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INSTITUTE OF OCCUPATIONAL MEDICINE

EXPERIMENTAL STUDIES ON DUST IN THE LONDON UNDERGROUND WITH SPECIAL REFERENCE TO THE EFFECTS OF IRON ON THE TOXICITY OF QUARTZ

by

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INSTITUTE OF OCCUPATIONAL MEDICINE

EXPERIMENTAL STUDIES ON DUST IN THE LONDON UNDERGROUND WITH SPECIAL REFERENCE TO THE EFFECTS OF IRON ON THE TOXICITY OF QUARTZ

EXECUTIVE SUMMARY

Introduction.

Airborne dust in the London Underground railway system can contain significant quantities of quartz (a type of silica), which is known to cause silicosis (scarring of the lungs). However, previous investigations of dust in the London Underground have suggested that the quartz content may be relatively non-toxic because it also contains iron. This hypothesis led to the commissioning of the study to examine (a) the effects on the lung of fine dust collected from the air of an underground station and (b) the extent to which iron may modify the harmful effects of quartz on the lung.

The Dusts Studied.

In this study the test dusts included a sample of fine dust collected from the tunnel air of a selected London Underground station. The particles were small enough to be breathed into the deep parts of the lung. Analysis showed that most particles contained iron, and that many of the quartz particles were contaminated with iron. Comparisons were made with prepared dusts containing pure quartz to which iron was added in varying proportions. For some experiments quartz particles were also coated with iron using solutions of iron salts.

The Biological Tests.

The possible damaging effects of these dusts were investigated in several ways. They included: (1) injecting a dose of dust into the windpipe of rats, then measuring various responses of the lung itself and of the scavenger cells and other cells found within the lung which cause inflammation; (2) examining the effects of iron coating of quartz particles on their harmfulness.

Results and Conclusions.

From the results of these investigations we have concluded that the London Underground tunnel dust was considerably less harmful to the lung than pure quartz, at doses equivalent to the quartz content of tunnel dust. This amelioration of the effects of quartz is likely to be due to the presence of iron in the dust, as shown by the modifying effects of iron on the activity of pure quartz in quartz/iron artificial mixtures. Tunnel dust, and also mixtures of quartz with particulate iron, have less of an effect than quartz on the release from the lung scavenger cells (macrophages) of tumour necrosis factor, a substance shown to be important in the development of inflammation and fibrosis.

However, tunnel dust was not completely harmless, as higher doses caused some inflammation, aggregations of dust and dust-filled scavenger cells in deep lung air spaces, and some thickening or breakdown of the walls of these air spaces. Tunnel dust was also more harmful than pure quartz to isolated lung scavenger cells in doses equivalent to the quartz content of the

tunnel dust, although this effect may simply be the result of larger numbers of particles. It should also be noted that the effects of the tunnel dust were only studied over 60 days, and it is not known whether prolonged residence in the lung causes the quartz particles to lose their associated, protective iron coating.

The implications for health risks are that airborne tunnel dust appears to be considerably less toxic than the quartz content would suggest, but is not completely without harmful effects in man if persistent exposure to high dust concentrations were to occur. We have only studied one selected sample of dust and thus possible differences in the compositions of other tunnel dusts should be taken into account before extrapolating the results to the London Underground system as a whole.

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SUMMARY

Introduction. Airborne dust in the London Underground railway system can contain significant quantities of quartz (also known as silica) a substance known to cause silicosis. However, previous investigations of dust in the London Underground have suggested that the quartz content may be relatively non-toxic because of its iron component. This hypothesis led to the commissioning of the study to examine (a) the effects on the lung of respirable dust collected from the air of an underground station and (b) the effects of iron on the toxicity of quartz to the lung.

The study dusts. In this study the test dusts included a sample of fine (respirable) dust collected from the air of a selected London Underground station. This dust contained 7.8% quartz and 35% iron. The particles were small enough to be breathed into the deep parts of the lung. The median diameter was 1.1 micrometers. Analysis of individual particles showed that most contained iron, and that many of the particles classed as quartz were contaminated with iron. Comparisons were made with prepared dusts containing pure standard quartz (DQ12) to which were added particulate iron (carbonyl iron) in varying proportions. For some experiments standard quartz particles were also coated with iron using solutions of the iron salts, ferrous or ferric chloride.

The biological tests. The possible inflammatory and damaging effects of these dusts were investigated by (a) instilling dust into the lungs of rats, and at intervals washing out the lung air spaces and counting the numbers of lung scavenging cells (macrophages) and inflammatory cells (neutrophils) in the wash; (b) measuring the release from these isolated cells of chemicals (tumour necrosis factor and superoxide) thought to participate in inflammation and lung damage; (c) measuring the rate at which the rat lungs cleared away the dusts residing in them; (d) assessing the appearance of the lungs, after an interval, for type and severity of damage; (e) measuring the amounts of dust required to kill normal lung scavenging cells; (f) studying the effect of iron coating on the electric charge and the production of hydrogen peroxide on the quartz surface (thought to be important in causing lung damage); (g) studying the effect of iron coating on the toxicity of quartz to normal lung scavenger cells; (h) studying inflammation induced by dusts in the abdominal (peritoneal) cavity of mice.

Results. The tunnel dust, instilled into the lung, caused a dose-related increase in the numbers of scavenger cells and inflammatory cells, but this was much less than the response to a dose of pure quartz equivalent to the quartz content of the tunnel dust.

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The responses to a 7.8% mixture of quartz in iron particles were also less than to equivalent amounts of quartz alone. In this test, coating pure quartz with solutions of iron salts did not diminish the response to quartz.

The longer term (60 day) effect of tunnel dust on the appearances (by microscope) of the lungs was to cause dose-related increases in numbers of dust-containing scavenger cells, and slight thickening of the walls of the air spaces (alveolar septa). After high doses, some large aggregations of dust occurred with breakdown of the walls of air spaces but no evidence of scarring (fibrosis). This was less than the response to the 7.8% mixture of quartz in iron which caused more inflammatory cells to accumulate around blood vessels, and in high doses, widespread thickening of the walls of air spaces, and nodules of aggregated cells characteristic of the effects of quartz. The effects of both these dusts were much less than the effect of equivalent doses of pure standard quartz which in low doses caused accumulations of abnormal, foamy scavenger cells, thickening of air space walls, and the filling of air spaces with fat and protein. In one case early silicotic nodules, characteristic of quartz, were present, and in some cases the lung lymph nodes contained areas typical of the effects of quartz. After high doses of quartz, additionally, there were, in some cases, large areas of consolidated lung with scarring (fibrosis) and destruction of the original lung structure, and replacement by masses of actively growing cells (fibroblasts). Many isolated silicotic nodules were also present. The response to particulate iron alone was minimal in low doses, but in high doses caused some large aggregations of dust-laden cells with some breakdown of the walls of air spaces and small amounts of scarring.

The lowest dose (1 mg) of tunnel dust was found to be clearing from the lungs more quickly than 1 mg of pure quartz. With the higher doses of dust there was no evidence of clearing over the selected time interval.

Cells washed out from the lungs following treatment with dust generally showed a dose-related, reduced secretion of the toxic chemical, superoxide anion, when compared to cells recovered from untreated lungs. This reduction was least for the iron alone treatment and greatest for quartz alone; tunnel dust and 7.8% quartz in iron produced intermediate results. In addition, for any dust the greater the dose of dust, the lower the production of superoxide. Scavenger cells washed from dust-treated lungs produced increased amounts of the inflammation and fibrosis promoting substance, tumour necrosis factor; those from tunnel dust and from 7.8% quartz in iron produced less of this factor than those from pure quartz treatment.

In additional bench-top experiments, isolated scavenger cells from normal, untreated lungs produced similar amounts of tumour necrosis factor when given no treatment, or treated with tunnel dust or 7.8% quartz in iron. The response was greater for pure quartz and quartz added to tunnel dust. In high doses tunnel dust appeared to be more toxic (killed more isolated normal scavenger cells) than pure quartz (in equivalent quartz dose) or 7.8% quartz in iron, but no more toxic than a mixture of 7.8% quartz in the relatively harmless control dust titanium dioxide. Titanium dioxide alone at the highest dose also showed some toxicity, and this effect may simply have been due to larger numbers of particles in some dusts and mixtures.

Coating of quartz with iron chloride solutions did not protect isolated normal lung scavenger cells from the toxic effects of quartz as measured by release of another substance, lactate dehydrogenase, nor reduce the inflammation caused by quartz in the mouse peritoneal cavity. Iron coating also failed to measurably alter the surface charge or surface hydrogen peroxide production of quartz particles.

Conclusions. From these investigations it can be concluded that the tunnel dust was considerably less toxic to the lung than pure quartz, in doses equivalent to the quartz content of tunnel dust, and that this amelioration of the effects of quartz is likely to be due to the presence of iron in the dust as shown by the modifying effects of iron on the activity of pure quartz in quartz/iron artificial mixtures. Tunnel dust, and also mixtures of quartz with particulate iron, have less of an effect than quartz on the release from lung macrophages of tumour necrosis factor, a substance shown to be important in the development of inflammation and fibrosis.

However, tunnel dust was not completely harmless in that high doses caused some inflammation, aggregations of dust and dust-filled scavenger cells in deep lung air spaces, and some thickening or breakdown of the walls of these air spaces. Tunnel dust was also more harmful to isolated lung scavenger cells than pure quartz in doses equivalent to the quartz content of the tunnel dust, although this effect may simply be the result of larger numbers of particles. It should also be noted that the effects of the tunnel dust were only studied over 60 days, and it is not known whether with prolonged residence in the lung the quartz particles lose their associated, protective iron.

The implications of this for health risks are that airborne tunnel dust appears to be considerably less toxic than the quartz content would suggest, but not completely without harmful effects in man if persistent exposure to high dust concentrations were to occur. We have only studied one selected sample of dust and thus possible differences in the compositions of other tunnel dusts should be taken into account before extrapolating the results to the London Underground system as a whole.

1. INTRODUCTION

1.1 Background

There is a long-standing recognition that the London Underground system is a dusty environment and that inhalation of such dust could present a potential health risk to London Transport passengers and staff. In 1967 concern over the possible health effects of dust in the London Underground (LU dust) led to investigations by London Transport scientific and medical staff and by a number of other bodies, which resulted in the publication of a comprehensive report (London Transport, 1982).

Particular attention was paid in these studies to the concentrations and likely health effects of two components of LU dust, asbestos and quartz, both mostly derived from brake blocks. The concentrations of asbestos were deemed to be too low to pose a threat to health. However, concentrations of quartz were in excess of the threshold limit values (TLV) at certain sites, notably Highgate Station and in the tunnels of the Bakerloo Line.

The medical investigations of staff working in the London Underground failed to show any adverse effects associated with dust. For example, sickness absences for staff working in relatively high airborne dust concentrations were no greater than for train crews generally, and absences due to bronchitis amongst train crews were no greater than in a matched population of bus crews. There was no excess lung cancer in underground workers compared to the general population of greater London.

Laboratory studies at the Medical Research Council's Pneumoconiosis Unit in which respirable LU dust was instilled into the lungs of rats or cultured with living cells concluded that LU dust was no more harmful than nuisance dusts which do not produce lung fibrosis in animals or pneumoconiosis in humans. The laboratory studies also showed that the quartz was covered in iron, which was thought to reduce quartz toxicity. Unfortunately, the report and the records of the investigations cannot be found. Iron in the form of oxides or metal particles can account for more than 50%, by mass, of the airborne dust in the London Underground. The possible role of iron in altering the toxic effects of quartz in the lung is discussed later.

Major surveys of the respirable and total inhalable dust in the air of the London Underground were conducted in 1975 and 1986/7 with a less extensive survey in 1980. Whereas the 1980 survey had shown some improvement in dust levels compared to the 1975 survey, the results from 1986/7 showed increased levels of both inhalable and respirable dust fractions compared to the earlier surveys.

Renewed interest in the possible threat to health of the reported dust concentrations and, in particular, the quartz content of the dust, led to the commissioning by London Transport of a literature review by the Institute of Occupational Medicine (IOM) entitled "The toxicity of quartz in mixed dust with special reference to the London Underground" (Donaldson *et al*, 1991). This review made several recommendations for future work which included the need for further experimental studies to re-examine the lung toxicity of LU dust and to elucidate the possible protective role of the iron content of the dust, studies which were eventually carried out by the IOM and which are reported here.

1.2 Concentrations of dust in the London Underground

In the 1986/7 survey of dust the total inhalable and respirable concentrations ranged from 0.56 to 3.22 mg/m^3 and 0.32 to 2.26 mg/m^3 respectively (London Transport Research Laboratory 1989). The quartz content of these dusts ranged from 4.3% to 11.8% by mass and the quartz concentrations of airborne quartz ranged from 0.02 to 0.17 mg/m^3. The present British Occupational exposure limit (OEL) for respirable quartz for an 8-hour time-weighted average exposure, measured using a personal sampler, is 0.1 mg/m^3 and the mandatory maximum exposure limit (MEL) is 0.4 mg/m^3 (HSE, 1992). The London Underground dust surveys were carried out using static samplers and so the measurements only give an indication of the likely personal exposure and probably had most relevance for platform staff. Nevertheless, it was clear that quartz concentrations in some locations might approach or even exceed the OEL.

A recent London Underground survey (London Transport Research Laboratory 1993) using personal sampling of 17 personnel in a variety of jobs, but excluding drivers and guards, in different locations found that, with one exception, the quartz concentrations were at or below the detection limit of 0.02 mg/m^3. The exception involved a "dirty job" (cleaning escalator trays) which produced a level of respirable quartz, 0.13 mg/m^3, which was greater than the OEL. Workers carrying out particularly dusty tasks such as this wear respiratory protection in the form of disposable masks, 3M type 8810.

1.3 Health effects of quartz inhalation

Inhalation of quartz, a crystalline form of silicon dioxide, can lead to the fibrosing lung disease, silicosis, characterised by whorled, nodular fibrous lesions in the lung which can be limited or progressive. Symptoms occur when silicosis advances and becomes complicated by infection, such as tuberculosis, and emphysema. Both restrictive and obstructive changes can occur resulting in the reduction of airflow and impaired gas transfer in the lungs (Morgan and Seaton, 1984; Seaton *et al*, 1987).

Exposure to quartz alone is rare and most exposures occur through the presence of quartz in other dusts produced as a consequence of activities such as mining, tunnelling, quarrying, stone grinding, and sandblasting. Thus silicosis is often combined with mixed dust pneumoconiosis. The importance of quartz exposure in occupational lung disease in these industries has been established in a number of studies reviewed in Morgan and Seaton, 1984 and Seaton *et al*, 1987.

1.4 Mechanisms of quartz toxicity in the lung

Although the pathology of silicosis is fairly well understood and cellular and biochemical models have been described which can explain the development of quartz-related disease, the reasons why quartz is toxic and fibrogenic are not so clear.

1.4.1 The nature of quartz and pathogenicity

Silica forms a major part of the earth's crust. It occurs in three main forms in nature; quartz, tridymite, and cristobalite. The crystals of these forms consist of three-dimensional SiO_2

tetrahedral networks of empirical formula $(SiO_2)n$. Quartz is not the most toxic of the silica types but is by far the most abundant, and hence the most important. Silica may exist in both crystalline and amorphous forms and, although both forms have been reported to be pathogenic, crystalline silica is generally more so.

The size of crystalline particles has been shown to be an important pathogenetic factor with those in the 1-2 μm diameter range being most fibrogenic in the lung (Goldstein and Webster, 1966).

1.4.2 Surface chemistry

Early research favoured the idea that soluble products of silica, such as silicic acid, were particularly important in its toxicity (Kettle, 1926; King and Belt, 1938). However, it was later established that although silicic acid was associated with toxic effects, solubility did not correlate with fibrogenicity (King *et al*, 1953; Seaton *et al*, 1987).

