliest stages of pulmonary fibrosis in experiments using fibrogenic dusts and their presence suggests that volcanic ash may well be such a material. However, 56 days is too short an exposure to cause definite fibrosis and if after this exposure the animals had been allowed to survive for their full lifespan (without further exposure), it is possible that the pulmonary lesions observed would have resolved completely.

In summary, the inhalation experiment showed the ash to be more toxic than a nuisance dust, and that within the 8 week exposure it produced the earliest stages of fibrosis that suggest that fibrosis would have been caused if the exposure had been prolonged.

6.2   INSTILLATION EXPERIMENT

The lung instillation experiment found no differences in cell recruitment 24 hours post-treatment between two samples of Montserrat ash, TiO_2, or saline. Only DQ12 caused a statistically significant increase in PMN numbers. The ash samples used were M4 from the inhalation experiment and that used in Professor Richards' instillation experiment (RMVA) described below (Section 6.3).

6.3   OTHER ANIMAL STUDIES ON VOLCANIC ASH

The findings of our study are similar to those found in a number of studies generated in the aftermath of the explosive eruptions of the Mount St. Helens volcano in Washington State, USA in 1980.

We summarise the results from one long-term and one short-term inhalation studies with a range of different end points, and two instillation studies with Mount St Helens Ash

The crystalline silica content of the Mount St. Helens ash was variously reported as 3 to 7% by weight (Dollberg *et al*. 1986), less than the approximately 18% for the sample of Montserrat volcanic ash used in the study reported here.

Two week inhalation exposure

Martin *et al*. (1983) exposed rats to Mount St. Helens ash or quartz at 100 mg.m^{-3} for two weeks and then observed them for up to nine months. After six months, ash-exposed animals had moderate interstitial thickening and fibrosis whereas those exposed to quartz had severe fibrosis. The quartz group, in contrast to the ash group, also had a marked increase in inflammatory granulocytes, protein, and phospholipids recovered by bronchoalveolar lavage. *In vitro* chemotaxis of lavaged macrophages was impaired in both the ash and quartz groups, a general finding in macrophages from particulate-exposed lungs (Donaldson *et al*. 1990b; Brown *et al*. 1992). We have also reported that another *in vitro* alveolar macrophage function, the production of superoxide, is markedly reduced following dust exposure *in vivo* (Donaldson *et al*. 1988b; Cullen *et al*. 1995). Other laboratories have also reported defects in macrophage function following inhalation of quartz (Dauber *et al*. 1982) or TiO_2 or carbonyl iron (Warheit *et al*. 1997).

Inhalation exposure for 12 months

Wehner *et al*. (1983) exposed rats to respirable aerosols of Mount St. Helens ash at 5 or 50 mg.m^{-3} for up to 12 months. Quartz at 50 mg.m^{-3} was used as a positive control; there was no inert particle control. Note that the 5mg.m^{-3} concentration is comparable to the occupational exposure limit for a low toxicity (nuisance) dust. Currently the UK limit is 4 mg.m^{-3}. After 12

months of exposure, the lungs of animals exposed to 5 mg.m^{-3} showed minimal pathological changes consisting of macrophage accumulation, lymph node granulomas, peribronchiolar lymphoid hyperplasia, and interstitial reaction. These changes were more marked in the 50 mg.m^{-3} ash group that also showed alveolar proteinosis. The quartz-exposed lungs had pronounced alveolar proteinosis and alveolar hyperplasia, neutrophil recruitment, and areas of fibrosis. Lung burdens were not measured in that study, nor were lungs investigated by bronchoalveolar lavage.

Instillation studies with Mount St. Helens ash

Differences between quartz and volcanic ash were also seen in an instillation study where ash, soil, or quartz were administered to rats at high doses, either as a single bolus of 40 mg or multiple weekly doses that amounted to 22 or 77 mg (Sanders *et al*. 1982, 1983). Animals were killed at various times up to 400 days following the single or initial treatment. The ash-treated animals developed simple pneumoconiosis, limited fibrosis, enlarged mediastinal lymph nodes, and moderate lipoproteinosis, changes that were more marked than in the lungs of soil-exposed animals. The quartz-treated animals had much more intense fibrosis, greater alveolar lipoproteinosis than the ash group.

Another rat instillation study used a smaller single dose of 10 mg volcanic ash and found granuloma formation by six months (Vallyathan *et al*. 1983). However, that study had no positive or inert controls.

Instillation with Montserrat volcanic ash

Richards and colleagues have conducted two instillation studies in rats with samples of respirable Montserrat volcanic ash. In the first study, 1 mg of instilled respirable ash, collected either from phreatic explosion (8.6% cristobalite) or pyroclastic flows (20.1% cristobalite), had little effect over a 9-week period compared to cristobalite or DQ12 quartz that induced marked inflammation and enlargement of lymph nodes (Housley *et al*. 2002). In the second study, rats were instilled with 1, 2.5 or 5 mg of respirable ash (20% cristobalite) and followed for up to 49 weeks (Richards 2002, personal communication). Anorthite and cristobalite served as inert and positive controls respectively. The anorthite and 1 mg ash treatments showed no toxic effects. Preliminary results showed that the 2.5 and 5 mg doses of ash caused enlargement of hilar lymph nodes, most noticeably at 49 weeks. Compared to controls, there were significant increases in total and PMN cell numbers in lavage at 49 weeks in the 5mg ash group, but no evidence of fibrosis. In contrast the cristobalite treatments caused progressively greater inflammation, cell proliferation, early enlargement of lymph nodes, and, by 49 weeks, fibrosis of lungs and lymph nodes. The late appearance of inflammation and lymph node pathology at 49 weeks in the 5 mg ash group suggests that there may be a delayed toxic effect. Delayed pathological effects have been seen with sand instilled into rat lungs (Le Bouffant *et al*. 1982), possibly due to the gradual leaching of aluminium that is known to reduce the toxicity of quartz (Brown *et al*. 1989). Such results suggest that short-term *in vivo* experiments and assays that essentially measure particle surface activity, might not be good predictors of longer-term pathological effects.

6.4   IN VITRO STUDIES

The present study also included *in vitro* experiments. The various ash samples and the quartz caused greater mean release of LDH from A549 lung epithelial cells, especially at higher dust concentrations, than the control dust, TiO$_2$, but the differences were not statistically significant. Only cristobalite was significantly more active than TiO$_2$. In a previous study we had found that three of the ash samples (M1, M2, M3), with differing cristobalite content,

were significantly more toxic (by LDH release) than TiO_2 and they were as active as quartz (Cullen and Searl 1998). Interestingly, the cytotoxicity did not correlate with the cristobalite content. Recent evidence from Horwell and colleagues (2001) shows that most of the reactive radical production by ash particles is not associated with cristobalite. See Section 6.6.