It is now believed that it is the characteristics of the surface of quartz particles which determine their interaction with biological molecules. The two most important chemical functionalities at silica surfaces are those involving siloxane bridges (Si-O-Si) and silanols (SiOH) (Iler, 1979). Reaction of the silica surface with water results in hydrolysis of the surface silicon-oxygen bonds producing surface silanol groups (Iler, 1979; Tsuchiya, 1982).

$$\equiv SiO^- + H_2O \rightarrow \equiv SiOH + OH^-$$

$$\equiv SiOH \rightarrow \equiv SiO^- + H^+$$

Silanol groups can form hydrogen bonds with cell membranes, thought to be the main site of toxicity. Silanol groups can progress to ionised silanol, also thought to be important in toxicity. Silanol groups can have an acidic pK which leads to dissociation at physiological pH. This dissociation contributes to a net negative charge on the silicate surface which, as discussed further below, can lead to adsorption of organic and inorganic cations.

The quartz surface can also generate a range of other reactive species such as hydrogen peroxide (H_2O_2) and the hydroxyl radical (·OH). These agents are both known to be toxic to cells and tissues (Fantone and Ward 1984). However, only freshly fractured quartz particles are able to take part in such reactions. This process may be important with regard to the London Underground where quartz will be ground during braking. It should be pointed out, however, that for most quartz samples fresh grinding is not necessary for toxic or fibrogenic activity. Thus, for example, the standard DQ12 quartz sample produced in Germany in 1973 (Robock, 1973) and used in this and other studies in our laboratory is still able to produce lung damage and fibrosis more than 17 years after storage in air.

The production of reactive oxygen species can be measured using electron spin resonance (ESR) techniques. Grinding of quartz is thought to break silicon-oxygen bonds and to produce silicon-based radicals which react with water to produce hydroxyl radical (reviewed by Shi et al, 1989). Allowing the freshly ground quartz to age reduced hydroxyl ion production. Silicon-based radicals and hydroxyl radicals decrease in abundance with aging of ground quartz in air by 30-50 % (Fubini *et al*, 1990; Dalal *et al*, 1989; Shi *et al*, 1988).

Freshly ground quartz was found to be more toxic to macrophages, caused more lipid peroxidation, and induced macrophages to produce more oxidants (Vallyathan *et al*, 1988).

Of particular relevance to the LU study, positively charged metal cations, such as iron or aluminium, can be coordinated (bound) by silanol groups. This binding of metals has been shown to reduce the toxicity of quartz (Nolan *et al.* 1981; MacNab 1960; Stalder and Stober 1965). Paradoxically, the interaction of iron with hydrogen peroxide can also produce hydroxyl radicals (·OH), extremely potent oxidants, through the Fenton reaction:

$$Fe^{2+} + H_2O_2 = Fe^{3+} + \cdot OH + OH^-$$

Hydrogen peroxide for this reaction can be produced at the quartz surface or be released from macrophages and neutrophils as described below.

1.4.3 Nature of the cell injury induced by reactive oxygen species

The mechanisms by which free radicals injure cells are not entirely clear but probably involve lipid peroxidation of cell membranes leading to the generation of fatty acid breakdown products, such as the hydroxyalkenals, which then trigger a cascade of detrimental intracellular reactions which include enzyme damage, depletion of glutathione and nicotinamide adenine dinucleotide, and DNA strand breakage (Bast *et al.* 1991; Heffner and Repine 1989). Lipid peroxidation has been demonstrated in quartz-induced lung damage in rats (Zsoldos *et al.* 1983).

1.5 Interactions of quartz with the lung

The fate of dust inhaled into the respiratory tree will depend on where it deposits. Particles which land on mucus within the airways will be quickly cleared from the lung via the mucociliary apparatus and eventually swallowed or expectorated. Particles depositing between areas of mucus, or which reach the unciliated parts of the lower respiratory tract will either be phagocytosed by macrophages or will penetrate the respiratory epithelium, perhaps by transport through the epithelial cells, to the interstitium (Brody *et al* 1980). Toxic particles can thus exert their effects directly on a variety of cells and structures within the lung. However, it is now believed that much of the toxicity observed with dusts such as quartz arise as a consequence of their interactions with macrophages.

1.5.1 Toxic effects of quartz on macrophages

The principal function of the macrophages in the alveolar region of the lung is to engulf microorganisms and other foreign particles and neutralise or remove them from the lung, mostly via the mucociliary apparatus but with some also transported to the hilar lymph nodes. Quartz can directly damage the macrophage membrane causing rapid cytotoxicity. The mechanisms involved are likely to be those described above, namely, hydrogen bonding of silanol groups, charge interactions, and free radicals. Coating of quartz particles with molecules which make up the lining fluids of the lung can delay these toxic effects (Allison 1971). Alveolar lining fluid comprises substances transudated from blood, such as immunoglobulins and components of the complement system, and lipids and proteins secreted by type 2 alveolar epithelial cells (Bell *et al.* 1982). It has been proposed that, once inside the

macrophage phagolysosome, any coating on the engulfed particle will be digested by proteases thus "refreshing" the toxic quartz surface and allowing damage of the membrane of the phagolysosome. Rupture of this membrane will lead to release of enzymes within the macrophage leading to death of the cell and possible damage to other cells and structures nearby (Harrington *et al.* 1975). Phagocytosis by another macrophage of the released quartz continues the cycle.

However, it has also been shown that coating of dust particles can enhance the production of inflammatory mediators (Scheule and Holian 1989). In addition, some foreign particles can activate the alternative pathway of complement and thus generate chemotaxins and inflammatory mediators which amplify the lung's response. This has been demonstrated for asbestos (Warheit *et al.* 1985) and there is some evidence that it occurs with quartz (Donaldson *et al.* 1987).

1.5.2 Stimulation of macrophage activity by quartz

From in vitro studies, low doses of quartz which do not kill macrophages are probably stimulatory to macrophage activity in the lung and such activity is considered to be extremely important in mediating the pathogenic effects of harmful dusts. The effects are mediated principally through the release of inflammogenic and fibrogenic factors such as interleukin 1, tumour necrosis factor (TNF), interleukin 6, leukotrienes, prostaglandins, and fibroblast growth factors and tissue-damaging proteases and oxidants (reviews by: Brandes and Finkelstein 1990; Fantone and Ward 1984; Sibille and Reynolds 1990).

The important immune and inflammatory mediators interleukin 1 (IL-1) and TNF have a similar range of effects and both are released by alveolar macrophages in response to quartz exposure (Driscoll *et al*, 1990a; Kusaka *et al.* 1990) and both have been implicated in a rat model of acute immune alveolitis (Warren 1991; Warren *et al.* 1989). TNF has also been shown to play a major role in two animal models: bleomycin-induced fibrosis and silicosis (Piguet *et al.* 1989 and 1990). It should be stressed that there are mediators, such as the cytokines IL-6 and transforming growth factor Beta, which can also act to reduce or reverse pulmonary inflammation (Ulich *et al.* 1991).

Lung leukocytes recovered by bronchoalveolar lavage from rats exposed to fibrogenic dusts, either by inhalation or lung instillation, and cultured in vitro, secrete raised amounts of IL-1 together with enzymes which can break down lung connective tissue (Kusaka *et al.* 1990; Brown and Donaldson 1988). Lavage of workers exposed to mixed, quartz-containing dusts provided leukocytes with enhanced production of hydrogen peroxide and superoxide anion (Rom *et al.* 1987).

Thus the interaction between quartz and macrophages can have three possible outcomes depending on the dose of quartz. High doses with no protein coating produce rapid toxicity and high doses with protein coating give a delayed toxicity, whereas low doses lead to the production of mediators of inflammation and oxidant radicals. The net result of these interactions is inflammation in the alveolar region of the lung followed by fibrosis and permanent damage. This progression has been described by Keogh and Crystal (1982). Inflammation has been found in the the lungs of workers exposed to quartz (Begin *et al.* 1986a; Rom *et al.* 1987) and in rats exposed by inhalation (Donaldson *et al.* 1988a) or instillation (Brown et al 1989). However, in the bleomycin-induced model of pulmonary fibrosis the neutrophil is not essential for the development of fibrosis (Thrall *et al.* 1981) and the intensity

of the initial neutrophil response does not correlate with the subsequent fibrosis (Phan *et al.* 1983).

1.5.3 Neutrophils and lung injury

As described in the preceding section, inflammation in the alveolar region may precede fibrosis. The presence of neutrophils within the alveolar spaces, detected by histology or alveolar lavage, provides a marker of inflammation. As for macrophages, neutrophils are professional phagocytes and potent producers of tissue-damaging enzymes and oxidants (reviews by: Brigham and Meyrick 1986; Sibille and Reynolds 1990). Of course, neutrophils are also subject to the toxic effects of quartz. The activities of neutrophils within the lung can be both enhanced and depressed by macrophage mediators. Neutrophils can also participate in the regulation of inflammatory and immune processes through their production of IL-1 (Kusaka *et al.* 1990), and the release of inhibitors of IL-1 (Tiku *et al.* 1986) and TNF (Seckinger *et al.* 1990).

It is likely that neutrophils are recruited to the dust-exposed lung by the chemotaxins interleukin 8 (Streiter *et al.* 1990) and leukotriene B4 (LTB4; Smith *et al.* 1980) produced by macrophages and by the activated complement component C5a (Hensen *et al.*1979).

1.5.4 Macrophages, quartz, and lymphocytes

Mediators such as IL-1, produced by phagocytes in response to quartz, influence lymphoctes to produce other mediators such as macrophage activating factor, interferon gamma, and macrophage migration inhibition factor. Helper T cells stimulated by IL-1 also produce interleukin 2 which induces proliferation and expansion of lymphocyte populations (Cantrell and Smith 1984). Such cell interactions can provide amplification of responses to quartz. The precise nature of these intercellular communications has not been established but it is known that both macrophages and lymphocytes appear in close proximity in silicotic nodules and increased numbers of lymphocytes are found in lavage fluid in both humans (Christman et al. 1985; Schuyler *et al.* 1980) and animal models (Callis *et al.* 1985). There are reports of both normal (Schuyler *et al.* 1977) and decreased (Dauber *et al.* 1976) cell-mediated immunity in silicotics. Immunoglobulin levels generally appear to be raised in silicosis (Doll *et al.* 1981) as they are in other occupational groups exposed to dusts (Robertson *et al.* 1984; Kagan *et al.* 1977). There is also evidence of autoimmunity, as anti-nuclear antibodies and rheumatoid factor, in quartz-exposed workers (Doll *et al.* 1980, 1981). The role, if any, of these humoral immune disturbances in the silicotic disease process is not clear.

1.5.5 Macrophages, quartz, and fibroblasts

Major features of the later stages of silicosis are the fibrotic nodules and the bands of fibrosis linking the nodules and extending into the surrounding lung parenchyma. A major component of this excess connective tissue is collagen produced by fibroblasts in response, it is believed, to signals from quartz-exposed macrophages (Bateman *et al.* 1982; Lugano *et al.* 1984). Mediators identified in this process include IL-1 (Schuyler *et al.* 1977) and macrophage-derived growth factor (MDGF; Kovacs and Kelley 1985a). Interferon produced by lymphocytes in response to signals from macrophages could stimulate macrophages to produce further MDGF (Kovacs and Kelley, 1985b). In addition to excess collagen laid down in the lung, other connective tissue matrix components are produced by macrophages and other cells. Thus fibronectin, a high molecular weight glycoprotein, important as a fibroblast growth competence factor and in cell adhesion and spreading (Bitterman *et al.* 1983; Mosher 1984), is

found in significant quantities in the conglomerate lesions of silicosis and other pneumoconioses (Wagner *et al.* 1982). Enhanced production of macrophage fibronectin has been found to correlate with increased lung fibrosis following instillation of quartz (Driscoll *et al.* 1990b). Fibronectin has also been shown to act as a chemotaxin for monocytes and fibroblasts (Postlethwaite *et al.* 1980).

1.6 Modulation of quartz toxicity by the presence of other minerals in mixed dusts

Extensive epidemiological studies in Britain and Germany have demonstrated that the risk of developing coalworkers' simple pneumoconiosis (CWP) and progressive massive fibrosis (PMF) is related to cumulative lifetime exposure to respirable dust and to the type of coal being mined (Hurley *et al*, 1982; Reisner and Robock, 1977; Walton *et al*, 1977). The composition of coalmine dust varies greatly from area to area, with non-coal components accounting for 5 to 60 % by weight. Quartz and aluminium-containing clays often form a major part of these other constituents. However, the quartz content of coalmine dust did not account for the differences in simple pneumoconiosis prevalence and incidence seen in different collieries.

There have been exceptions to this where exposure to high levels of quartz was shown to be related to rapid progression of simple pneumoconiosis (Seaton *et al*, 1981; Hurley *et al*, 1982) and a relatively frequent occurrence of PMF (Hurley *et al*, 1982). The study by Seaton found that 21 cases out of 623 workers surveyed at a Scottish mine had been exposed to 13 % quartz compared to 8% in matched controls. However, the incidence of CWP at a high quartz coal mine in Nottinghamshire was much lower (Walton *et al*, 1977). It was thought that minerals from clays such as illite could bind to quartz particles and reduce their biological activity and that this might explain the differences in lung disease seen at different collieries (Walton *et al*, 1977; Le Bouffant *et al*, 1977).

A recent experimental study in which rats inhaled dust from the Scottish or Nottinghamshire mines showed that this explanation did not always apply. Both dusts produced inflammation and interstitial fibrosis, but the Scottish dust also caused a profuse development of fibrotic nodules (Davis *et al*, 1991). Both dusts had a relatively high illite clay content and so the explanation for the differences in the incidence of lung disease in miners and pathology in rats is now thought to lie in the fact that the quartz grains in the Nottinghamshire dust were small and unfractured by coal cutting and were probably coated with other minerals, whereas the Scottish dust particles consisted of freshly fractured fragments of larger quartz grains which were probably free of contaminating metals (Miller *et al*, 1993). These studies show that the nature of the quartz particles needs to be taken into account in epidemiological studies, not just the overall quartz content of a mine dust.

Of particular interest to the LU study, in view of the high iron content of LU dust, are those researches in workers exposed to dusts containing significant quantities of iron such as in iron ore mining and foundries. Several studies have confirmed silicosis (mixed dust fibrosis) associated with silica in iron ore (Stewart and Faulds, 1934; Jorgensen 1986) and materials used in foundries (Bailey *et al*, 1974; McLaughlin and Harding, 1956). A major study on the levels of quartz in the lungs of haematite (Fe_2O_3) miners found that lungs with the greatest degree of fibrosis had no more quartz than those with no fibrosis (Faulds and Nagelschmidt, 1962). As the amounts of haematite in the lungs did not vary with degree of fibrosis a protective role of iron could not be judged. However, by comparing the levels of quartz and fibrosis in the lungs of haematite miners with the quartz and fibrosis seen in coalminers, the authors concluded that, with the same level of quartz, there was less pneumoconiosis in

haematite workers. This suggested a protective effect of other components, including iron, in haematite mine dust. The oxidative state of iron, for example whether it is in the ferrous (Fe^{2+}) or ferric (Fe^{3+}) form, is thought to be extremely important in determining its effects in the body (Aust *et al* 1985; Ghio *et al* 1990).

Similarly, in a study of quartz in the lungs of German iron ore miners who did not have pneumoconiosis, Reichel *et al* (1977) argued that the amounts of quartz found would have caused silicosis in coal miners and thus that the presence of iron hydroxide had a protective effect.

Both quartz and total dust exposure were associated with the development of pneumoconiosis at an iron ore mine in Labrador (Moore *et al*, 1987). As in the Scottish coal mine studies referred to above, the quartz relationship was only apparent when quartz formed at least 13% of the mine dust. Thus iron may be protective up to that proportion of quartz.

Significant quantities of iron can be found in pneumoconiotic lungs. This iron content is related to the mineral and coal contents of lungs and also to the numbers of years worked underground (Bergman and Carswell 1972). There was good correlation between simple pneumoconiosis and coal and other minerals, including iron (Bergman and Casswell 1972; Rossiter 1972). Because iron has a high absorption coefficient for x rays, the iron content of lungs correlates strongly with radiological category of pneumoconiosis (Casswell *et al*, 1971). At least some of the iron in pneumoconiotic lungs is endogenous, that is it arises from the coating of foreign particles in the lung with an iron-containing protein, haemosiderin. There is no evidence that iron has a modulating role in the body's reaction to coalmine dust.

The evidence from these human studies that the presence of iron in a mixed dust can lessen the pathological effects of quartz is suggestive, but not conclusive. A number of animal experiments have provided mostly corroborative grounds for iron protection. Kettle (1932) reported that quartz particles coated with iron were non-fibrogenic. Gardner (1938) showed that quartz-containing iron ores produced less fibrosis than quartz alone when instilled into rabbit lungs. In another early study, Naesland (1940) found that aluminium was more effective than iron in reducing silicosis in guinea pigs and rabbits exposed by inhalation.

Gross *et al* (1960) used quartz which had become contaminated with iron (8%) during the preparation of quartz particles from flint. Control quartz had the iron removed chemically. The process of silicotic nodule formation was greatly slowed in rat lungs instilled with the iron-containing quartz compared to the control quartz. Histological assessment showed that collagenisation was accompanied with a loss of iron. In guinea pigs exposed by inhalation for 1 year to the iron-contaminated quartz there was fibrosis in the alveolar walls but no nodule formation. Unfortunately, no guinea pigs were exposed to iron-free quartz and so a judgement on the protective role of iron cannot be made.

Renne *et al* (1985) found the same dose-related levels of lung fibrosis in hamsters whether instilled with quartz alone or with quartz mixed with iron oxide.

A number of studies have also examined the effects of iron or iron oxides alone on the lung. Stacey *et al* (1959) found only a mild lung response to instillations of two iron hydroxides, goetite and lepidopracite, and iron phosphate ($FePO_4$). In another study, bronchoalveolar lavage of animals instilled with iron oxide also showed increased numbers of neutrophils and other indications of an inflammatory response such as elevated concentrations of albumin and

cytoplasmic enzymes (Beck *et al*, 1982). However, the authors went on to state that they did not believe that these changes were precursors of chronic pulmonary damage. This view was supported by Wright *et al* (1988) who found no pathological change in rat lungs that had been instilled one month earlier with 10 mg iron oxide.

In summary, the evidence from both human and animal studies tends to show that iron can ameliorate the toxic effects of quartz in the lung; iron or oxides of iron appear themselves to be relatively harmless to lung tissue. On the other hand, as described in section 1.4 above, it has been argued that iron from iron-binding proteins in the body plays an important role in the pathological process of fibrosis whether caused by chemical means (Chandler *et al*, 1988; Bus and Gibson, 1984) or silicates (Ghio *et al*, 1990).

2. OUTLINE OF THE STUDY

Concern by London Transport over the levels of respirable quartz in parts of the London Underground system led to the commissioning of a literature review entitled "The toxicity of quartz in mixed dusts with special reference to the London Underground (Donaldson et al 1991). This review made several recommendations including the need to assess the lung toxicity of London Underground (LU) dust in an animal model and to determine the modulating effect, if any, of the dust's iron component on that toxicity. London Underground Ltd commissioned the IOM to conduct such a biological study and the findings are reported here.

The main elements of the study are outlined as follows:

2.1 To collect and analyse airborne respiratory dust from a station in the London Underground.

2.2 To determine the effects of the instillation of various concentrations of the collected LU dust on the lungs of rats as determined by histopathology and the nature and activities of leukocytes recovered from lungs by bronchoalveolar lavage. Leukocyte activities studied were the *in vitro* release of the toxic agent superoxide and the inflammation-promoting cytokine tumour necrosis factor (TNF).

2.3 To determine the effects of LU dust *in vitro* on alveolar macrophage viability and ability to produce TNF.

2.4 To study the effect of iron on quartz toxicity in the lung, either as particles or coatings on the quartz surface as described in 2.2 above.

2.5 To study the effect of coating quartz particles with iron on their production of surface radicals, on their toxicity to macrophages in vitro, and on their stimulation of the macrophage production of the toxic agent hydrogen peroxide.

2.6 To study the effect of particulate iron or iron surface coating on the inflammatory response to quartz in the peritoneal cavity of mice.

3. MATERIALS AND METHODS

3.1 Animals

Specific pathogen-free (SPF) male Wistar rats weighing about 250 g and 12 week old, SPF, male C57Bl6 mice were obtained from Charles River UK Ltd, Margate, England.

3.2 Respirable dust from the London Underground

3.2.1 Selection of a site for sampling airborne dust

An underground dust sampling location was selected by London Underground Ltd Safety Services Department in conjunction with I.O.M staff using a number of criteria. A location was required which was, as far as possible, representative of the London Underground system as a whole. In addition, stations were excluded which had steep track gradients, which might have resulted in higher dust concentrations associated with increased braking, or which did not have a sufficiently large and secure area in which to set up the sampling apparatus. Such considerations led to the selection of room 3/122 on platform 2 of Holland Park station on the Central line.

Room 3/122 is situated at the left hand end (when facing the track) of the platform 4 metres from the tunnel exit at a point where the rear carriages of London-bound trains are braking heavily to stop at the platform. The room has a false wooden floor with a 150 mm gap around the edge open to the track 2.5 m below. The wall along the track side is brick to a height of 1.68 m above platform level, with a 1.45 m high, open metal grille above reaching to the tunnel wall. The room is 0.94 m wide and 3.78 m long.

It was observed that airborne dust was generated by the trains due to the rapid displacement of air as the trains exit the tunnel. The dust-laden air rushed into room 3/122 through the gaps around the false floor and wall grill as each train arrived.

3.2.2 Dust collection apparatus

In order to obtain a sufficient quantity of dust with the minimum amount of attendance from the occupational hygienist, sampling was carried out using three German-designed vT/BF 50 gravimetric dust samplers (Breuer, 1971). The vT/BF 50's were positioned centrally on the tunnel wall of the room at a height of 1.17 m above the false floor, i.e. about 3.6 m above track level. Removing the sub-respirable fraction filters made it possible to connect two samplers to a single rotary vane pump using independent valves. The samplers were run at the recommended flow rate of 50 L/minute using their in-built flowmeters. A third vT/BF 50 was run using a Rotheroe Mitchell high flowrate sampling pump.

and connected to the same time switch. Samplers were switched off and dismantled on Monday 11 May 1993.

As the aim of the sampling was simply to collect dust and not to calculate the airborne dust concentration, the sampling time was only recorded to the nearest day. Total sampling time was approximately 2016 hours (18 hours/day for 56 days) for the first two samplers and 522 hours (18 hours/day for 29 days) for the third sampler. The total volume sampled was thus approximately 7614 m^3. A total of 1.2 g of dust was collected.

3.2.4 Bulk analysis of LU dust

Approximately 3mg of LU dust was accurately weighed into a crucible then ashed for 3 days at 380°C. The weight loss was determined and the proportion of ash calculated. Two aliquots of ash (approximately 1mg each) were accurately weighed and analysed for quartz, kaolinite and illite/mica by infrared spectrophotometry using the potassium bromide technique (HSE, 1984). The proportion of quartz in sample was calculated from the mass of quartz in ash and the proportion of ash in sample. A qualitative elemental analysis of the LU dust was undertaken by scanning electron microscopy/energy dispersive X-ray spectrometry. Following this iron, manganese, lead, cadmium, zinc, copper and chromium were analysed quantitatively by atomic absorption spectrometry following procedures based on those described by the HSE (1981). For this approximately 2mg of dust was dissolved in 50% hydrochloric acid and the solution diluted to 25ml. Analytical wavelength and instrument conditions were those supplied by the manufacturer (Thermoelectron Inc).

3.2.5 Size and composition analyses of individual particles

This was determined using scanning electron microscopy (STEM) with elemental analysis by energy dispersive X-ray spectrometry (EDXS). The methods used were those described by Addison et al (1990).

Samples of the dust to be studied were dispersed in 50:50 methanol water mixtures and deposited on 0.2 μm pore size polycarbonate filters which were then carbon coated. Three mm x 3 mm squares of the carbon film with the entrained particles were cut out and transferred to 150 mesh or 200 mesh copper transmission electron microscope grids using the Jaffe Wick method with chloroform (Chatfield, 1982).

These grids were examined using STEM. The image signal was inverted and passed to the image processor (Link AN10000 image processor). The 15 x 15 cm square screen image was digitised in a 512 x 512 point matrix so that at 5000X magnification the point (pixel) resolution was 0.06 μm. The processor was set up to recognise particles occupying at least 10 pixels (or about 0.035 μm^2). Up to 30 diameters were measured to define the particle size and the mean diameter was used in this work to define size.

The electron beam scanned each particle for 5 seconds to generate X-ray for the EDXS analyses. Ten particle classifications were used in the study: quartz, kaolinite, illite/mica, iron carbonate, iron sulphate, iron, iron silicate, iron oxide, organic and others. To achieve this carbon, oxygen, iron, sulphur, calcium, titanium, aluminium silicon and potassium contents of each particle were measured. The percentage of total X-rays emitted from the various elements were used to categorise individual particles. The ranges of these percentages for each class are given below:

	Quartz	Kaolin	Mica	Iron Carbonate	Iron Sulphate	Iron	Iron Silicate	Iron Oxide	Organic
%									
Carbon	0-30	0-30	0-30	10-40	0-30	0-30	0-30	0-30	10-100
Oxygen	10-100	10-100	10-100	15-100	10-50	0-10	10-30	10-50	5-100
Iron	0-10	0-10	0-10	10-100	10-100	20-100	10-30	20-100	0-10
Sulphur	0-6	0-10	0-10	0-10	10-100	0-10	0-10	0-10	0-10
Calcium	0-6	0-6	0-6	0-6	0-6	0-6	0-6	0-6	0-6
Titanium	0-6	0-6	0-6	0-6	0-6	0-10	0-6	0-10	0-6
Aluminium	0-15	10-100	10-100	0-10	0-10	0-10	0-10	0-10	0-15
Silicon	25-100	10-100	10-100	0-15	0-15	0-15	10-35	0-10	0-15
Potassium	0-6	0-6	6-20	0-6	0-6	0-6	0-6	0-6	0-6

These are not elemental compositions and have been selected to enable a non-standard or mixed mineral or particle to be classified according to the composition of the predominant type present. In particular, it was necessary to allow, for classification purposes, particles to contain significant proportions of iron. Otherwise many particles would have been unclassifiable. In addition to the above classification, particles were also categorised according to their iron content as assessed by the emission of X-rays.

3.3 Control dusts

Three respirable mineral dusts, the pathogenic quartz (DQ12 standard) and the relatively non-pathogenic titanium dioxide (TiO_2; rutile; Tioxide, Stockton-on-Tees, UK) and particulate iron (reduced pentacarbonyl iron; Sigma) were used as control dusts.

In order to study the effects of the presence of iron on the toxicity and pathogenicity of quartz, particulate iron was mixed in various proportions with quartz. In a number of experiments we used a mixture which reflected the concentration of quartz in our sample of LU dust. For some experiments quartz particles were actually coated with iron as described in the next section.

3.4 Coating of quartz with iron

Samples of DQ12 quartz were coated with iron using ferrous or ferric chloride dissolved in distilled water to 1M concentrations. Quartz was suspended at 1 mg/ml in the iron chlorides and mixed by hand from time to time throughout the day and then left overnight at room temperature. The treated quartz samples were then washed twice in distilled water by centrifugation. The pellets of quartz were allowed to dry and finally stored at room temperature in a closed container until required for experimentation. Three batches of iron-treated quartz prepared in this way plus a fourth batch prepared by mixing on a rotary mixer for 1 hour were sent to Dr Vallyathan (NIOSH, USA) for experiments on radical production (section 3.15 below).

3.5 Confirmation of iron coating of quartz

The presence of iron on quartz particles treated with iron chlorides was confirmed using an iron staining kit (Sigma, Poole, U.K.). This kit is based on the Prussian blue reaction in which ionic iron reacts with acid ferrocyanide producing a blue colour. Ten mg samples of treated and untreated quartz were suspended in 2 ml staining solution (1:1 mixture of 4% potassium ferricyanide solution and hydrochloric acid at 2 mmol/L) and left at room temperature for 10 minutes. Quartz particles were then washed three times by centrifugation in distilled water. Both ferrous and ferric chloride-treated quartz particles stained blue indicating that iron was bound to the quartz, untreated quartz was not stained.

3.6 Coating of quartz with aluminium

Samples of DQ12 quartz were suspended at 1 mg/ml in 1% aluminium lactate in water and mixed by hand from time to time throughout the day and then left overnight at room temperature. The treated quartz samples were then washed twice in distilled water by centrifugation. The pellets of quartz were allowed to dry and finally stored at room temperature in a closed container until required for experimentation. Three batches of aluminium-treated quartz prepared in this way plus a fourth batch prepared by mixing on a rotary mixer for 1 hour were sent to Dr Vallyathan (NIOSH, USA) for experiments on radical production (section 3.15 below).

3.7 Endotoxin

The principal active component of endotoxin, lipopolysaccharide (LPS) was used in this study to stimulate alveolar macrophages *in vitro* to produce tumour necrosis factor. LPS from E.coli strain 0127:B8 (phenol extraction) was obtained from Sigma (catalogue number L3880).

3.8 Injection of dusts

3.8.1 Preparation of dusts for injection

Before injection all dusts were suspended in phosphate-buffered saline (PBS) and sonicated for about 1 minute. London Underground dust was flushed several times through a 19 gauge hypodermic needle to break up clumps prior to sonication.

3.8.2 Intratracheal administration in rats

Animals were anaesthetised by fluothane inhalation. A small area of the trachea was then exposed by cutting the overlying skin and muscle. Test dusts, suspended in 0.5ml phosphate-buffered saline(PBS), were injected with a hypodermic syringe and blunted needle into the lung through a small incision between two cartilage rings made with a sterile 21 gauge needle. Wounds were closed with two Michel clips.

3.8.3 Intraperitoneal administration in mice

Dusts (2.5 mg) suspended in 0.5 ml PBS were inected into the peritoneal cavity of C57Bl6 mice.

3.9 Recovery of cells by lavage

3.9.1 Bronchoalveolar lavage cells

Animals were killed by intraperitoneal injection of an overdose (70mg per Kg body weight) of sodium pentobarbitone (Nembutal; Sanofi Limited, Watford, UK). Lungs were removed and the bronchoalveolar space lavaged with 4 x 8ml volumes of warm (37°C) saline. Bronchoalveolar lavage cells were recovered by centrifugation and resuspended to $1x10^6$ in Ham's F10 medium (Gibco, Paisley, UK) supplemented with 0.2% bovine serum albumin (Sigma). This medium contained less than 0.1 ng/ml endotoxin (Coatest;) Differential cell counts were made on stained cytocentrifuge preparations. One million cells were cultured overnight in 24-well plates, 1 ml per well (Greiner Labortechnic Ltd, Cam, Glos., UK) at 37°C in an atmosphere of 5% CO_2. Cell-free culture fluid from these dishes was prepared by centrifugation and stored at -70°C until required for cytokine assay.

3.9.2 Peritoneal lavage cells

Mice were killed by intraperitoneal injection of an overdose (70mg per Kg body weight) of sodium pentobarbitone (Nembutal; Sanofi Limited, Watford, UK). Peritoneal cavities were washed out with 3 sequential injections of PBS containing 10 units/ml heparin. Recovered cells were counted and identified on stained cytocentrifuge preparations.