The haemolysis results from the present study were similar to those found previously for the samples M1, M2 and M3, with the M1 ash sample and quartz showing significantly greater toxicity than the control TiO_2, (Wilson *et al.*, 2000). By contrast, the M4 ash used in our inhalation experiment was not significantly more haemolytic than the control dust in the present *in vitro* study.

Previous *in vitro* tests on other volcanic ashes have produced comparisons which show some similarity in the toxicity relative to quartz, and in the range of response among ash samples. *In vitro* experiments on Mount St. Helens ash have shown that it was less toxic to alveolar macrophages and A549 cells than quartz (Martin *et al*. 1984). Vallyathan *et al*., (1984) carried out a rare comparison of ash samples from three different volcanoes, El Chichón, Galunggung, and Mt. St. Helens, using haemolysis and alveolar macrophage cytotoxicity assays. All samples were toxic to macrophages but ash from Galunggung was the most potent. There were wide differences between the ashes for haemolysis, but activity was less than that of quartz and the toxic effects correlated with the surface area of the ash particles.

6.5 COMPARISONS WITH OTHER DUSTS

Although this report shows that Montserrat volcanic ash is clearly more toxic than a benchmark low toxicity dust, TiO_2, it is valuable to compare the ash response with that seen with other well-studied dusts such as quartz and coal mine dust. Using data from previous IOM studies (Jones et al 1988; Donaldson *et al*. 1988a, 1990a) and one conducted at NIOSH (Porter *et al*. 2002), we have been able to make such comparisons.

The previous IOM studies used a pure quartz (Sikron F600, A9950) and coal mine dusts with differing quartz content, over a longer inhalation period, and examined lung inflammation in an inbred strain of rat (PVG) (Donaldson *et al*. 1988a, 1990a) but measured lung burden (Jones *et al*. 1988) in similar exposures in HAN Wistar rats, the strain used in the present volcanic ash study. During that previous exposure to pure quartz, the numbers of PMN increased steadily from one assay time point to the next. For quartz exposure producing a lung burden (in Han Wistar rats) of 2 mg, the number of PMN (in PVG rats) was 5×10^6.

PMN relative to lung burden results from the IOM quartz study (Jones *et al*. 1988; Donaldson *et al*. 1988a, 1990a) are compared to the volcanic ash burden-PMN results in Figure 6.1. Minusil-5 quartz in Fischer 344 rats in the NIOSH study (Porter *et al*. 2002) appeared to be slightly more toxic than the Sikron F600 in the IOM study but show a similar trend (data not shown). It can be seen that, weight for weight, the quartz is considerably more inflammogenic than Montserrat ash.

The comparisons made with the coal mine dust data indicate that it is similar to the ash in terms of numbers of PMN for given mass burden, as also illustrated in Figure 6.1. These coal mine dust data were produced from inhalation experiments lasting up to 18 months. Coal mine dust A was from an anthracite mine and dust L was from a low rank mine (Jones *et al*. 1988). The two coal mine dusts had very similar activity despite having different levels of quartz.

Although some assumptions were made about the quartz and coal dust data in producing Figure 6.1, it does indicate that the volcanic ash is considerably less toxic than quartz dusts

and that it may be regarded as having similar toxicity to coal mine dust. Therefore, coal mine dust may be a useful benchmark dust, especially as there is a wealth of exposure-response data from coal miners.
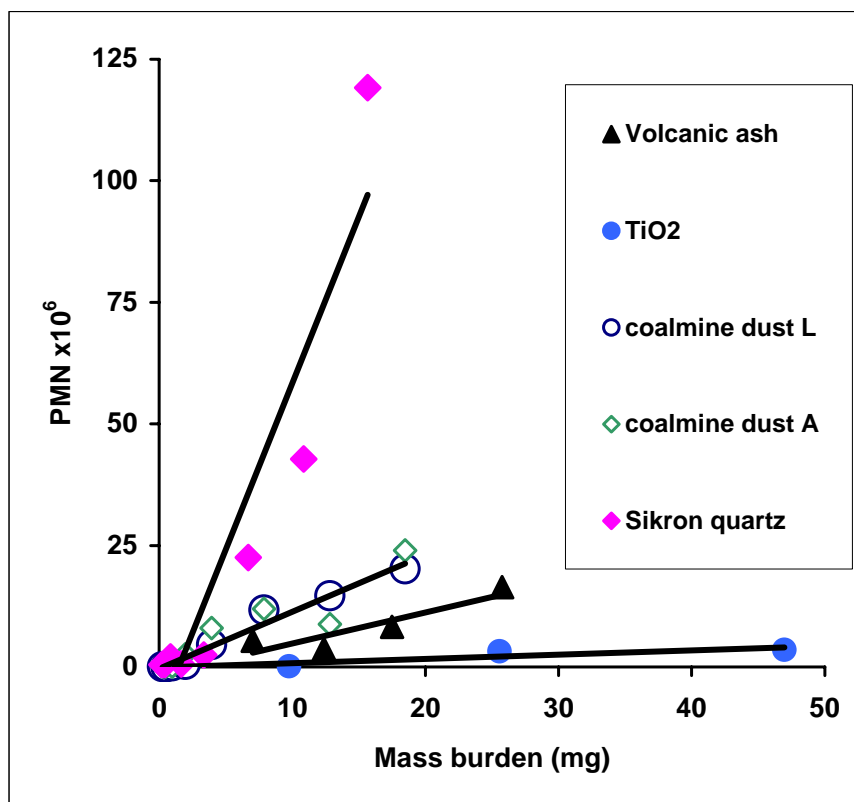

Figure 6.1 PMN numbers plotted against lung mass burden. Data from two quartz and two coal mine dust studies compared with Montserrat volcanic ash and TiO_2.

6.6   MECHANISMS OF TOXICITY

6.6.1 Oxidative stress

The explanation for the enhanced inflammogenicity and toxicity of the Montserrat volcanic ash, compared to the benchmark dust TiO_2 is uncertain and some possibilities are discussed below. The difference in activity was not due to differences in surface area. An important issue is whether the toxicity was due to the presence of cristobalite. It is believed that it is the characteristics of the particle surface of cristobalite and other crystalline silicas that determine their biological activity. The two main surface groups on particles are siloxane bridges (Si-O-Si) and silanols (SiOH). Mechanical fracture (e.g. by grinding) can generate silicon-based free surface radicals such as $Si^\bullet$, $SiO^\bullet$, and $SiOO^\bullet$ (Fubini *et al*. 1987). In water, these surface radicals generate hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and hydroxyl radical ($^\bullet OH$) (Shi *et al*. 1998). Radicals cause damage to cells through lipid peroxidation of cell membranes and damage to DNA (Hardy and Aust 1995; Shi *et al*. 1998). Damage to lung epithelial cells leads to increased permeability of the epithelial layer allowing particles to pass into the interstitium where they may contribute to further inflammation and lung damage (Vincent and Donaldson 1990; Ferin *et al*., 1992). Interstitialised particles may eventually be cleared to the lymph nodes.