3.10 Tumour necrosis factor (TNF) assay

TNF activity was determined using the mouse L929 fibroblast lysis assay (Flick and Gifford, 1984). L929 cells were plated in 96-well microtitre plates at $3x10^4$ cells per well in Eagle's minimal essential medium (Gibco, Paisley, UK) and cultured for 20 hours at 37°C. Serial dilutions of test supernatants were then added in medium containing 1 µg/ml actinomycin D (Sigma) but free of all other antibiotics. Eighteen hours later remaining cells were stained with crystal violet (0.5% in 20% methanol) and the absorbance of wells read at 540 nm using a Dynatech MR650 plate-reader (Dynatech LaboratoriesLtd). The amount of TNF required to mediate half-maximal (50%) cytotoxicity was assigned the value of 1 unit. TNF activity was standardised using a human recombinant TNF alpha preparation (Genzyme Diagnostics, West Malling, Kent, UK) shown to have cross reactivity with rat TNF (Phan *et al*, 1992; Savic *et al*, 1990).

3.11 Superoxide assay

The production of superoxide anion by bronchoalveolar lavage cells was measured using a method adapted from Johnstone (1981) as described previously (Donaldson *et al*, 1988). The reaction buffer consisted of phosphate-buffered saline (PBS) supplemented with 2 mg/ml dextrose and the substrate cytochrome c (from horse heart, Sigma, Poole, UK). BAL cells were adjusted to $5x10^6$/ml and 50 µl of the cell suspension added to 1.5 ml of the reaction buffer in 4.5 ml plastic tubes. Test dusts were prepared in PBS and added to the cells to give

final dust concentrations of 50 μg/ml. Other tubes were set up containing, additionally, the membrane stimulant phorbol myristate acetate (PMA; Sigma) at 1 μg/ml in order to examine the possible priming of lung leukocytes for superoxide production by the in vivo dust treatments. All treatments were set up in triplicate. Control tubes were set up for each assay condition containing superoxide dismutase at 360 units/ml (SOD; Sigma), an enzyme which inactivates superoxide. Assay tubes were cultured for 2 hours at 37 °C and then centrifuged to provide particulate-free supernatants. The absorbance of each supernatant at 550 nm was read against a reagent blank in a scanning spectrophotometer (model SP8/400; Pye Unicam, Cambridge, UK). Subtracting any non-SOD inhibitable activity, absorbance was converted to nanomoles of cytochrome C reduced per $2.5x10^5$ cells.

3.12 Cytotoxicity

3.12.1 Macrophage Labelling

Alveolar macrophages were obtained from rats by BAL as previously described (section 3.9). The cytotoxic assay was based on that by Donaldson and Brown (1988). Cells were resuspended at 5 x 10^5 cells/ml in F-10 medium (Gibco, Paisley) containing 0.2% BSA (Sigma) and 200 ul of this cell suspension were added to microtitre plate wells (Flow Labs., High Wycombe). Plates were incubated at 37°C in 5% CO_2 for 1 hour to allow the macrophages to adhere and then non-adherent cells were removed from the wells with two washes of PBS. The PBS was replaced with 200 μl of F10 medium containing 0.2% BSA and ^{51}Chromium (Amersham, Bucks.) to a final concentration of 0.36 MBq/ml. Plates were incubated for 18 hours at 37 °C and unincorporated ^{51}Cr washed away using two washes of PBS.

3.12.2 Cytoxicity of test dusts

Test dust suspensions in F10 + 0.2% BSA were added to the radio-labelled macrophages to give final concentrations of 25, 50, 100, and 200 μg/ml with a volume of 200 μl/well. Plates were incubated for 24 hours at 37°C in 5% CO_2 and then centrifuged for 5 minutes at 1000 rpm. One hundred microlitres of supernatant were removed from each well and counted in a gamma counter. Release of ^{51}Cr into the supernatants provides a measure of macrophage damage. The assay was performed in triplicate for each treatment and the experiment was repeated 5 times. The detergent Tween 20 was used to provide a total release control. Spontaneous release was the amount of radioactivity released in the absence of any treatment.

3.13 Statistical analysis

The work was carried out as a series of experiments and assays for which different methods of statistical analysis were necessary. Data descriptions, by means of tables and graphs, were carried out for each assay and, where necessary, data were transferred to the log-scale prior to analysis. Statistical methods used included analysis of variance and regression methods. Full details of the statistical analyses are given in the results sections to which they refer. The analyses were done using the facilities of the statistical software packages Genstat, version 5.1 (Genstat 5 Committee, 1987) and Minitab, release 8.2 (Minitab Inc, Pennsylvania, USA).

3.14 Digestion of lung tissue to recover quartz

In some lung instillation experiments, lungs were stored frozen, following lavage, and later assayed for total dust and quartz burden. The lung digestion method used was based on that of Sebastien et al., 1989. From storage, lungs were thawed at room temperature, dried with paper tissue, and weighed. The lungs were chopped into small pieces with a scalpel and transferred to glass centrifuge tubes. Lungs were then defatted using mixtures of ethanol and ether by sequential mixing, sonication and centrifugation over a period of about 24 hours.

After a final exposure to ether alone the lung pieces were dried, re-weighed and added to 100 ml bleach in a plastic container. The containers were agitated continuously until the lung tissue had been digested; this process can take up to 18 hours. Following digestion the contents of the containers were washed onto a pre-weighed Nuclepore filter (0.2 μm pore size) using 0.2M hydrochloric acid, the dried filters were re-weighed and the residue washed in a platinum crucible with methylated spirit under ultrasonication. The quartz content of the lung residue was assayed as described in the next section.

3.15 Measurement of quartz recovered from lung tissue

Quartz was assayed according to the Health and Safety Executive's recommended method, MDHS 38 (HSE, 1984) based on the paper by Dodgson and Whittaker (1973). After drying at 140 °C a weighed aliquot of the residue was mixed with potassium bromide and pressed into a disc suitable for infra-red spectrophotometry. The quartz content of the discs was derived from a standard curve produced using DQ12 quartz.

3.16 Experiments carried out by Dr. V. Vallyathan, NIOSH, USA

3.16.1 Preparation of alveolar macrophages for experiments with iron and aluminium-treated quartz

Normal, male, pathogen-free, Sprague-Dawley rats were anaesthetised by intraperitoneal injection of sodium pentobarbital and exsanguinated by cutting the left renal artery. Lungs were lavaged ten times with 8 ml volumes of calcium and magnesium-free Hanks balanced salt solution (HBSS). Recovered cells were treated with hypotonic 0.2% sodium chloride to lyse red blood cells. Hypertonic NaCl (1.6%) was then added to equilibrate the osmolarity. Lavage cells were centrifuged in complete HBSS (containing Ca and Mg) and the red cell lysis step repeated. After a final centrifugation, alveolar macrophages were suspended in complete HBSS for the superoxide dismutase (SOD) and lactate dehydrogenase assays and in HEPEs buffer containing 5.5 mM glucose for the hydrogen peroxide assay. Differential cell counts were made using an electronic cell counter equipped with a cell sizing attachment (Coulter model ZB1 with a Channelizer 256; Coulter Electronics Inc., Hialeah, Florida, USA) and the cell concentration adjusted appropriately for the selected assay (see below). Cell viabilty and purity were determined microscopically using the trypan blue dye exclusion technique. Ninety six per cent of cells were viable alveolar macrophages.

3.16.2 Hydrogen peroxide assay

Hydrogen peroxide (H_2O_2) production by alveolar macrophages in vitro was measured using the scopoletin method (Van Scott et al. 1984). Macrophages at 1 million/ml were incubated with 0.5 mg/ml treated or untreated quartz in buffer containing 5 mM HEPES, 5.5 mM

glucose, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 6 U/ml horseradish peroxidase, and 0.72 mM scopoletin for 20 minutes at 37 °C . Reaction tubes were spun at 2500 rpm for 5 minutes and the supernatants assayed in duplicate on a IDEXX fluorescence concentration analyser at a gain of 25 and with the excitation filter set at 365 nm and the emission filter set at 450 nm. H_2O_2 concentration was derived from a standard curve and the results expressed as nmoles per million cells.

3.16.3 Superoxide dismutase assay

Intracellular and extracellular superoxide dismutase activity was measured in cultures of macrophages exposed to treated and untreated quartz in vitro. Enzyme activity was determined as the amount of inhibition of superoxide produced by the xanthine/xanthine oxidase reaction based on the method of McCord and Fridovich (1969) with the modification adapted by Elstner *et al.* (1983). Macrophage supernatants (extracellular SOD) or macrophage lysates (intracellular SOD) obtained by disrupting the cells with ultrasonication were added to a 0.05M carbonate buffer, pH 10.0 containing cytochrome C (0.248 mg/ml), and 0.5 μM xanthine. Xanthine oxidase was then added to a final concentration of 0.004 U/ml. The assay was performed using the automated COBAS FARA II system (Roche Diagnostic Systems Inc., Montclair, New Jersey, USA) and the concentrations of SOD determined from a standard curve produced with pure superoxide dismutase (Sigma Chemical Co.; S-2515).

3.16.4 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a cytosol enzyme whose release from cells provides a reliable measure of cell injury. Macrophages at 1 million/ml were incubated, with shaking, together with 1 mg/ml treated or untreated quartz in complete HBSS for 1 hour at 37 °C. Supernatants from treated alveolar macrophages were prepared by centrifugation at 2500 rpm for 7 minutes at 2 °C and refrigerated until assayed.

The basis of the assay is the reversible LDH-mediated reaction between lactate and NAD (nicotinamide dinucleotide) to form pyruvate and the reduction of NAD to NADH (Gay *et al.* 1968). Since the reduction of of NAD proceeds at the same rate as the oxidation of lactate, the reaction can be followed spectrophotometrically by measuring the increase in absorbance at 340 nm due to the formation of NADH. The substrate reagents used were 2-amino-2-methyl-1,3-propanediol (125mmol/L) and L-lactic acid (lithium salt; 85mmol/L) with NAD at 10.6 mmol/L (all reagents supplied by Roche, USA). The assay was carried out using a COBAS FARA II instrument and the results expressed as LDH units per litre per million cells.

3.16.5 Electron Spin Resonance measurements on coated quartz

Electron spin resonance (ESR) spectra were obtained from iron or aluminium-coated quartz using a Varian E109 ESR spectrometer at X-band (9.7 GHz) The magnetic field was calibrated and the following typical settings were used: receiver gain $1x10^4$, modulation amplitude 2G, scan time 120 seconds, and magnetic field 3380 ± 200 G. Silicon band radicals were characterised by the ESR spectra centred around 2.00015 G. The microwave power and magnetic field modulation amplitude were adjusted to obtain optimum ESR signal resolution and intensity.

As described in sections 3.7 and 3.9 above, four sets of quartz samples were prepared, three coated for 24 hours and one coated for 1 hour. Fifty mg of dry quartz dust were added to NMR tubes for ESR readings. ESR settings were the same for all samples.

Hydroxyl radical production by the treated quartz samples was measured in the presence of hydrogen peroxide (H_2O_2) and the spin trap agent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO; Aldritch Chemical Co, Milwaukee, USA). One hundred ul of 1M DMPO and 100ul 1M H_2O_2 were added to 10 mg quartz in 800 µl phosphate buffered saline. All samples were read 3 minutes after the the addition of H_2O_2.

3.17 Histology

Rat lungs were inflation fixed with 10% formalin in PBS at a pressure of 30 cm water and later embedded in paraffin. Peripheral sections and deep sections were cut and stained with haematoxylin and eosin for light microscopy. Some additional sections were stained with Van Gieson's stain to highlight collagen, or silver to show reticular connective tissue (reticulin).

4. RESULTS

4.1 Analyses of LU dust

4.1.1 Bulk analysis

The London Underground dust sample was found to contain 7.8% quartz, 34.5% iron, 0.24% manganese and relatively minor amounts of lead, zinc, copper and chromium (Table 4.1). This quartz content is within the range found in previous analyses of dusts collected in the London underground system (London Transport Research Laboratory, 1989). As a result of this bulk analysis artificial dusts were prepared consisting of particulate iron and 7.8% quartz, by weight, for study in animal experiments.

4.1.2. Size of the LU particles

Particles in the LU dust sample were measured using electron microscopy (STEM) as described in the Materials and Methods section. The mean of up to 30 measured diameters were used to define particle size and a total of 896 particles were measured. The number size distribution is shown in Table 4.2. The median diameter was 1.1 μm and the 25% and 75% quartiles were 1.8 μm and 0.5 μm respectively. The size distribution of the LU dust is thus within the respirable range and is similar to coalmine dust samples collected using vT/BF 50 samplers (Robertson et al, 1992) but coarser than DQ12 quartz (Robock, 1973). The distribution is skewed because the vT/BF 50 samplers collect only a small proportion of the airborne dust particles whose aerodynamic diameters are less than 1 μm.

This skew is apparent in Figure 4.1 which is the size distribution of LU dust, suspended in aqueous media, carried out by Malvern Instruments (Malvern, England) using a Zeta-sizer 4 instrument. This machine determines the velocities of particles in suspension as they undergo Brownian motion using the Stokes-Einstein equation:

$$D = kT / 9.43\ V\ S$$

where diffusion (D) is related to particle diameter (S), k is Boltzman's constant, T is temperature, and V is viscosity. The machine reports size distributions in 24 logarithmically related size bands spread over a pre-selected size range, 0 - 7 μm in the case of the LU dust.

The results show that the LU dust is heterogeneous with particles ranging in size from less than 0.15 μm to greater than 6.9 μm and, as for the electron microscope analysis, the median diameter was 1.1 μm.

4.1.3 Mineral and chemical classification of particles

The mineral and chemical classification of the individual particles are summarised in Table 4.3. Only 4% of particles were classified as quartz. A further 55% of the particles were iron or iron compounds with iron, iron carbonate and iron oxide accounting for almost all of these. Thirty seven percent were not classified using the methods described earlier. Almost all particles

contained more than 5% iron (from X-ray emission) with the median iron content being around 26% (Table 4.4). Many particles classified as quartz contained significant proportions of iron. Table 4.5 shows the percentages of iron associated with quartz particles in the LU dust. It is not known whether this was surface contamination or if the iron was part of the particles' structures. Tightening the quartz classification rules reduced the numbers of particles classified as quartz. Reducing the upper limit of iron emitted X-rays from 10% to 5% had little effect but a further reduction to 2.5% halved the number and further tightening caused greater reductions. Note that pure quartz would not contain iron.

Direct comparison of results from these analyses and the bulk analyses of the dust are difficult. The two sets of results are clearly compatible. In the dust analyses mass proportion of quartz was 7.8% and the mass percentage of iron 34.5% These compare with 4.2% of particles being classified as quartz and the median iron content of individual particles (as determined by intensity of emitted X-rays) of 26%.

4.2 The effect of iron on the pulmonary cellular response to instilled quartz

The majority of the particles in the London Underground dust sample contained iron and thus experiments were set up to study the influence of iron on the possible toxicity of the LU dust's quartz component.

4.2.1 The effect of iron particles on the pulmonary response to quartz

Rat lungs were instilled with 1 mg of quartz, particulate iron, or the non-pathogenic dust titanium dioxide (TiO_2), or 1 mg quartz mixed with 1 mg of particulate iron or TiO_2. Three animals per treatment group were used and, with the exception of TiO_2, the experiment was repeated four times. Lungs were lavaged 7 days following instillation and differential counts were made on the recovered cells. The data were analysed using linear regression methods to look at the effects of differences between experiments, treatments, and their interactions. Preliminary examination of the cell count data indicated that transformation to the log scale would provide a better fit in the regression models. Geometric means and geometric standard deviations for total cells, macrophages, and neutrophils are shown in Table 4.6. The effects of treatment varied with experiment, mainly because experiment 1 differed from the other three experiments, and it was not possible to draw general conclusions about differences between treatments. However, with the exception of experiment 1, iron had no effect on the inflammatory response to quartz. In the absence of quartz, particulate iron and TiO_2 produced very little response.

4.2.2 The effect of varying the proportions of quartz and iron

The 1 mg dose of quartz used in the above experiments produced a strong inflammatory response which might have masked any modulatory effects of iron. Therefore, further experiments were conducted using smaller amounts of quartz. Rat lungs were instilled with 1 mg of mixtures of quartz and particulate iron containing 1, 5, 10, or 25%, by weight, of quartz. These percentages of quartz reflect the range of quartz levels found in airborne dust sampled at different sites in the London Underground (London Transport Research Laboratory, 1989).

Control animals were treated with quartz alone at 10, 50, 100, or 250 μg. Two additional treatments comprised one to one mixtures (by weight) of quartz and iron containing 10, or 100 μg quartz. Three experiments were conducted with one animal per treatment group per experiment. Lungs were lavaged 32 days following instillation and total cell numbers and numbers of macrophages, neutrophils, and lymphocytes were recorded and log-transformed prior to analysis of variance.

Total cell, macrophage, and neutrophil numbers all increased significantly with dose for the quartz alone controls (Table 4.7). Among the quartz/iron mixtures total cell and macrophage numbers for the 5% (50 μg) quartz in iron were generally lower than for the other mixtures, but there was no trend with increasing quartz content. Neutrophil numbers increased with dose of quartz in both the quartz alone and quartz/iron groups. There were also more neutrophils in lavage from animals treated with the higher dose 1:1 mixture (100 μg quartz + 100 μg iron) compared to those treated with the lower dose 1:1 mixture. Lymphocytes tended to be absent or at low levels (1% or less of total cells) for most treatment groups but their numbers did increase with quartz dose in the quartz alone group (data not shown).

Overall there was no significant effect of iron on any of the cellular responses to quartz.

4.2.3 The effect of particulate iron or TiO_2 on the pulmonary response to a fixed dose (100 μg) of quartz

Rat lungs were instilled with 100 μg quartz with or without mixture with 25, 50, or 100 μg of the non-pathogenic dusts TiO_2 or particulate iron, making 7 treatments in all. Four experiments were conducted on different days with one rat per treatment per experiment. Lungs were lavaged 32 days following instillation and, as before, total cell numbers and numbers of macrophages, neutrophils, and lymphocytes were recorded and log-transformed prior to analysis of variance. There were no major differences between experiments.

Results for total cell, macrophage, and neutrophil numbers (as geometric means) are shown in Table 4.8. For total cells there was no overall effect of TiO_2 or iron. There was some evidence that macrophage numbers declined with increasing dose of TiO_2 or iron ($p=0.06$). Neutrophil numbers were not influenced by the TiO_2 or iron treatments. Lymphocytes were often absent in lavages or formed only a small percentage of total cells (3% or less) but compared to the quartz alone treatment, lymphocyte numbers were significantly lower for the 100 μg TiO_2 treatment and for each of the iron treatments (data not shown).

4.2.4 The effect of coating quartz with iron or aluminium on the pulmonary cellular response to 1 mg instilled dust

The effect on the pulmonary leukocyte response of coating quartz particles with ferrous or ferric iron was also studied. Aluminium lactate-treated quartz was included as a positive control as it has previously been shown to reduce quartz toxicity in the lung (Brown et al, 1990). Control quartz was subjected to the same coating procedure using distilled water in place of the metal salts. Rat lungs were lavaged 7 days or 32 days following intratracheal instillation of 1 mg of quartz or quartz coated with ferrous or ferric iron, or aluminium lactate and the inflammatory response was assessed by counting the total number of leukocytes and the proportions of different cell types recovered in the lavage fluid . Three rats per treatment were

used and the experiment was repeated four times for the 7 day time point and, with the exception of the aluminium treated quartz which was done once, repeated twice for the 32 day time point. Total cell numbers and numbers of macrophages, neutrophils, and lymphocytes recovered in lavage were log-transformed prior to analysis of variance. Cell counts were significantly higher at day 32 than at day 7 for all treatment groups. Geometric means of total cell counts, pooled from all experiments, are shown in Figure 4.2. Note that, although the data were pooled to assist in illustration, there was some interaction between experiment and treatment with the magnitude of the differences between treatments varying between experiments. Nevertheless, in all the experiments the strong cellular response to quartz was significantly reduced only by the aluminium lactate treatment, pre-treatment with iron had no effect. Similar statistically significant reductions were seen with aluminium treatment for macrophage, neutrophil, and lymphocyte numbers (data not shown).

4.2.5 The effect of coating quartz with iron on the pulmonary cellular response to smaller quantities of instilled dust

Additional animals were instilled with 25, 50, or 100 μg quantities of quartz or quartz coated with ferrous or ferric iron since it was thought that the strong response to 1 mg quartz may have obscured any modulatory effect of the iron treatments. Four experiments were conducted on separate days with one rat per treatment/dose combination per experiment. Lungs were lavaged 32 days following instillation. Total cell numbers and numbers of macrophages, neutrophils, and lymphocytes recovered in lavage were log-transformed prior to analysis of variance. Results (as geometric means) are shown in Figure 4.3 for total cells, macrophages and neutrophils. For total cells, macrophages, and lymphocytes there were no significant differences between experiments, doses, or treatments. There was limited evidence that total cell numbers increased with increasing dose but this was not statistically significant at the (5% level). For neutrophils there were large and significant differences between experiments, doses, and treatments, but no interactions between these factors. Numbers were much higher in experiment 2 than in the other experiments, while numbers for experiment 4 were lower. Neutrophils increased with dose and were much higher for the ferrous (Fe^{2+}) and ferric (Fe^{3+}) treated quartz than for the water-treated quartz. Iron showed no protective effect in these experiments.

4. 3 Modulation of the inflammatory response to quartz in the mouse peritoneal cavity

The potential of iron to modify the inflammatory response to DQ12 quartz was also explored using the mouse peritoneal model. Peritoneal cavities were lavaged two days following injection of 2.5 mg quantities of test dusts and recovered cells were identified and counted. Two types of experiment were conducted, the first studied the effect of coating quartz with iron or aluminium and the second examined the effect of co-injection of quartz with the particulate dusts iron or titanium dioxide. Each experiment type was repeated three times and there were 4 mice per treatment per repeat experiment.

4.3.1 The effect of coating quartz on its inflammogenic potential

Three coating treatments, ferrous chloride, ferric chloride, and aluminium lactate, were compared with quartz treated with water. The response variables total cells, macrophages, neutrophils, eosinophils and lymphocytes were transformed to the log scale prior to analysis of

variance. Neutrophil and eosinophil numbers were also combined to give a 6th variable designated granulocytes. Neutrophil and granulocyte numbers provide indices of the inflammatory response in the peritoneum. Results from the three experiments are shown in Table 4.9 for total cells, macrophages, and granulocytes. For all variables, except lymphocytes, there were significant differences between treatments. In all cases levels for quartz/aluminium were much lower than for the other treatments ($p<0.01$). Differences between quartz/water, quartz/Fe^{2+}, and quartz/Fe^{3+} were much smaller and there was little consistency in their relative values. These results thus show no modulatory effect of iron coating on the response to quartz in this mouse peritoneal model.

4.3.2 Co-injection of quartz with iron or TiO_2

For all of the response variables there were significant differences between experiments and the relationships between the treatments varied depending on the experiment. It was not, therefore, possible to make any general conclusions about the relative effects of the various treatments and the results for this group of experiments have not been included in this report.

4.4 Experiments carried out by Dr. V. Vallyathan, NIOSH, USA on untreated quartz and quartz pre-treated with iron or aluminium

4.4.1. Hydrogen peroxide production by alveolar macrophages exposed in vitro to untreated quartz or to quartz coated with iron or aluminium.

Hydrogen peroxide (H_2O_2) production in vitro by alveolar macrophages incubated for 20 minutes with 0.5 mg/ml untreated quartz or quartz particles coated for 24 hours with iron or aluminium is summarised in Figure 4.4. H_2O_2 levels were greatest with the water (control) and aluminium lactate treatments. Production was lowest with Fe^{2+}-treated quartz, even lower than the spontaneous H_2O_2 production by untreated macrophages. When all results were compared to the untreated macrophage (basal) level using Student's t test, the water, Fe^{2+}, and aluminium treatments gave significantly more ($P< 0.001$;) H_2O_2 production whereas the untreated quartz and the Fe^{3+} treatments gave similar amounts to the basal level. Comparison of untreated quartz results with those of treated quartz dusts showed no difference for the Fe^{2+} and Fe^{3+} treatments but significantly more production for the water ($p< 0.02$) and aluminium ($p< 0.01$) treatments.

4.4.2 Measurements of intracellular and extracellular superoxide dismutase following incubation of alveolar macrophages with untreated quartz or with quartz coated with iron or aluminium.

As part of the body's defence system against oxidant injury, cells can produce the enzyme superoxide dismutase (SOD) which converts superoxide to oxygen and hydrogen peroxide. Two million normal alveolar macrophages were incubated with 0.5 mg untreated quartz or quartz pre-treated with iron or aluminium for 1 hour and both the intracellular and extracellular (supernatant) concentrations of SOD assayed.

Compared to untreated (no dust) macrophages, only untreated quartz caused a significant ($p < 0.05$) drop in the intracellular level of SOD (Figure 4.5A). All the quartz dusts caused some release of SOD extracellularly into the culture supernatant (Figure 4.5B). When the extracellular results were compared using Student's t test, untreated quartz was no different to the treated quartz dusts.

4.4.3 Release of lactate dehydrogenase by alveolar macrophages exposed in vitro to untreated quartz or to quartz treated with iron or aluminium

Lactate dehydrogenase (LDH) is a cytosol enzyme whose release from cells provides a reliable measure of cell injury. Normal alveolar macrophages were incubated for 1 hour with 1 mg/ml untreated quartz or quartz pre-treated with ferrous or ferric iron or aluminium lactate. All quartz samples, treated or untreated, caused LDH release from macrophages (Figure 4.6). Some of the differences in the amounts of LDH released by the various treatments were statistically significant. For example, untreated quartz produced more than water or aluminium treated quartz, p values by Student's t-test 0.004 and 0.007 respectively. There was no difference between untreated quartz and ferrous or ferric-treated quartz. However, despite the statistical significance of some of the results the differences were relatively small and it is clear that all the quartz samples were cytotoxic in this assay.

4.4.4 Measurement of radicals on the surface of quartz particles treated with iron or aluminium

Electron spin resonance measurements were made on 4 batches of quartz treated with water, ferrous or ferric chloride, or aluminium lactate. Quartz was treated overnight for batches 1 to 3 and for 1 hour for batch 4. Batch 1 was measured in a separate session to that of the other batches.

Measurements on dry dust.

ESR spectra, summarised as peak heights, for dry dust are shown in Figure 4.7. All the treated quartz samples produced radical activity, and although there were slight differences between treatments, these were not consistent from batch to batch. Comparison of results from batches 2 and 3 with those of batch 4 indicate that the length of the treatment period, 24 hours as opposed to 1 hour, did not affect radical production. By analysis of variance of results pooled from the 4 batches, there were no significant differences between the four treatments.

Measurements in liquid in the presence of DMPO and hydrogen peroxide

Hydroxyl radicals, measured as an adduct of the spin trap agent DMPO, were produced by all treated quartz samples and results are shown in Figure 4.8. Previous published work by Dr Vallyathan and colleagues (Shi et al 1988) has shown that DMPO alone does not produce a detectable ESR spectrum. There were slight differences in radical concentration between the various treatments but, as for the ESR measurements on dry dust, these differences were not consistent from batch to batch. Comparison of results from batches 2 and 3 with those of batch 4 indicate that the length of the treatment period, 24 hours as opposed to 1 hour, did not

affect hydroxyl radical production. There were no significant differences between the four treatments as shown by analysis of variance of results pooled from the 4 batches.

4.5 Dose-dependent stimulation by quartz of tumour necrosis factor production by alveolar macrophages

Normal alveolar macrophages were cultured overnight *in vitro* with various concentrations of quartz and production of the pro-inflammatory cytokine tumour necrosis factor (TNF) was assayed. Quartz stimulated TNF production in a dose-dependent manner (Figure 4.9).

4.6 Production of tumour necrosis factor by alveolar macrophages cultured with London Underground dust or various control dusts

Normal alveolar macrophages were cultured overnight in vitro with 50 μg/ml of LU dust, quartz alone, or TiO_2 or particulate iron mixed with quartz to 7.8% by weight. The choice of dust concentration was based on the results of the previous section (4.5) which showed that quartz at 50 μg/ml provided a good stimulus to TNF production. TNF was measured in triplicate in each of the cell-free culture supernatants. Cultures were set up in duplicate for each treatment and the experiment was repeated three times to give a total of 36 supernatants. In order to assess the repeatability of the TNF assay, the supernatants were tested on three separate occasions.

Initial analyses and residual plots suggested that the data be log-transformed. The data were then subjected to an analysis of variance. Four outlying data points were identified which differed markedly from corresponding replicate measurements and thus the data were analysed both for the whole data set, and for the data treating these four points as missing and estimating them by the least squares principle. The ordering of the treatment means is the same in the two analyses, with the means differing only slightly and thus the four excluded data points did not affect the outcome of the analyses.

Results for the amended data set are summarised in Figure 4.10 for the three experiments as geometric means of the three assay results. Differences between dusts were, overall, highly significant. This finding differed little between assays, but there was a significant interaction between dust type and experiment, suggesting that the differences between dusts were not constant over the experiments. Overall, the non-treatment controls, the quartz with iron, and the LU dust treatments all produced similar levels of response. The response was slightly greater than these for quartz and the quartz/TiO_2 mixture and greater still for the quartz/LU dust mixture. Taking a mean of the three experiments the dusts can be ranked: control < quartz/iron < LU < quartz/TiO_2 < quartz < quartz/LU.

4.7 Experiments on the in vitro toxicity of quartz and London Underground dust to alveolar macrophages

Damage to alveolar macrophages through the phagocytosis of toxic dusts could lead to impaired dust clearance from the lung and to heightened inflammatory responses. The toxicity of LU dust and quartz were studied by culturing radiolabelled alveolar macrophages from

normal rats with various concentrations of dusts in vitro and assessing cell death as the amount of radioactivity released. The role played by the iron component of LU dust in this model was examined by using particulate iron mixed with quartz at the same proportion (7.8%) as found in our sample of LU dust. A mixture of 7.8% quartz and the relatively inert dust TiO_2 was used as a control for any specific effect attributed to iron. The effect of quartz alone at 7.8% of the mass used for LU dust was also studied. Finally, we included mixtures of quartz with LU dust or TiO_2 in the ratio of 1:1.

The data, as radioactive counts per minute, from five experiments were log-transformed and examined by analysis of variance methods. Tables of pooled means and of accumulated analysis of variance are given in Appendix 1. Results have also been summarised in Figure 4.11 as the pooled geometric means from the five experiments. Note that the concentrations of the quartz alone controls in Figure 4.11 were 2, 4, 8, and 16 μg/ml. Data from the 1:1 mixtures of dust are shown separately in Figure 4.12.

The analysis showed that there were large differences between experiments and treatments and that there was also some interaction between experiment and treatment. The lack of consistency from experiment to experiment has made interpretation of the data difficult. Thus some dusts only showed toxic effects in some experiments. Adding to the interpretational difficulties was the fact that the 100% lysis control result was, in some instances, considerably lower than some treatment results. In addition, in several experiments, some doses and treatments gave a lower release of chromium than with the medium alone controls. This was most marked with quartz, iron, and the quartz/iron mixture, although the lowest concentrations of the remaining treatments also showed this effect in some experiments. The reason for this effect is not known.

Despite these difficulties, the higher doses of two treatments, LU dust and 7.8% quartz in TiO_2 appeared, overall, to be more toxic than other treatments. Pure TiO_2 was toxic at 200 μg/ml. With the 1:1 mixtures there was a more consistent, dose-dependent, toxic effect (Figure 4.12). Results for the higher quartz control (5, 12.5, and 25 μg/ml) were substantially lower than for the mixtures and showed less of a dose effect.