However, Horwell *et al*. (2001) has recently shown that the cristobalite particles in samples of Montserrat ash have very little radical activity. Thermal treatment (>800 ^{o}C) of crystalline silica causes annealing of the surface radicals transforming hydrophilic silanols into hydrophobic siloxanes (Fubini *et al*. 1995). Heating cristobalite at 1300 ^{o}C removed its cytotoxicity (Fubini *et al*. 1999). Radical activity, and the associated toxicity, can also be neutralised by coating silica particles with polymers (Wiessner *et al*. 1990; Mao *et al*. 1995) or treating them with aluminium lactate (Brown *et al*. 1989). Natural coating or binding of metal ions (e.g. aluminium) in the environment may account for the toxicity of some quartz-containing materials such as coal mine dust being less than would have been expected from their quartz content (Hurley *et al*. 1982; Miller *et al*. 1993; Donaldson and Borm 1998).

The cristobalite crystals in lava are very small and may not be subject to fracture in the way that other mineral components and glass would be. A crystalline core in the cristobalite particles may be covered by an amorphous, non-reactive layer that, within the lung, is eventually leached away to reveal a reactive surface. This could account for the late development of pathology seen in the Cardiff instillation study (Richards 2002, personal communication).

Nevertheless, particles in the ash, other than cristobalite, do have radical activity and this has been associated with the presence of iron (Horwell *et al*. 2001). A role for iron has also been suggested in the pathogenic effects of coal mine dust (Tourmann and Kaufmann 1994; Dalal *et al*., 1995). The significance of iron is in its ability, in the correct oxidant states, to act as a catalyst in generating toxic radicals. Thus surface iron, or leachable iron, as Fe^{2+} on particles can reduce molecular oxygen to superoxide anion which can dismutate to hydrogen peroxide. Hydrogen peroxide in the presence of Fe^{2+} leads to the generation of hydroxyl radicals and Fe^{3+}. In the presence of superoxide or a reductant such as ascorbate, Fe^{3+} is recycled to Fe^{2+} (the Fenton reaction) to continue the catalytic reactions. Trace amounts of iron are sufficient to produce the toxic radicals. Other transition metals can also take part in these reactions. For more details of the reactions see Aust *et al*. 1985 or Hardy and Aust 1995. Surface radical activity could account for short term toxic effects of volcanic ash both in *in vitro* and *in vivo* experiments.

Macrophages and PMN can also produce reactive oxygen radicals in response to dust particles, for example, coal mine dust (Wallaert *et al*. 1990) or quartz (Vallyathan *et al*. 1992). These phagocytic cells can also produce the reactive nitrogen species nitric oxide and peroxynitrite (Blackford *et al*. 1994). Cellular production of these radicals provides another potential source of toxicity within the lung.

From the above discussion, the toxic effects seen in our inhalation and *in vitro* studies could be due to iron-containing, non-cristobalite, ash particles leading to the release of reactive radicals in the lung and the possibility of oxidant stress. Oxidant stress leads to the heightened activation of transcription factors, such as nuclear factor-κB (NF-κB), that regulate transcription of a wide range of genes, many of which are associated with inflammation (Blackwell and Christman 1997; Driscoll *et al*., 1997; Rahman and MacNee 1998; Schins *et al*., 2000).

6.6.2 Other cell products involved in pathogenesis

The generation of reactive radicals in the lung is not the only mechanism contributing to lung injury and fibrosis following exposure to dusts and so simple tests of radical activity may not be predictive of toxicity. The alveolar macrophage appears to play a pivotal role in dust pathogenesis through its ability to initiate and regulate inflammatory and fibrogenic factors

such as interleukin-1 (Schmidt *et al*., 1984), interleukin-6 (Ulich *et al*. 1991), PMN and monocyte chemokines (Blackwell *et al*., 1994; Driscoll *et al.*, 1996), TNF (Driscoll *et al*. 1997), and fibroblast growth factors (Brandes and Finkelstein 1990). Lung epithelial cells and PMNs recruited to the lung can also produce some of these factors (Kusaka *et al*. 1990; Xing *et al*. 1994; Driscoll *et al*. 1996). Macrophages and PMN are also involved in the toxic manifestations of dusts through the release of proteases (Sibille and Reynolds 1990).

The net result of these many interactions can be the persistence of inflammation in the alveolar region of the lung with subsequent fibrosis and permanent damage.

6.7 EXTRAPOLATIONS TO HUMANS

An important question is whether the ash sample studied here is likely to be representative, in terms of toxicity, of ash exposures to humans or animals on Montserrat and neighbouring islands. The *in vitro* and instillation experiments reported here and those from previous studies (Cullen and Searl, 1998; Wilson *et al*. 2000) provided evidence of both differences and similarities between different ash samples. This needs to be studied further. Horwell *et al*. (2001) also showed that the levels of oxidative activity, and hence the toxic potential, does vary between ash samples, for example, ash from explosive events as compared to ash from pyroclastic flows.

Extrapolating from animal toxicity data to humans is a key issue in risk assessment. Previously we have compared the relative toxicity for inhaled insoluble dusts as assessed in animal studies with indicators of relative hazard for humans (Soutar *et al*, 1997) and found that the animal studies were a good indicator of relative hazard for humans for such dusts.

One of the differences between rats and humans lies in the aerodynamic selection of particles that can penetrate to and deposit on the alveolar lung. The difference is in one sense remarkably small compared to the huge difference in body size, but nevertheless it could affect dose (in the lung) relative to exposure. (Airborne concentrations are usually measured by instruments sampling to the conventions for human respirable dust). In this study, we have compared dusts relative to dose in the lung, which should have minimised the influence of this factor on relative hazard for given concentrations of respirable airborne dust.

The influence of factors that affect the extrapolation from rats to humans could be quantified. This can conveniently be done using mathematical modelling of the exposure, dose and response relationships. Mathematical models have been used to relate dose effect models developed in rats to humans (Tran *et al.* 1999, 2000; Kuempel *et al*. 2001). However, such calculations would go beyond the scope of the present study.

6.8 PRE-EXISTING DISEASE

Most toxicity studies examine effects in normal, healthy, adult animals but environmental exposures in humans can affect the whole population, including the very young, the elderly and the sick. It is believed that those with pre-existing lung or cardiovascular disease are likely to be the most vulnerable when exposed to particulate or gaseous pollution. One study addressed this issue following the Mount St. Helens eruption by comparing the effects of inhalation to volcanic ash (9.4 mg.m^{-3}) for two hours daily for 5 days in normal rats and rats with elastase-induced emphysema (Raub *et al*. 1985). There were no immediate or long-term effects on histology. These authors also studied the effects of combined exposure to sulphur dioxide (SO$_2$) and ash in the emphysema model but again found no treatment-associated changes. However, the exposures were relatively low in this study and, so, effects at higher concentrations, or over a longer period, cannot be ruled out.