4.8 The pulmonary leukocyte response to instilled London Underground dust

4.8.1 Protocol

London Underground (LU) dust and various control dusts were instilled into the lungs of rats at doses of 1, 10, or 50 mg. As our sample of LU dust contained 7.8% quartz and a significant quantity of iron, we prepared a control dust consisting of 7.8% DQ12 quartz mixed with particulate iron. Other control dusts included particulate iron alone, DQ12 quartz alone (the high quartz group) and lower doses of DQ12 quartz alone equivalent to the amount of quartz instilled in the LU dust-treated animals (i.e. 7.8% of 1, 10, or 50 mg; the low quartz group). Untreated animals were used as an additional level of control. Lungs were lavaged 7, 32, or 60 days later and the cells recovered in lavage were identified, counted and cultured. Tumour necrosis factor and superoxide anion were then measured in the culture fluids.

4.8.2 High doses of quartz (1, 10, or 50 mg)

Quartz alone instilled at doses of 1, 10, or 50 mg (the high quartz controls) produced considerably greater numbers of total cells and neutrophils than the other treatments and results from these animals were excluded from the main statistical analysis. Results for the high quartz group are summarised in Figure 4.13. Numbers of total cells, macrophages and neutrophils recovered in lavage increased with time post-instillation. The effect of dose was more complex. Numbers of cells (all types) were lower with the 1 mg dose than for the 10 or 50 mg doses. However, cell yields with the 10 mg dose tended to be higher than with the 50 mg dose, particularly, at the 32 and 60 day time points. These differences between the two higher doses were not statistically significant by analysis of variance (analysed separately from the other dust treatments). The reason for the lower yields of cells from the 50 mg treatment may have been due to less efficient lavage because of the increased lung damage associated with the higher dose (see the histology results below).

4.8.3 All other treatments

Total cells

Total cell numbers recovered in lavage increased with dose of dust administered. Results are summarised in Figure 4.14. More detailed results giving geometric means and ranges are provided in tables in Appendix 2, together with details of the statistical analysis. With the 1 mg dose there was no difference in cell numbers between the different treatments (Figure 4.14a). However, at 10 and 50 mg, LU dust produced significantly greater numbers than particulate iron, but significantly lower numbers than the low quartz control (Figures 4.14b and 4.14c). Responses to LU dust and the quartz/iron mixture were similar, at all three doses. Comparison of low quartz results with those of iron/quartz at the higher doses indicates a marked effect of particulate iron in reducing the cell response to quartz.

Time effects were more complex but overall the statistical analysis showed that time only had a comparatively small two-way interaction with dose and a three-way interaction with dust type and dose. This suggests slight differences in the relationship between total cells and dust and dose at the different post-instillation times. For example, at 32 days the effect of LU dust was greater than that of the quartz/iron mixture. For the higher doses of the low quartz group, cell numbers were greatly elevated at day 32 compared to day 7. There was no 60-day time point for this dust. For LU dust and iron/quartz at 50 mg there was an approximate doubling of cell numbers between day 7 and day 60.

Macrophage numbers

Macrophage numbers tended to mirror the total cell results with dust type and dose being the most important variables and the effect of dose varying with dust type. Results from the 1 mg dose show no significant differences between treatments (Figure 4.15). At the higher doses the macrophage response to LU dust was similar to that with iron/quartz, greater than with iron alone, but significantly lower than with the low quartz treatment. Again, particulate iron is seen to reduce the response of quartz. As with total cells, significant interactions between dust dose and time, and between dust type, dose and time were evident, but small in comparison to

the main effects. Further details of the statistical analysis together with tables of means are given in Appendix 2.

Neutrophil numbers

As for the analyses for total cell and macrophage number, the important effects for neutrophil numbers were those from dust type and dose and their interaction. See Appendix 2 for further information on the analysis and tables of means. In general, the neutrophil content of lavages from animals treated with the iron/quartz mixture or the low quartz control was greater than that from LU dust-treated animals at each of the three doses (Figure 4.16). At the 10 and 50 mg doses the effect of LU dust was significantly greater than for particulate iron. At 10 and 50 mg the low quartz results were considerably higher than those with the iron/quartz mixture. Similar to the results for total cells and macrophages, the effects of time were minor.

The relative proportions of neutrophils and macrophages in lavage may be important in determining the activities of both cell types. Table 4.10 summarises the percentage of neutrophils in lavage for the various treatments. The pattern of results was similar to that described for total neutrophils with the percentage rising with dose. Note that neutrophils are rare in the lavage from untreated lungs, generally forming less than 1% of cells. For the 10 and 50 mg doses the percentage of neutrophils found with LU dust treatment was higher than with iron but lower than with the other treatments. For any one dose for any dust the percentages tended to be similar at the three time points.

Summary of the lavaged leukocyte data

For total cells and macrophages, the effects of LU dust were comparable to the quartz/iron mixture but greater than those with iron alone. The neutrophil response was lower with LU dust than with the quartz/iron mixture.

Comparison of the low dose quartz control and the quartz/iron dust indicated a very marked ameliorating effect of iron at the two higher doses.

4.9 Weights of lungs instilled with LU dust or other quartz-containing dusts

As part of the process to assess the clearance of quartz from dust-treated lungs, lungs were weighed prior to mincing and de-fatting (see below, section 4.12). Lung weights 7 and 32 days following the instillation of the quartz-containing dusts are shown in Table 4.11. The results were examined by analysis of variance. There was little difference between the treatments at the 1 mg dose except that the low quartz lungs at day 32 were lighter than the lungs treated with LU dust ($p=0.034$) or quart/iron ($p=0.027$). However, lungs treated with 10 mg (high dose quartz) were significantly heavier than lungs from other treatments at either time point ($p=0.001$). At 50 mg the high dose quartz group was significantly heavier than the other groups at both time points (day 7, $p=0.015$; day 32, $p=0.008$). The difference in lung weights between 50 mg high quartz at day 7 and day 32 was not significant. Lung weight can thus provide a crude index of severe lung inflammation.

4.10 Production of superoxide by cells recovered in lavage from animals instilled with LU dust or control dusts

4.10.1 Protocol and analysis

Cells recovered in lavage from dust-treated rats were cultured for 2 hours with and without the membrane stimulant phorbol myristate acetate (PMA) and the production of the toxic agent superoxide was measured. Results were transformed to the log scale prior to analysis of variance. Data resulting from the high quartz treatment, which caused a much lower superoxide response than other dusts, were excluded from analysis. In addition, all results for the low quartz group at 3.9 mg (equivalent to 50 mg of the LU and mixed dusts) and without PMA, were found to be zero and were also excluded. Further details of the statistical analyses are described in Appendix 2.

4.10.2 Spontaneous production of superoxide

The results for the three time points are shown in Figure 4.17. More detailed results giving geometric means and ranges are provided in tables in Appendix 2. In general, superoxide decreased with increasing *in vivo* dose of dust. The extent of this effect depended on the treatment. Thus the decrease in superoxide production for the low dose (7.8%) quartz controls between "1 mg" and "50 mg" was significantly greater than the corresponding decrease seen for LU dust.

With the exception of the lowest dose of quartz, 0.078 mg in the low dose quartz controls, superoxide production was markedly depressed in cells from animals treated with quartz alone.

4.10.3 Production of superoxide in the presence of the stimulant, PMA

In general, when the membrane stimulant PMA was present in the cultures more superoxide was produced. Results are shown in Figure 4.18. There was still a marked effect of dose. There was also a tendency for results from the 1 and 10 mg dosed animals to decrease with time whereas those from 50 mg treated animals increased with time.

The presence of PMA produced a response in many cultures from quartz alone-treated animals which had given no superoxide in its absence. These cells therefore still had the capacity to produce superoxide provided they were given sufficient stimulus. The reason why some *in vivo* treatments, in particular quartz alone, should result in a depressed spontaneous response is not known.

4.11 Production of tumour necrosis factor (TNF) by cells recovered in lavage from animals instilled with LU dust or control dusts

Alveolar macrophages lavaged from dust-treated lungs were cultured overnight with or without 10 μg/ml LPS (endotoxin) and their production of TNF assayed. Initial examination of TNF results indicated that they should be transformed to the log scale prior to statistical analysis. Results were adjusted for assay to assay differences, and average results calculated for each animal. A regression analysis was then carried out in a similar way to the cell and superoxide

data described above. The controls (no treatment) were given a weight of 2, as opposed to 1 for the other groups, in the analysis since these results were based on cells pooled from two animals. Further information on the analysis methods together with analysis of variance tables can be found in Appendix 3.

In the absence of LPS, the analysis showed that there were no significant differences between different treatments for any dose at any time point. Estimated geometric means, adjusted for the effects of experiment, are shown in Table 4.12.

In the presence of LPS there was considerably greater production of TNF. Two results were excluded from the analysis as outliers. These were from an untreated control animal and an animal treated with 1 mg iron/quartz at the 60 day time point. The analysis was also conducted with and without results from an experiment (Experiment 70: 10 mg dust/ 60 days) in which the LPS, unusually, had no effect on TNF production. Exclusion of these results did not alter the outcome of the analysis.

Although results from the 32-day experiments tended to be higher than those of the other time points, there was no significant effect of time. Estimated geometric means of TNF concentration, including the results from experiment 70, are shown in Table 4.13 for the different doses. Macrophages from untreated animals produced significantly less TNF than those from treated animals. There was a significant effect of dose, TNF being higher with 10 mg treatments than with 1 mg treatments for all dusts except high quartz where the 1 mg treatment produced the greatest activity. TNF values for macrophages from the LU dust and iron treatments were significantly lower than those from the quartz/iron treated group.

4.12 Weights of total dust and quartz recovered from lungs instilled with LU dust or other quartz-containing dusts

In order to assess the amount of dust clearance from treated lungs during the time elapsed since instillation, the total dust and quartz contents of instilled lungs were measured following lavage at the day 7 and day 32 time points. Only measurements for the LU dust, quartz/iron mixture, and the high dose quartz treatments were made. The amounts, in mg, of dust or quartz recovered are shown in Table 4.14. For the 1 mg inoculum there was evidence that dust had been cleared from the lung between days 7 and 32 for each of the dusts. There was little evidence that dust had been cleared with the 10 and 50 mg doses.

The clearance of total dust or quartz for the 1 mg treatments is illustrated further in Figures 4.23A and 4.23B respectively in which the dust, or quartz, content at day 32 was calculated as a percentage of the dust, or quartz, present at day 7. By day 7 any dust within the trachea and major airways would have been cleared and the day 7 baseline should therefore reflect the dust content of the lung tissue. Thus, on average, about 80% of the high quartz, 74% of the quartz/iron mixture and 58% of the LU dust remained in lungs 32 days after instillation of 1 mg (Figure 4.19A). Significantly more LU dust was cleared than quartz alone ($p=0.046$, Student's t test). For the quartz retention data (Figure 4.19B) this comparison was only significant at the 10% level ($p=0.07$). There was no difference between the clearance of LU dust and quartz/iron for both the total dust and quartz weights.

4.13 Histology of lungs 60 days following instillation of London Underground dust or various control dusts

4.13.1 Quartz

The effects of 1 mg and 0.78 mg (the quartz equivalent dose of 10 mg of LU dust) of quartz were very similar. There were patchy responses, mainly around terminal bronchioles where there was thickening of alveolar septa, and with the alveoli containing many free cells. These free cells ranged in appearance from normal to large foamy cells. Some alveoli were filled with lipoproteinaceous material. Figure 4.20 shows some of these features. In two of the 1 mg lungs, some of the peribronchiolar lesions had progressed with an accumulation of cells around blood vessels in the centre of the acini. In one lung the aggregates had enlarged to the point where they could be called early silicotic nodules. Where present in sections, lymph nodes were normal in the 1 mg group but showed quartz typical areas in the 0.78 mg group.

Lungs treated with 3.9 mg quartz (the quartz equivalent of 50 mg LU dust) showed more widespread and larger accumulations of foamy macrophages, particularly around the terminal bronchioles, and greater thickening of alveolar septa. Many alveoli were filled with lipoproteinaceous material. One animal had several very early silicotic nodules. Lymph nodes had quartz typical areas.

With the 10 mg quartz treatment the response was much more marked overall than at the lower doses but was variable due to the uneven distribution of the dust. Much of the lungs of two rats were relatively normal but there were large areas of quartz consolidation. This was of fibrotic type with the original lung structure destroyed and replaced by masses of actively growing fibroblasts (Figure 4.21). There were a few outliers, nodules of silicotic type close to the main masses. In the other two lungs there were no massive quartz lesions but there was a lot of alveolar lipoproteinosis. Some small silicotic nodules are present even in areas of lipoproteinosis. Where lymph nodes were present in sections from the 10 mg group they showed typical "quartz-type lesions" (Figure 4.22).

The two animals treated with 50 mg quartz had massive quartz-type lesions in their lungs. Areas of lung tissue had been transformed into fibrosing granulation tissue with loss of alveolar structure, as shown in Figure 4.21. Where recognisable lung tissue was present it contained silicotic nodules (Figure 4.23). Silicotic areas and nodules stained positive for both reticulin and collagen. Lymph nodes showed widespread quartz damage. Figure 4.24 shows a cytocentrifuge preparation of foamy macrophages and neutrophils lavaged from a lung 7 days following instillation of 50 mg quartz.

4.13.2 Particulate iron

There was a minimal lung response to 1 mg particulate iron. There were relatively few macrophages with dust although some were aggregated to fill individual alveoli.

Macrophages with dust were more numerous, some filling occasional alveoli, with the 10 mg dose. Where dust aggregations were greatest there was some thickening of alveolar walls, but this was minimal. Hilar lymph nodes were normal in 10 mg-treated animals.

Treatment with 50 mg iron produced dust impaction in some areas forming quite large lesions. These consisted of dust-containing macrophages with a few other cells (Figure 4.25). Reticulin staining suggested breakdown of alveolar walls in these lesions with a small amount of new reticulin deposited. There was also minimal new collagen staining in these areas. Lymph nodes were normal in these sections.

4.13.3 7.8% quartz in particulate iron

In lungs treated with 1 mg of the quartz/iron mixture, dust deposition was uneven, but where dust accumulated most densely in macrophages there was thickening of alveolar septa and some perivascular accumulation of inflammatory cells. Some of these perivascular accumulations might be called "micronodules" and some were found in the characteristic subpleural position where quartz lesions are often found.

With the 10 mg dose there were areas of alveolar thickening and perivascular accumulations of inflammatory cells were more widespread (Figure 4.26). However, no lesions were present that could be called silicotic nodules. Lymph nodes showed no quartz-related lesions. The highest dose (50 mg) of this mixed dust caused widespread areas of alveolar wall thickening and nodular lesions of silicotic type (Figure 4.27). The nodules stained positive for reticulin but only minimally for collagen. They were typical early silicotic nodules.

4.13.4 London Underground Dust

No pathological changes were found in lungs treated with 1 mg LU dust. Macrophage numbers were slightly raised, and these cells often contained dust particles. Dust-containing macrophages were much more evident in lungs instilled with 10 mg LU dust. Some alveoli were full of these cells and where this occurred there was slight thickening of alveolar walls (Figure 4.28). Lymph nodes appeared to be normal. Figure 4.29 shows a cytocentrifuge preparation of dust-laden macrophages and one neutrophil lavaged from a lung 7 days following instillation of 10 mg LU dust.

Treatment with 50 mg LU dust resulted in widespread dispersion of dust, almost all of which appeared to be in alveolar macrophages. In areas with many dusted cells there was frequently some thickening of alveolar septa, but this reaction was not very great. Macrophages containing dust frequently filled alveoli (Figure 4.30). In some areas of the lung dust "masses" occurred which were many times the diameter of a single alveolus. Within these masses reticulin staining indicated that there was a breakdown of alveolar walls but that neither new reticulin nor collagen had been produced.

4.13.5 Summary of the histology findings

Although the quartz content of the LU dust is the same as in the artificial iron/quartz mixture, the dust has produced less inflammatory response and no silicotic lesions. At the highest LU dose, dust masses were present in which the lung structure had been destroyed but not replaced by fibrosis. Comparison of the 10 mg dose of dust with the 0.78 and 1 mg doses of quartz demonstrated that quartz produced more pathological change and that macrophages in the LU dust-treated lungs appeared normal whereas those in the quartz-treated lungs were foamy and often necrotic. Comparison of the 3.9 mg quartz lungs with the 50 mg LU dust lungs was more

difficult because the very large mass of LU dust had, in some areas, almost obliterated large parts of the lung. Nevertheless, it was felt that the reaction to the quartz is one of definite pathological change, with early silicotic nodules present in one animal, whereas there was only a non-toxic (non-fibrotic) reaction to a very high dose of dust in the case of the LU dust. The implication of these findings is that the quartz in the LU dust is not as active as the DQ12 standard quartz.

4.14 Summary of the main findings of the research

4.14.1 Our London Underground dust sample was found to contain 7.8% quartz and 34.5% iron by bulk analysis. Analysis of individual particles showed that most contained iron and that many of the particles classed as quartz were contaminated with iron. This agrees with previous studies on LU dust conducted in other laboratories.

The results of the bulk analysis led to the incorporation in animal experiments of an artificial control dust consisting of 7.8% quartz mixed in particulate iron.

4.14.2 In experiments where London Underground (LU) dust was instilled into lungs, the total numbers of cells recovered from lungs by lavage were similar to those from animals treated with the quartz/particulate iron control dust. The numbers of neutrophils recovered in lavage were lower with LU dust than with the quartz/iron mix. LU dust is thus less inflammatory than the control dust. Treatment with quartz alone generally produced much greater numbers of cells in lavage than the other treatments.

Comparison of quartz alone at the levels seen in LU dust with the quartz/iron mixture showed that iron had a marked ameliorating effect on the cell response.

4.14.3 Histology of lungs instilled with the test dusts supported these findings. Compared to the quartz/iron dust, LU dust was less inflammogenic and produced no silicotic lesions. The quartz in LU dust would appear to be either less toxic than DQ12 or the presence of other components in the dust reduce the quartz activity.

4.14.4 Cells recovered in lavage were assayed for the production of superoxide a toxin released by leukocytes which may be involved in dust-mediated damage to lungs. Both spontaneous and stimulated production of superoxide were similar for cells from LU dust, iron, and quartz/iron treatments. Treatment with the higher doses of quartz resulted in lower spontaneous superoxide release than with the other dusts.

4.14.5 Macrophages recovered in lavage were also assayed for the production of the inflammation-promoting cytokine, tumour necrosis factor (TNF). There was evidence that treatment with LU dust led to similar production of TNF to that with iron but less than that resulting from quartz/iron treatment.

4.14.6 The addition of the test dusts to cultures of macrophages showed that although the LU dust was more toxic than the quartz/iron dust it was no more toxic than a mixture of 7.8% quartz in titanium dioxide.

4.14.7 Coating of quartz with iron chloride solutions did not protect macrophages from the toxic effects of quartz as measured by release of the enzyme lactate dehydrogenase.

4.14.8 There was no evidence that coating quartz particles with iron chloride solutions could alter the inflammatory response to quartz. Production of hydrogen peroxide by alveolar macrophages was reduced by ferrous chloride treatment of quartz but unaffected by treatment with ferric chloride.

4.14.9 Overall conclusion.

London Underground dust is considerably less harmful than DQ12 quartz, and is no more pathogenic than the artificial dusts containing equivalent proportions of quartz mixed with particles of iron or titanium dioxide. There was evidence that the presence of iron can ameliorate the harmfulness of DQ12 quartz in the lung. The close association of quartz with iron in the LU dust may account for it being less toxic than might have been predicted from its quartz content.

5. DISCUSSION

5.1 Dust in the London Underground

Environmental and occupational exposure to mineral dusts such as coal, asbestos, or quartz (silica) can lead to chronic lung disease and even cancer (Morgan and Seaton, 1984; Seaton *et al* 1987). Although there has been no epidemiologic evidence of excess lung disease associated with working in the London Underground system (London Transport 1982), there has been continuing watchfulness over the general levels of dust there, in particular, the quartz component of the dust. Regular surveys of airborne dust and personal sampling have been carried out over the years by the company (e.g. London Transport 1982; London Transport Research Laboratory, 1989; London Underground Ltd, 1991, 1993) and the Institute of Occupational Medicine was commissioned to undertake a literature review (Donaldson et al, 1991) and the laboratory study reported here. This laboratory study has examined the characteristics of a dust collected from the air near a platform on the Central Line at Holland Park underground station and its effects on the lung and phagocytic cell responses using animal models.

5.2 The effects of LU dust on the lung and on alveolar leukocytes

Our sample of London Underground (LU) dust was tested in several ways. Three concentrations of dust were instilled into rat lungs and, at various times later, lungs were washed out and the recovered cells counted and tested in functional assays *in vitro*. Additional investigations included histological examination after 60 days and the measurement of the dust and quartz contents of lungs on days 7 and 32 post-instillation. The cytotoxicity of LU dust to alveolar macrophages was examined *in vitro*. Instillation is, of course, an artificial way in which to deliver dust or other environmental or occupational contaminants into lungs, but inhalation, the preferred, natural way in which to administer such substances is expensive and requires large amounts of test material. As we only had 1.2 g of respirable dust from Holland Park station, instillation was the only option.

The numbers and types of cells recovered in lavage fluid from LU dust-treated lungs were similar to those from lungs treated with iron containing 7.8% quartz but greater than those arising from treatment with iron alone. The lower responses seen with particulate iron are confirmed by a number of other studies showing that iron or iron oxide are relatively harmless in the lung (Warheit *et al* 1991; Stacey *et al* 1959; Wright *et al* 1988). Numbers of neutrophils, a marker of inflammation, were lower in the LU dusted lungs than in the quartz/iron-treated lungs. Treatment with pure quartz produced greater leukocyte responses than for any of the other treatments.

With the higher doses, neutrophil numbers tended to remain elevated at all time points for all dusts, indicating a chronic inflammatory state. Chronic inflammation and fibrosis have been seen in animal lungs exposed to high concentrations, similar to those used in this study, of relatively non-pathogenic "nuisance" dusts such as titanium dioxide (Lee *et al* 1986; Vincent and Donaldson 1990; Driscoll *et al* 1990b). It is thought that even with non-toxic dusts the clearance mechanisms in the lung become overloaded at higher treatment doses which leads to these pathogenic effects (McClellan 1990; Warheit *et al* 1990).

In general, histological examination of lungs 60 days following treatment confirmed the lavage cell results except that the LU dust appeared to be less harmful than the quartz/iron mixture. There were no silicotic nodules in sections from LU dusted lungs and no quartz-typical areas in pulmonary lymph nodes. As expected from previous work in our laboratory and many others (Brown *et al* 1989; Driscoll *et al* 1990 a, b; Ziskind *et al* 1976) quartz on its own proved to be the most pathogenic dust with evidence of lipoproteinosis and fibrosis. It must be pointed out that the instillation of 10 or 50 mg of dust makes for a very severe test and it was not surprising that all the dusts, even iron, produced some lung damage at these higher doses.

Dust clearance from the alveolar region is believed to be accomplished mainly through the action of macrophages phagocytosing dust particles and then transporting them to the mucociliary apparatus, the principal means of removal of particles from the airways (Lehnert *et al*, 1989). While this is the main route for the elimination of dust from the lung, a proportion of free particles, or particles contained in macrophages, are removed via the lymphatic system to the hilar lymph nodes (Vincent *et al*, 1987). Other particles can be retained by alveolar epithelial cells or by the interstitium (Heppleston and Young, 1974; Adamson and Bowden, 1981). We examined the clearance of dust and quartz between days 7 and 32 for rats instilled with LU dust, quartz, or the quartz/iron mixture. We were only able to detect clearance with the 1 mg dose. There was no difference in clearance rates of total dust or of quartz between rats instilled with LU dust or the quartz/iron mixture. Quartz was cleared less slowly than the other two dusts.

The fibrotic changes occurring in the lungs following exposure to quartz, or other harmful dusts, are well documented although precise mechanisms have not been established (Davis, 1986). Through its interaction with quartz particles, the alveolar macrophage appears to play an important initiating and regulatory role through the release of inflammatory and fibrogenic factors such as interleukin-1 (Schmidt *et al* 1984), tumour necrosis factor (TNF) (Dubois *et al* 1989), fibroblast growth factors (Brandes and Finkelstein, 1990), and interferon (Elias, 1988). However, it is also known that macrophages can inhibit fibroblast activity through the release of prostaglandin E2 (Brown *et al* 1988).

Neutrophils from quartz-inflamed lung have also been shown to produce the fibroblast-regulating and immunomodulatory cytokine interleukin-1 (Kusaka *et al* 1990). Thus the generation of silicotic lung lesions may reflect an imbalance in fibroblast regulation, favouring fibrosis.

Of the plethora of possible leukocyte factors which might be important in determining the outcome of dust inhalation, two were selected for *in vitro* measurement in our study; production of the cytokine TNF, since it appears to play a key role in silica-induced fibrosis models (Piguet *et al* 1990), and superoxide, since oxidants have been implicated in lung damage due to dusts (Mossman and Marsh 1989; Voisin *et al* 1985; Weitzman and Graceffa 1982).

As with other studies (Driscoll *et al* 1990a) we showed that intra-tracheal instillation of mineral dusts enhanced the *in vitro* production of TNF by macrophages recovered by bronchoalveolar lavage. In the absence of the macrophage stimulant lipopolysaccharide (LPS), there was no difference in TNF production by macrophages from the different treatment groups. However, in the presence of LPS, LU dust and iron were associated with lower TNF production than with quartz/iron. TNF production was also greater in the 10 mg treatments than in the 1 mg treatments, except for the 0.78 and 1 mg doses of quartz which were higher

than with the other doses. We also showed that quartz and the various test dusts could stimulate normal alveolar macrophages *in vitro* to produce TNF.

Driscoll *et al* (1990a) found that TNF production by alveolar macrophages in the absence of LPS was only raised following instillation of relatively high amounts (10 and 20 mg) of TiO_2 or quartz. They also found that TNF production was significantly greater with quartz than with TiO_2 and remained raised throughout the experiment (28 days), whereas TNF production in the TiO_2 group had returned to normal levels by day 28. In the presence of LPS, macrophages from the quartz and TiO_2 groups produced similar amounts of TNF. There are, thus, several differences between our results and those of the Driscoll study. The reasons for this are not known but may reflect differences in rat strains (they used Fischer 344 rats), culture media, and the sensitivities of the TNF assays.

Spontaneous production of superoxide by unseparated leukocytes recovered in lavage showed an inverse relationship to the dose of dust instilled and that most results were below the level produced by cells from untreated lungs. Apart from results from the high dose quartz treatments where superoxide concentrations were extremely low, or even zero, there were no real differences between the treatments. In the presence of the membrane stimulant phorbol myristate acetate (PMA) there was greater production of superoxide but the pattern of results was very similar. There was even some superoxide activity in cultures from the high dose group which had given no activity in the absence of PMA; thus these cells still had the capacity to produce superoxide.

The reason for the decrease in superoxide activity *in vitro* with increasing dose of dust *in vivo* is not clear. The lavaged cells were not enriched for macrophages in these experiments and thus there were variable proportions of neutrophils present in the cultures, which might affect superoxide levels. However, a comparison of the superoxide results with Table 4.6, percentages of neutrophils, shows that this cannot be the explanation. There are a number of other possible reasons. Within the lung, leukocytes may have already been stimulated by dust to produce reactive oxygen species and thus their energies in this capacity were depleted. Toxic effects of the dusts, particularly by quartz, might also explain the results, although the response to PMA indicates that the cells were still active. We have shown previously that leukocytes from the lungs of rats instilled with 1 mg DQ12 quartz 5 days before also have a depressed capacity to produce spontaneous or PMA-stimulated superoxide and hydrogen peroxide compared to macrophages from untreated lungs (Donaldson *et al*, 1988b). In addition, both normal and quartz-treated cell populations failed to respond to quartz in vitro. This finding thus questions whether the production of superoxide and H_2O_2 plays a significant role in quartz-exposed lungs. We have also shown depressed spontaneous and PMA-stimulated H_2O_2 responses 1, 4, and 12 weeks following lung instillation of 1 mg quartz (Brown *et al*, 1989). This paper also reported that pre-treatment of quartz with aluminium lactate resulted in H_2O_2 responses similar to macrophages from untreated animals.

Another explanation for depressed oxidant production may be that certain activities of the alveolar leukocytes were down-regulated by signals from macrophages, or other cells, in response to the dust burden within the lung. The macrophage superoxide response has been shown to be abrogated by pre-treatment, *in vitro*, with PMA (Berton and Gordon 1983) or immune complexes (Yagawa *et al*, 1985). Macrophages isolated from a mouse tumour produced superoxide *in vitro* in response to a particulate stimulus (zymosan) but not to PMA

(Ghezzi *et al*, 1987). Thus the induction of anergy in macrophages can depend on the *in vivo* environment and be selective in terms of the subsequent responses to stimuli.

The toxicity of LU dust for alveolar macrophages was tested in vitro. Although the type of assay used, release of radioactive chromium (^{51}Cr), is a standard one, it has proved extremely variable in our hands and the resulting strong experiment/treatment interaction made interpretation of the data difficult. Nevertheless, LU dust, the quartz/TiO_2 mixture, and TiO_2 at the highest dose (200 μg) emerged as toxic dusts. The phenomenon where some dust treatments and doses led to a lower release of ^{51}Cr has been seen with fibres and other dusts (Brown and Donaldson 1991) but the reason is unknown. To further confound interpretation of the results the various dusts would have had differing densities and particle size distributions and thus the numbers of particles per unit weight and the area of the culture well which they covered would be different for each dust. Obviously further work is required on the cytotoxic effects of LU dust.

5.3 The effect of iron on the inflammatory and toxic effects of quartz

A central part of the project was the work on the role of iron in protecting against the toxicity of quartz. A previous study conducted for London Underground Ltd by the Medical Research Council, and referred to in the 1982 London Transport report (London Transport 1982), in which dust was instilled into the lungs of animals or added to cultured cells *in vitro,* concluded that the dust in the London Underground was no more harmful than "nuisance" dusts, and that this was probably due to the close association of iron with quartz particles. Unfortunately, copies of this report have not been traced to allow more detailed comparison with the studies reported here. The analysis of our sample of London Underground dust confirms the MRC finding that the majority of quartz particles are associated with iron.

We tackled the iron hypothesis in several ways. As described in the introduction the quartz surface is thought to play an important part in its biological effects. Thus with the help of Dr Val Vallyathan from the USA we examined the surface charges on quartz particles pre-treated with ferrous iron or ferric iron. We also determined the effects of these iron coating procedures on the inflammatory effects of quartz in the peritoneal cavity of mice and in the lungs of rats and on the functional activity of leukocytes in culture. Finally we studied the effect of iron particles on the inflammatory response to quartz in the mouse and rat models.

5.3.1 The effect of coating quartz particles with iron

There was no evidence that coating of quartz with ferrous or ferric iron could alter the leukocyte (inflammatory) response to quartz at 7 or 32 days in the rat lung model or at 2 days in the mouse peritoneal model. By contrast, treating quartz with aluminium lactate did significantly depress the inflammatory response in both models. Metal treatment of quartz produced only marginal changes in the toxicity of quartz to macrophages, as shown by the release of the enzyme lactate dehydrogenase. The *in vitro* production of H_2O_2 by alveolar macrophages was found to be affected by iron treatment of quartz with Fe^{2+} producing a marked reduction, and water and aluminium both producing an increase. In addition, only untreated quartz caused a reduction in intra-macrophage levels of superoxide dismutase. The reduction in the presence of Fe^{2+}-treated quartz may indicate decomposition of H_2O_2 by Fe^{2+}

in a Fenton reaction (see below). Metal treatment of quartz did not affect the level of surface radical activity in ESR measurements.