Emphysema is a disease of the lung parenchyma but volcanic ash might also affect the airways. Weister *et al*., (1985) studied airway reactivity to histamine in guinea pigs exposed for two hours to Mt. St. Helens ash at 10 mg.m^{-3}. Greater amounts of histamine were required to reduce dynamic lung compliance (C_{dyn}) in the ash-exposed animals than in normal animals, i.e. the ash was preventing airway contraction. No positive or negative particulate controls were included in this study. Reduction in airway reactivity caused by volcanic ash was also seen in an *ex-vivo* study of rat trachea (Fedan *et al*., (1981). These findings are in contrast to the finding that inert dusts tend to increase airway resistance in humans (Dubois *et al*., 1958).

There is some evidence that exposure to the ash has had an effect on the respiratory health of people on Montserrat. A postal questionnaire was conducted among Montserratians who had relocated to the UK following the volcanic eruptions (Cowie *et al*. 2001). Prevalence of respiratory symptoms in this group was higher than in the general UK population. This may have been due to moving to a new country and Montserratians with pre-existing respiratory disease would be more likely to want to escape from further ash exposure. There was also evidence of an association between the occurrence of symptoms and exposure from heavy ash cleaning activities on the island, but no evidence that house cleaning increased symptoms. In a second study on workers on Montserrat, (Cowie *et al*. 2002), it was found that exposure to ash had some mild effects on respiratory health, but there were no radiological signs. There was no evidence that residential exposure or domestic cleaning tasks had had an effect.

6.9   TOXIC POTENTIAL OF NON-PARTICULATE VOLCANIC EMISSIONS

In addition to ash, volcanoes produce a number of hazardous gases and vapours such as hydrogen chloride (HCl), sulphur dioxide (SO_2), carbon monoxide, oxides of nitrogen, and hydrogen sulphide. Gases such as HCl and SO_2 are respiratory irritants and can cause breathing difficulties, especially in asthmatics (Utell *et al*. 1984). Allen *et al*. (2000) measured gases during a period of modest volcanic activity on Montserrat in 1996 and found only low concentrations of these two gases in the town of Plymouth, 5km downwind of the volcano were not deemed to be of public health concern. However, gas concentrations could be higher during periods of greater volcanic activity.

The toxicology of mixtures of gases or of gases with particles, has not, in general, been well studied. As described above, Raub *et al*. (1985) found that, in an emphysema animal model, co-exposures to ash and SO_2 had no greater effect than ash alone. A combined exposure of H_2SO_4 and nitrogen dioxide (NO_2) by rats for up to 7 days, induced a greater inflammation and protein levels in lavage than either NO_2 or H_2SO_4 alone (Last 1991). At a dose of 5 ppm NO_2, this gas alone induced a significant increase in lung collagen which was not increased any further by the inhalation of H_2SO_4. However, reducing the dose of NO_2 to 2 ppm and then combined with H_2SO_4 revealed that the co-treatments could enhance collagen synthesis above that for either treatment alone (Last, 1991).

Very soluble gases, such as HCl, tend to be absorbed in the upper airways and have less impact on the alveolar region. However, gases can become adsorbed onto particles and, by this means, the acidic activity can be carried into the deep lung. For example, inhalation by rats of carbon black particles coated with SO_4^{2-} for four hours led to a decrease in the phagocytic index of alveolar macrophages which was not seen in rats exposed to carbon black particles without a sulphate coating (Jakab *et al*. 1996). Similarly, co-exposure of rats to ozone and carbon black led to greater inflammation and more marked depression of alveolar macrophage phagocytosis than with exposure to either agent alone (Jakab and Hemenway 1994). Such synergistic effects have been reported for other combinations of gases and particles and animal models (e.g. Last and Warren 1987; Jakab 1993; O'Neill *et al*. 1995; Farman et al 1999; Madden *et al*. 2000). Synergism was found in acute exposures to ozone

and sulphuric acid but not in chronic exposures. Interestingly intermittent exposures to ozone caused greater lung damage than continuous exposure (Last and Pinkerton 1997).

The acidic gases, particularly HCl, result in local acidic rainfall (Stoiber *et al*. 1986), and it is thought likely that this was the cause of the pre-ash fall damage to vegetation on Montserrat following the eruption (Allen *et al*. 2000). There was also evidence that ash particles had absorbed acids present (A. Searl, personal communication).

7. CONCLUSIONS, GUIDANCE, RECOMMENDATIONS

7.1 CONCLUSIONS

1. The *in vitro* and lung instillation experiments confirmed that the Montserrat ash sample was considerably less toxic than pure samples of quartz or cristobalite, and suggested that the ash might be more active than the benchmark low toxicity dust, titanium dioxide. These results are thus similar to those reported for volcanic ash from the Mount Saint Helens eruption.

2. The 8-week inhalation experiment showed that Montserrat volcanic ash was more toxic than titanium dioxide.

3. Comparison of the inhalation data with those from previous studies suggests that the toxicity of the Montserrat ash may be comparable to that of coal mine dust, and that it is considerably less toxic than samples of pure quartz.

4. The level of toxicity of the ash suggests that using coal mine dust as a benchmark comparison would be appropriate when considering exposure limits and human risk on Montserrat.

7.2 DERIVING GUIDANCE FROM THE RESULTS

The evidence from the inhalation experiment allows the toxicity of the Montserrat volcanic ash to be related to the toxicity of other dusts, and thereby enables guidance given for those other dusts to be used as a basis for advising the people and authorities on Montserrat.

The evidence from the inhalation experiment indicates that the volcanic ash sample studied is more toxic than a benchmark, insoluble, low toxicity dust. That is a basis for recommending that it would be inappropriate to set guidance limits for exposure to volcanic ash on Montserrat as high as the existing UK occupational exposure limits for exposure during normal working hours to "low toxicity" dusts i.e. 4 mg.m^{-3} respirable dust. However, on Montserrat, those workers exposed during the working day may be further exposed at home when clearing ash from their properties. Exposure to volcanic ash on Montserrat could be for 24 hours a day for many individuals, depending on local ash conditions.

The volcanic ash appears much less toxic than pure quartz, so the evidence does not support a case for guidance as stringent as would have been appropriate for quartz. We understand that the current guidance is based upon an assumed cristobalite content of 20%. From our comparisons between the Montserrat ash and pure quartz this would appear to be adequately protective. The volcanic ash appears to have a similar level of toxicity to the coal mine dusts, so coal mine dust may be a useful benchmark for setting standards or guidelines for exposure to the ash. However, the comparisons made between the Montserrat ash and coal mine dust in the above discussion were approximations based upon datasets from experiments with potentially important differences in the protocols (e.g. exposure durations). Therefore, we recommend that a more rigorous examination of IOM and other published data using mathematical modelling be carried out to confirm the value of using coal dust as a benchmark for volcanic ash.

7.3   RECOMMENDATIONS

1. The oxidative activity of the ash sample used in the experiments reported here, should be determined using the techniques employed by Horwell *et al.*, (2001).

2. Given that the Montserrat volcanic ash appears to have similar toxicity to coal mine dusts, data on the risks from coal mine dust could be used as a benchmark to estimate the risks from ash exposure. Mathematical modelling would probably be needed to take account of differences in exposure scenarios, and to allow for the differences in the age and health of the exposed population.

3. The comparison between the toxicity of volcanic ash and coal mine dusts should be explored further experimentally using sensitive *in vitro* techniques to measure toxicity and cell function. The IOM has appropriate samples of coal mine dusts in storage.

4. The inhalation experiment used only one sample of ash. The question remains open as to whether the effects seen in the inhalation experiment are representative of all Montserrat ash samples. Sensitive *in vitro* assays should be used to strengthen the comparison of the toxic potential of several different ash samples.

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9.   ACKNOWLEDGEMENTS

APPENDIX

IN VITRO AND INSTILLATION STUDIES

(NAPIER UNIVERSITY)

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A.1   INTRODUCTION

Five samples of Montserrat Volcanic Ash (MVA) were compared with three control dusts. These dusts are listed in Table A.1. The purpose of these tests was to assess the relative toxicity of various samples of Montserrat volcanic ash.

Three toxicological assays were carried out to determine biological reactivity:

1. the haemolysis of sheep red blood cells was used as an indicator of surface reactivity and potential cytotoxicity;

2. lactate dehydrogenase (LDH) release from cells was used as an indicator of membrane damage of the human type II epithelial cell line, A549;

3. ash particles were instilled into rat lungs and the inflammatory profile in the bronchoalveolar lavage (BAL) determined.

A.2   MATERIALS AND METHODS

A.2.1 Particles

Table A.1 gives details of samples used in this study.

Table A.1 Particle types used in the Napier University experiments

Particle type	Description
M1, M2 and M3	MVA samples used in the study of Wilson *et al*., 2000.
M4	MVA sample used in the IOM inhalation study. Supplied by the IOM.
RMVA	MVA sample supplied by Professor Roy Richards, Cardiff.
TiO$_2$	Relatively inert control dust. Heat-treated and supplied by the IOM.
DQ12	Reactive quartz sample supplied by the IOM.
Cristobalite	Reactive silica sample supplied by Professor Roy Richards, Cardiff.

A.2.2 Haemolysis Assay

Haemolytic activity of the particles was determined using a 10% erythrocyte solution utilising a modified method from Razzaboni *et al*. (1990). Sheep erythrocytes were obtained from Diagnostics Scotland and were washed twice in 0.9% sodium chloride solution (Baxter, UK) and centrifuged at 90 g for 5 minutes. The erythrocyte pellets were re-suspended and diluted in 0.9% sodium chloride solution to give a 10% erythrocyte preparation. Suspensions of MVA, along with DQ12 and TiO$_2$, were prepared in 0.9% sodium chloride solution to give final concentrations ranging from 0 to 20 mg/ml. These were mixed by vortexing and sonicated for 10 minutes. The particle suspensions (225 μl) were mixed with 113 μl of 10% erythrocyte preparation. Sodium chloride solution (0.9%) was used as a negative control and the positive control was 0.1% Triton X-100 (Sigma, UK) in 0.9% sodium chloride solution. The mixtures were incubated at room temperature for 10 minutes on an orbital plate shaker before centrifugation at 180 g for 5 minutes. The supernatants (112 μl) from each sample were transferred to a 96 well plate and the optical densities read at 540 nm (MRX plate reader; Dynatech Labs, UK). To convert optical densities to percentage haemolysis, the equation of the straight line was used, y=mx+c;

$$x=(y-c)/m \quad \text{where:}$$

x = percentage haemolysis
y = optical density
c = mean negative control optical density
m = (mean positive control optical density-mean negative control optical density)/100

A.2.3 LDH measurement of A549 cells

Confluent A549 cells were removed from a culture flask using trypsin and diluted to give a cell number of $2X10^5$ cells/ml. To each well of a 96 well plate 200 μl of cells was added giving a final cell number of $4X10^4$ cells/well. The cells were then incubated overnight at 37^0C, 5% CO$_2$. The particles were suspended in DMEM (10 % foetal calf serum) to give final concentration of 400 μg/ml, vortexed and sonicated for 10 minutes. These suspensions were double-diluted three times to give 200, 100 and 50 μg/ml, added to the cells and incubated at 37^0C, 5% CO$_2$ for 24 hours.

LDH release from the A549 cells was determined by comparing to an NADH/pyruvate standard curve. Standards were prepared to give final concentrations of between 0 and 2000 Units/ml of LDH. An NADH (Sigma, UK) solution was prepared in sodium pyruvate (Sigma, UK) and diluted in distilled water to give desired standard concentrations. In a 96 well plate, 60 μl/well of each standard was added in triplicate and 50 μl/well of NADH solution was added to the remaining well. The samples were then incubated at 37^0C, 5% CO_2 for 5 minutes. The plates containing the cells plus particle solutions were centrifuged at 900 g for 10 minutes and 10 μl of supernatant from the treated cells was added to the corresponding wells of the test plate containing 50 μl/well of NADH solution. The samples were incubated at 37^0C, 5% CO_2 for 30 minutes before adding 50 μl of 2,4-dinitrophenylhydrazine (Fluka, UK) in 1M HCl (BDH, UK) solution to each well and incubating at room temperature for 20 minutes. A volume of 50 μl 4M sodium hydroxide (Sigma, UK) was added to each well, mixed by pipetting and allowed to stand for 5 minutes at room temperature before reading the absorbance at 550 nm using a "MRX" plate reader (Dynatech Labs, UK).

A.2.4 Instillations and BAL

Male Wistar rats (Charles River, UK) were used for the instillation experiments. The animals were housed under standard conditions, weighed between 300 and 350g and were approximately 3 months old.

Particles were prepared in 0.9 % sterile saline (Baxter, UK) and saline was used as a negative control. A mass dose that delivered a surface area dose of 50 cm^2 of particles was used for either M4 or TiO_2. Surface area data was unavailable for RMVA therefore an equal mass dose (1.53 mg), compared to M4, was instilled.

Wistar rats were anaesthetised with halothane and then instilled intratracheally with 500 μl of treatments using sterile saline as a control. At 24 hours following instillation the animals were sacrificed and the lungs removed. Saline (8 ml) was injected through the cannula and the lungs were massaged firmly for approximately two minutes. This primary lavage fluid was withdrawn from the lungs and kept separate from three further washes of 8 ml each, which were pooled to form a secondary lavage. Following centrifugation (900g for 2 minutes) the cells were resuspended in 1 ml sterile saline and suspensions from the primary and secondary lavages pooled. A cell count was performed, cytospots prepared and differentially stained with "Diff Quik" (Lamb, UK) before counting.

Results were compared to those obtained 18 hours following instillation of DQ12, at a surface area dose of 25.3 cm^2 (250 μg), carried out in a previous study by Rodger Duffin (Duffin *et al*. 2001, 2002)

A.2.5 Statistical analysis

Each of the assays was repeated on three separate occasions using separate aliquots of particles, blood and cells. Three observations were made for each *in vitro* experiment and the mean calculated from these observations. The results represent the mean of the three experiments ± standard error of the mean (SEM). The LDH data were analysed using one way analysis of variance with Tukey's pairwise comparisons. All other data were analysed using a Students t-test assuming equal variance.

A.3   RESULTS

A.3.1 Haemolysis

The haemolysis induced by DQ12 was significantly greater than that induced by TiO_2 at all doses tested ($p<0.01$; Figure A1.1). M1 exhibited a significantly greater haemolytic activity compared to TiO_2 at the highest dose of 20 mg/ml ($p<0.01$; Figure A.1). The haemolysis induced by M4 was not significantly different to that induced by TiO_2 at any of the doses tested.
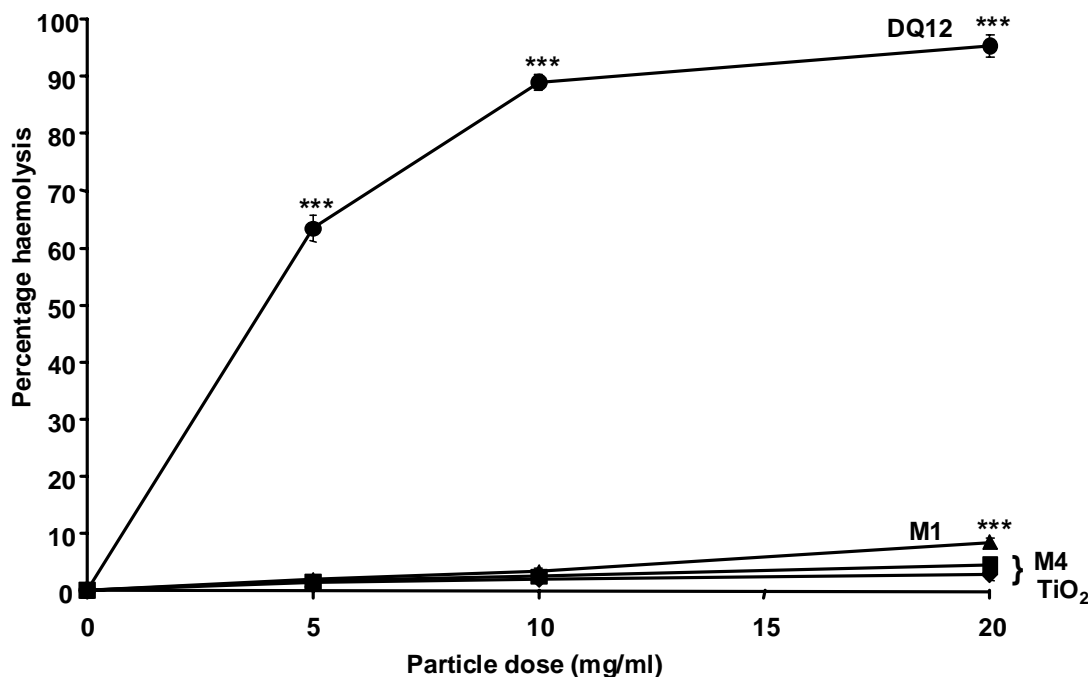

Figure A.1 Percentage haemolysis of sheep red blood cells induced by increasing doses of particles; ash samples M1 and M4, DQ12 quartz and TiO2. *** $p<0.01$ comparing haemolysis induced by DQ12 at all doses and M1 at 20 mg/ml versus TiO2 at corresponding doses.

A.3.2 LDH release from A549 cells

The only particle treatment that caused significant ($p<0.01$) release of LDH compared to TiO_2 treatment at any dose was cristobalite at the doses of 100 and 200 μg/ml (Figure A.2a - LDH release as Units/ml).   Figure A.2b shows the LDH release as percentage of total releasable LDH and shows the same significant differences as Figure A.2a. However the distinct impression gained from Figures A.2a and A.2b is that there is some activity in the MVA samples, as the dose response curves do not cluster around the control but spread up towards the positive controls. It is especially notable that M4 and RMVA are at the extremes of the range of effects with M4 being close to TiO_2 and RMVA being closest to cristobalite (see inset). It is emphasised, however, that neither RMVA nor M4 differ significantly from the TiO_2 control.
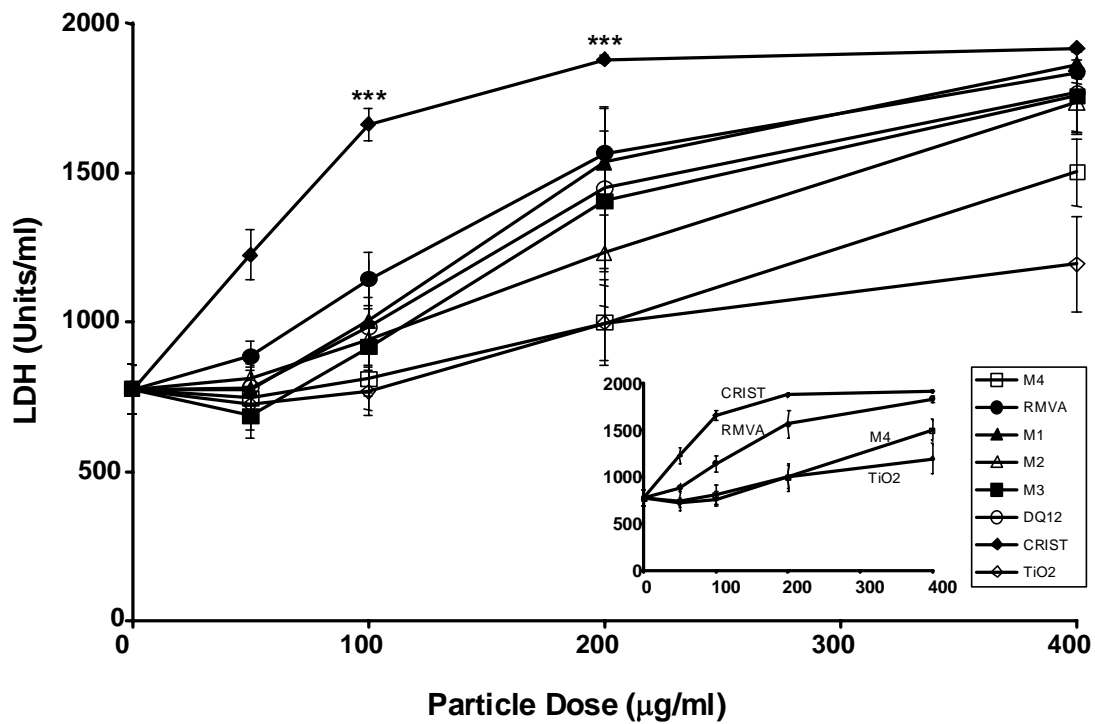
Figure A.2a Lactate dehydrogenase (LDH; Units/ml) release from A549 cells following exposure to increasing doses of Montserrat volcanic ash samples, DQ12 quartz, cristobalite and TiO2. *** $p<0.01$; 200 and 100 g/ml cristobalite vs TiO2 (n=3, 9 observations). The inset shows the same data as Figure A.2a with omission of M1, M2, M3 and DQ12 for clarity.
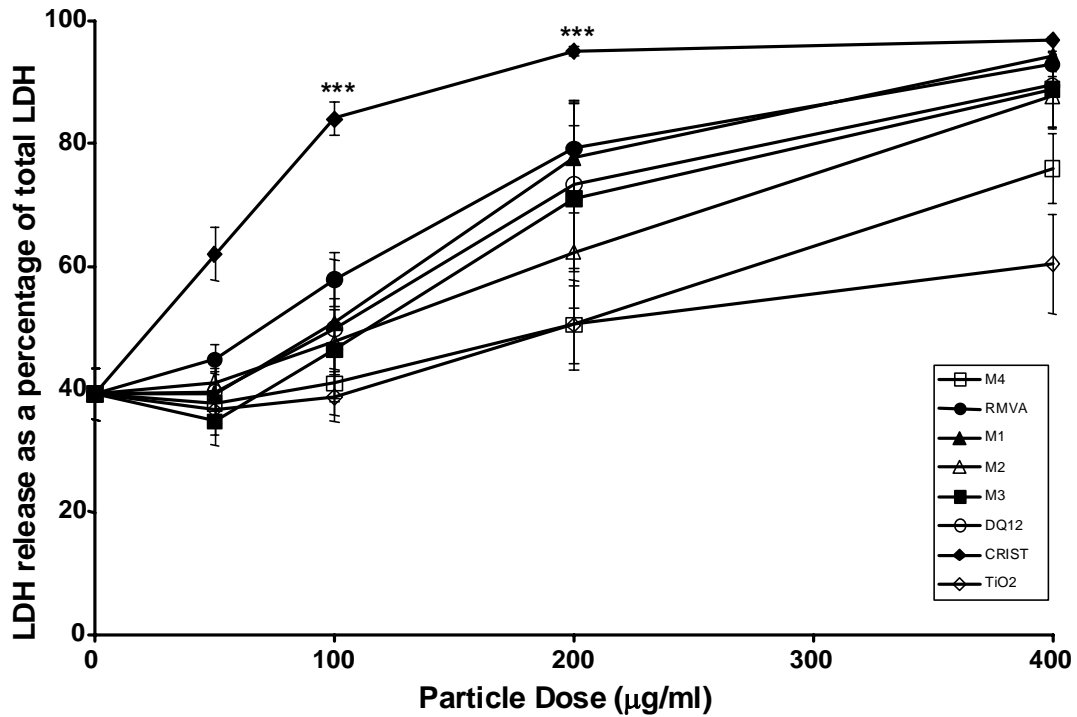
Figure A.2b Percentage of total releasable lactate dehydrogenase (LDH) from A549 cells following exposure to increasing doses of Montserrat volcanic ash samples, DQ12 quartz, cristobalite and TiO_2. *** $p<0.01$; 200 and 100 μg/ml cristobalite vs TiO_2 (n=3, 9 observations).

A.3.3   Inflammatory effects of Montserrat volcanic ash

The BAL inflammatory profile is summarised in Tables A.2 and A.3. The only significant ($p<0.01$) differences from control BAL cell profile were with DQ12 total cells, macrophages and neutrophils; (Table A.2); Table A.3 shows proportions of macrophages and PMN and confirms that the only significant changes compared to controls were in the DQ12-treated rats.

Table A.2 BAL inflammatory profile from rat lungs following exposure to MVA and control particles: mean total cell number ± SEM from three rats

Treatment	Total Cells ($X10^6$ cells)	Total Macrophages ($X10^6$ cells)	Total PMN ($X10^6$ cells)	Total Others ($X10^6$ cells)
Control	3.92±0.40	3.89±0.40	0.02±0.01	0.01±0.01
M4	5.92±1.25	5.40±0.89	0.48±0.40	0.03±0.02
RMVA	2.42±0.85	2.22±0.72	0.18±0.13	0.01±0.01
DQ12	21.60±1.00 ***	1.93±0.00 ***	19.70±1.00 ***	0
TiO$_2$	5.53±0.44	4.94±0.62	0.59±0.22	0

The control used was 0.9% sterile saline. M4 and TiO$_2$ were instilled at a surface area dose of 50 cm^2 for 24 hours. RMVA was instilled on a mass basis equal to that of M4 (1.53 mg) for 24 hours. DQ12 was instilled at a surface area dose of 25.3 cm^2 (250 µg) for 18 hours.
*** p<0.01 comparing corresponding cells counts of DQ12 versus control.

Table A.3 BAL inflammatory profile from rat lungs following exposure to MVA and control particles: mean percentage of total cell number ± SEM from three rats

Treatment	Macrophages (% of total cells)	PMN (% of total cells)	Others (% of total cells)
Control	99.27±0.13	0.53±0.29	0.20±0.20
M4	93.10±4.66	6.30±4.65	0.60±0.35
RMVA	93.53±2.39	5.94±2.56	0.53±0.39
DQ12	9.00±1.53 ***	91.00±1.53 ***	0
TiO$_2$	88.67±4.93	11.33±4.93	0

The control used was 0.9% sterile saline. M4 and TiO$_2$ were instilled at a surface area dose of 50 cm^2 for 24 hours. RMVA was instilled on a mass basis equal to that of M4 (1.53 mg) for 24 hours. DQ12 was instilled at a surface area dose of 25.3 cm^2 (250 µg) for 18 hours.
*** p<0.01 comparing corresponding cells percentages of DQ12 versus control.

A.4   DISCUSSION

A.4.1 Haemolysis

Only M1 and M4 were tested in this assay due to the large amount of dust required for this assay and the limited availability of the other MVA samples. As previously reported (Wilson *et al*. 2000), DQ12 was potently and significantly haemolytic at all concentrations. The only case where an MVA sample was significantly different from TiO_2 was at the highest dose tested in the haemolysis assay (20 mg/ml). We do not believe this to be biologically significant since it was a very small effect at a very high dose.

A.4.2 LDH release

The only significant increases in LDH release were seen with cristobalite. However there is a strong impression gained from visual inspection of Figs A.2a and A.2b that the ash samples did have some biological activity in the LDH assay, even though the effects were not significant. In particular, in contrast to the general findings in other studies, the RMVA sample that was found to have little short-term activity in the study by Housley and colleagues (2002), was closest to the cristobalite control; M4 on the other hand, which was found to have significant inflammogenic activity in the IOM inhalation study, had approximately the same ability to cause LDH release as TiO_2.

A.4.3 Inflammation

As expected, when instilled into the lungs, the 25.3 cm^2 (250 µg) dose of DQ12 caused a large scale influx of inflammatory cells (Duffin *et al.*, 2001) . A surface area dose of 50cm^2 of M4 was instilled but because surface area data were unavailable for RMVA, an equal mass dose (1.53 mg), compared to M4, was instilled. The PMN influx was modest for both of these samples and when the data is viewed along with the same index of response for a number of low toxicity dusts (Figure A.3) it is apparent that both of these samples can be seen as falling into the same class as these low-toxicity dusts in that their surface area predicts the extent of the PMN influx. This figure highlights the importance of equalising to surface area dose when comparing the short-term inflammatory effects of low toxicity dusts .
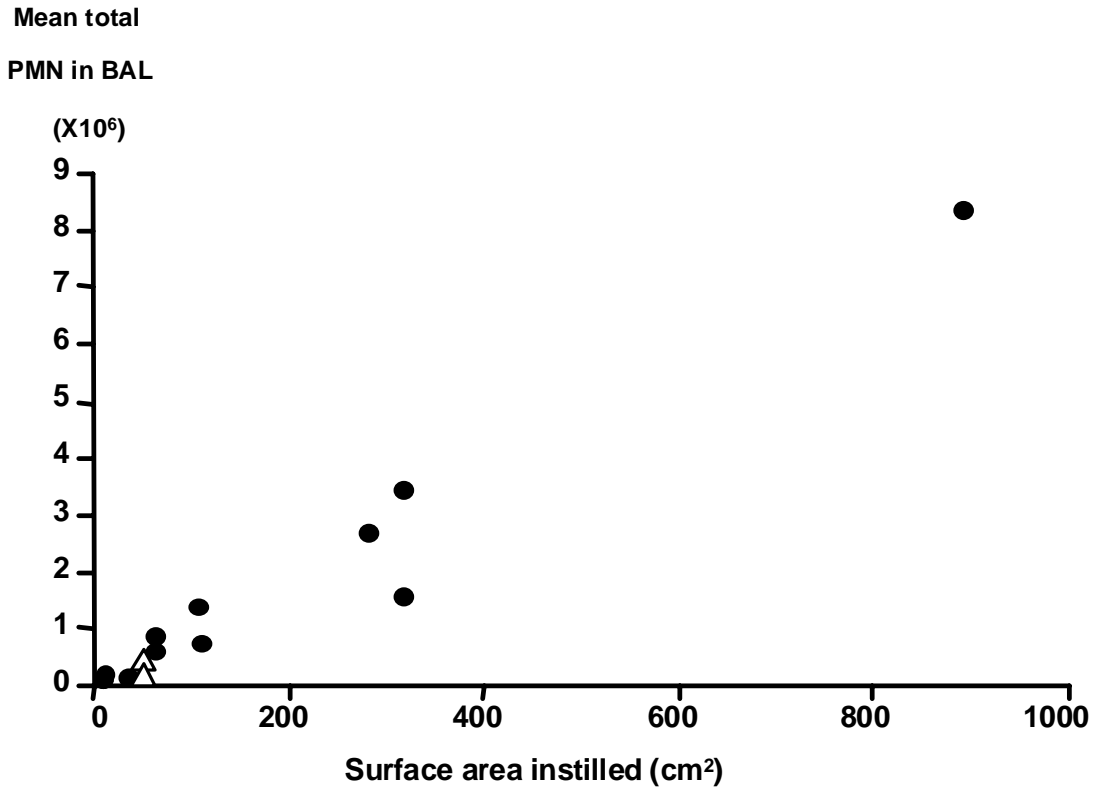
Figure A.3 M4 and RMVA PMN data from Table A.2 (open triangles) included in a graph that shows the pulmonary PMN influx at 18-24 hours, per unit surface area for a range of low toxicity particle types after instillation of various surface area doses. Particle types include, fine and ultrafine carbon black, TiO_2 and polystyrene latex (Duffin *et al.*, 2002). Note that the lower triangle represents RMVA with the surface area derived from an assumption that it has the same surface area per unit mass as M4.

A.5   CONCLUSIONS

A.5.1   Toxicity of volcanic ash

We conclude from these studies that different assays give different indications of the likely toxicity of volcanic ash. The haemolysis and *in vivo* short-term PMN recruitment assays following instillation into the rat lung indicate that the volcanic ashes have little activity. The LDH assay of epithelial cell membranolysis suggests (with no statistically significant data) that the MVA samples are more toxic than TiO_2; they are however, significantly less toxic than cristobalite but the degree of this effect is not known. It should be pointed out that, for large particles with little toxic activity (at least as indicated by the PMN data), like the volcanic ashes, macrophage clearance should be effective and there should be little interaction between the particles and epithelial cells. Therefore less weight could be accorded to results from this assay under these circumstances, because prolonged interaction between particles and epithelial cells may never arise.

A.5.2   M4 and RMVA

There were no differences between these two samples in their ability to cause PMN influx in the short-term instillation study. However, the RMVA instillation was based on an estimate of the surface area and Figure A1.3 shows the importance of surface area in predicting short

term PMN influx. If the RMVA was in fact much larger and had less surface area than we assumed, then we may have underestimated its inflammogenicity – however, this seems unlikely. The epithelial toxicity assay did show an indication (not significant) that the RMVA was more toxic than the M4. Again the caveat described above regarding the unlikelihood of prolonged interaction with epithelial cells for large low toxicity particles diminishes the weight we may place on this impression. In any case the LDH results suggesting that RMVA is more toxic than M4 goes against the pattern seen in the Cardiff instillation study (Housley *et al*. 2002) and the IOM inhalation study reported here, where the short-term effects of M4 (inhalation) were much greater that those of RMVA (instillation).

A.6   REFERENCES

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The Institute of Occupational Medicine

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