The protective effect of aluminium has been shown previously in rat lungs by our laboratory (Brown *et al* 1989) and in the lungs of sheep (Begin *et al* 1986). Although there are a number of publications on the effects of iron particles and iron containing mixed quartz dusts in altering the pulmonary toxicity of quartz (Gardner 1938; Gross *et al,* 1960; Kettle 1932; Naesland 1940), we are not aware of any published work in this area in which quartz has been pre-treated with iron chloride solutions. Nolan and colleagues (1981) reported the ameliorating effects of treatment with ferric chloride, aluminium chloride, zinc chloride, or polyvinyl-pyridine-N-oxide (PVPNO, a hydrogen-bonding polymer) on the lysis by quartz of red blood cells, once a standard *in vitro* toxicity assay. As described more fully in the introduction to this report, in the presence of water metal cations bind to ionised silanol groups on the quartz surface thereby reducing the negative surface charge, thought to be important in mediating the biological toxicity of quartz. It has been suggested that PVPNO coats quartz particles with a sufficiently thick layer of polymer to effectively "bury" the original quartz surface (Nolan *et al* 1981). It may be that much of the quartz in the LU dust is also buried beneath other dust components.

The reason for the lack of effect of iron coating, as opposed to aluminium treatment, in our *in vivo* assay is not known. Aluminium may be bound more strongly than iron and be better able to resist leaching from the quartz surface within the body because of its lower solubility. Geometrical considerations would indicate that ions with a similar charge to radius ratio to that of silicon, and a similar preference for tetrahedral sites, would fit more readily into the quartz surface. Aluminium^{3+} and, to a lesser extent, Fe^{3+} can both replace silicon in silicate minerals. Iron^{2+} being larger and with less charge would not fill tetrahedral sites or be held as strongly by electrostatic forces (Iler 1979).

In sharp contrast to considerations of the protective properties of iron with regard to quartz toxicity there is a considerable body of evidence that iron and other transition metals play a leading role in potentially damaging oxidative reactions (reviewed by Aust et al 1985).

A number of mechanisms for these metal effects have been described. Iron can promote the generation of the extremely reactive ·OH hydroxyl radical, considered to be the most toxic of the oxygen species, through the redox chains of the Haber-Weiss and Fenton reactions:

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

(Haber-Weiss)

$$O_2^- + HO_2\cdot + H^+ \rightarrow O_2 + H_2O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH \quad \text{(Fenton)}$$

Reactions at the quartz surface and interaction between quartz and leukocytes can provide superoxide (O_2^-) and H_2O_2 for these reactions. A number of other redox reactions involving iron are possible (Aust et al 1985) including the production of toxic iron/oxygen complexes such as the ferryl radical ($FeOH^{3+}$ or FeO^{2+} ; Koppenol and Liebman 1984).

Iron can also promote membrane damage through lipid peroxidation (Bast *et al,* 1991; Aust *et al,* 1985) and accelerate the non-enzymatic oxidation of molecules such as epinephrine (Misra and Fridovich 1972) and glutathione (Hamed *et al,* 1983).

Control of the extracellular availability of iron is seen as a major strategy of the body to restrict generation of hydroxyl radicals and is mediated by the iron-binding glycoproteins transferrin and lactoferrin (Gutteridge *et al,* 1981; Gutteridge 1986) and ceruloplasmin (Al-Timimi and Dormandy 1977). Within cells most iron is bound to ferritin and is thus not available for free radical reactions (Aisen and Litkowsky 1980). On disruption of a cell, ferritin-bound iron can become mobilised from the protein on interaction with ascorbic acid, organic radicals, or superoxide released from phagocytes (Biemond *et al,* 1984).

In view of all the possible complex interactions between iron, quartz and biological systems it was difficult to predict the outcome of our iron treatments of quartz in the lung. The lack of effect in the *in vivo* experiments may indicate that any protective effect is countered by promotion of oxygen radical production by iron, or simply that the iron was leached from the quartz surface.

5.3.2 The effect of particulate iron

Experiments in which iron particles were mixed with quartz showed no effect of iron on inflammation in the mouse peritoneal model. Initial experiments in the rat lung using 1 mg iron mixed with 1 mg quartz indicated that iron had some ameliorating effect on the leukocyte response at 7 days post-instillation in some animals, but that overall the results were not statistically significant. It was thought that the very strong response to 1 mg quartz might be obscuring any effect of the iron and thus mixtures with smaller proportions of quartz were used. Again there was weak evidence of a protective effect with leukocyte numbers at day 32 reduced only for the mixture containing 5% quartz. Further studies also found some reduction in macrophage numbers when 100 μg quartz was mixed with 100 μg iron. These early experiments thus produced no convincing evidence that particulate iron could reduce the toxic effects of quartz.

The effects of particulate iron were further studied in the main part of the project (see above) which compared the effects of intratracheal administration of LU dust with those of various control dusts. Comparison of data from the animals instilled with the 7.8% quartz in iron mixture or the low dose (LU dust quartz equivalent) quartz showed that with the higher doses (10 and 50 mg for quartz/iron; 0.78 and 3.9 mg for quartz) total cell, macrophage and neutrophil numbers were lower in the quartz /iron group at the 7 day and 32 day time point. There was no difference between the treatments at the 1 mg dose. The 60 day time point was not studied for the low quartz group. The reduction in both the number and percentage of neutrophils in the quartz/iron group is important because it indicates that there is less inflammation in the lungs of these animals despite having 12.8 times more total mass of dust instilled. Histological examinations showed that there were no quartz typical areas in lung lymph nodes from animals treated with 10 mg quartz/iron in contrast to the nodes from animals given 0.78 mg pure quartz, again indicating a protective role of iron.

These experiments show that particulate iron can have a marked ameliorating effect on the leukocyte response in the lung to instilled quartz. The reasons why the earlier experiments failed to show an iron protective effect probably lie in the differences, between the early and

late sets of experiments, in both the total amounts of quartz and iron injected and in their relative proportions. Thus, in the later experiments there was an effect of iron with the 10 and 50 mg doses but not with 1 mg since the amount of quartz in the 1 mg dose (0.078 mg) was insufficient to produce a response greater than that produced by iron alone. This explanation also applies to some of the earlier experiments. In addition, the ratio of iron to quartz in the later experiments was 11.8:1 by weight whereas in the first group of experiments the ratios were generally much less. For example in the second experiment (section 4.2.2, table 4.7) where the ratios were 99, 19, 9, and 3 to 1, only the 9:1 ratio gave any suggestion of an effect. The amounts of quartz used for the 99 and 19 to 1 ratios, 0.01 and 0.05 mg respectively, were probably insufficient to produce a sustained inflammatory response and with the the 3:1 ratio there would have been too little iron to influence the quartz effect. In conlusion, the ability of iron particles to counteract the effects of quartz depends on there being sufficient iron present; a ratio of at least 11.8:1 by weight is indicated.

5.4 Significance of the study findings for London Underground Ltd

This study has confirmed the findings of the MRC study, summarised in a previous report on the dust hazard in the London Underground system (London Transport 1982), that our sample of LU dust is less harmful than might be predicted from its quartz content. The study also confirms that the quartz in the LU dust is closely associated with iron. This association of iron and quartz may account for the LU dust being less toxic than the pure, control quartz (DQ12). However, our lung instillation studies only spanned a two month period and thus we do not know if LU dust would, in time, show greater pathogenic effects. It is possible that the iron component of the LU dust would be leached over time to leave exposed, toxic quartz surfaces in the lung. Gross et al (1960) found that iron disappeared more quickly than quartz from fibrotic areas in rat and guinea pig lungs exposed to iron-contaminated quartz. Further work would be required to study this.

The site of dust sampling for this project was carefully selected so that the dust sampled would be as representative as possible of the dust found in most underground stations in the network. Nevertheless, we cannot be certain that this is the case. Studies carried out by London Transport Scientific Services Division have shown that the quartz content of airborne respirable dust in stations can vary between 4.0% and 11.8% (by mass) depending on the station and line sampled (London Transport Research Laboratory 1989). A more recent study of air sampled in drivers' and guards' cabs showed that the quartz content of respirable dust varied from 1% to 9.5% (London Underground Ltd 1991). Our dust sample having 7.8% quartz therefore lies at the upper end of this distribution.

Because of the considerable heterogeneity in the composition of LU respirable dusts with regard to quartz content, some caution is required in extrapolating the findings in this report to the whole of the underground system. If the quartz particles in dust elsewhere in the network are also closely associated with iron, and this would appear generally to be the case according to the HSE report on dust in the LU (London Transport 1982), then it could be predicted that such dust samples would be no more toxic in our assays than the sample reported on here. Testing of samples from other locations would be required to investigate this prediction.

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Table 4.1. Bulk analysis of LU dust

% in LU Dust								
Ash	Quartz	Fe	Mn	Pb	Cd	Zn	Cu	Cr
81.0	7.8	34.5	0.24	0.03	none	0.03	0.06	0.02

Table 4.2. Cumulative size distribution of LU dust

	Particle diameter (μm)									
	0.25	0.5	0.75	1.0	2.0	3.0	4.0	5.0	6.0	7.0
% Greater than specified size	90.5	75.9	67.0	54.8	18.9	6.4	2.0	0.2	0.1	0.0

Table 4.3. Classification of particles in LU dust

Classification	Percentage of particles
Quartz	4.2
Kaolin	0.2
Mica	0.0
Iron carbonate	20.1
Iron sulphate	0.2
Iron	20.0
Iron silicate	2.1
Iron oxide	13.1
Organic	3.2
Unclassified	36.8

Table 4.4. Iron contents of individual particles according to the percentages of X-rays emitted during EDXS analysis

	% Iron									
	<5	5-10	10-15	15-20	20-25	25-30	30-35	35-40	40-45	>45
% of particle with specified iron content	8.5	6.5	12.6	13.2	7.5	6.6	6.6	4.8	5.2	28.6

Table 4. 5. Iron content of quartz particles in LU dust

	% Iron							
	0-0.5	0.5-1.0	1.0-2.0	2.0-3.0	3.0-4.0	4.0-5.0	5.0-6.0	6.0-10.0
Number of quartz particles	5	1	8	11	7	3	1	3

Table 4.6. The lung cellular response to 1 mg quartz, particulate iron, or TiO_2, or to 1 mg quartz mixed with 1 mg iron or TiO_2. Geometric means (standard deviation) of total cells, and neutrophils recovered in lavage 7 days post-instillation from 4 experiments.

Cells	Treatment	Experiment number 1	2	3	4
Total cells	Quartz alone	41.5(1.2)[a]	23.8(1.2)	26.6(1.3)	15.1(1.2)
	Iron alone	7.2(1.4)	5.8(1.6)	11.7(1.3)	5.2(1.1)
	Quartz + Iron	11.0(1.1)	20.4(1.4)	25.8(1.4)	17.6(1.3)
	TiO2 alone	not done	not done	not done	3.8(1.3)
	Quartz +TiO2	not done	26.2(1.3)	26.8(1.3)	20.0(1.4)
Macrophages	Quartz alone	22.4(1.4)	13.0(1.2)	17.8(1.3)	9.8(1.2)
	Iron alone	6.7(1.3)	4.9(1.4)	11.3(1.3)	5.1(1.1)
	Quartz + Iron	7.8(1.1)	11.9(1.2)	18.4(1.6)	10.7(1.3)
	TiO2 alone	not done	not done	not done	3.6(1.3)
	Quartz +TiO2	not done	16.1(1.4)	16.5(1.5)	14.0(1.5)
Neutrophils	Quartz alone	16.3(1.2)	9.7(1.2)	5.9(1.6)	5.0(1.3)
	Iron alone	0.3(2.4)	0.4(5.1)	0.1(2.0)	0.1(3.8)
	Quartz + Iron	2.5(1.5)	7.3(1.9)	3.6(1.7)	6.6(1.3)
	TiO2 alone	not done	not done	not done	0.1(3.0)
	Quartz +TiO2	not done	9.1(1.2)	8.3(1.3)	5.6(1.2)

a. Results are given as the geometric means with the geometric standard deviations in parentheses. Note that to calculate confidence intervals geometric means should be multiplied/divided by the standard deviation raised to the relevant power (1.96 for 95% confidence interval).

Table 4.7. The lung cellular response to instilled quartz or mixtures of quartz and particulate iron. Geometric means (standard deviation) of total cells, macrophages, and neutrophils recovered in lavage at day 32 post-instillation.

Treatment				
Quartz	Iron	Total cells	Macrophages	Neutrophils
10 μg	0 μg	3.29(2.16)[a]	3.22(2.18)	0.03(2.56)
50	0	7.96(1.23)	7.69(1.24)	0.18(2.49)
100	0	10.21(1.48)	8.31(1.29)	1.36(4.21)
250	0	12.69(1.21)	9.28(1.05)	2.68(2.05)
10	990	7.73(1.21)	7.63(1.21)	0.06(2.83)
50	950	4.78(1.13)	4.67(1.14)	0.08(2.59)
100	900	6.40(1.35)	5.84(1.31)	0.44(2.91)
250	750	9.41(1.54)	7.64(1.46)	1.56(2.05)
10	10	5.80(1.20)	5.72(1.19)	0.05(3.78)
100	100	5.85(1.62)	5.46(1.57)	0.23(5.61)

a. Results are given as the geometric means with the geometric standard deviations in parentheses. Note that to calculate confidence intervals geometric means should be multiplied/divided by the standard deviation raised to the relevant power (1.96 for 95% confidence interval).

Table 4.8. The effect of particulate iron or TiO_2 on the lung cellular response to 100 μg quartz. Geometric means (standard deviation) of total cells, macrophages, and neutrophils recovered in lavage at 32 days post-instillation.

Treatment	Total cells	Macrophages	Neutrophils
Quartz alone	10.92(1.34)[a]	8.78(1.18)	1.51(3.21)
Quartz + 25 μg Iron	8.26(1.35)	7.29(1.23)	0.42(8.41)
Quartz + 50 μg Iron	8.73(1.40)	6.94(1.30)	1.62(2.00)
Quartz + 100μg Iron	7.50(1.22)	5.85(1.28)	1.40(1.88)
Quartz +25 μg TiO2	10.97(1.29)	9.01(1.19)	1.53(2.50)
Quartz +50 μg TiO2	10.61(1.19)	8.54(1.13)	1.88(1.63)
Quartz +100μg TiO2	9.35(1.11)	7.55(1.10)	1.61(1.50)

a. Results are given as the geometric means with the geometric standard deviations in parentheses. Note that to calculate confidence intervals geometric means should be multiplied/divided by the standard deviation raised to the relevant power (1.96 for 95% confidence interval).

Table 4.9. The effect of coating quartz with ferrous or ferric iron or aluminium on the leukocyte infiltrate in the mouse peritoneum 2 days following injection of 2.5 mg dust. Geometric means (standard deviation) of total cells, macrophages, and neutrophils recovered in lavage are shown for 3 experiments.

Cells	Quartz Treatment	Experiment number 1	2	3
Total cells	Water	4.0(2.1)[a]	4.7(1.1)	7.2(1.6)
	Iron (Fe^{2+})	5.2(1.6)	3.5(2.1)	6.3(1.6)
	Iron (Fe^{3+})	5.8(1.8)	4.6(1.3)	6.3(1.1)
	Aluminium	2.9(1.4)	3.5(1.3)	3.9(1.3)
Macrophages	Water	3.0(2.0)	3.3(1.1)	4.0(1.6)
	Iron (Fe^{2+})	3.4(1.6)	2.3(2.1)	3.8(1.6)
	Iron (Fe^{3+})	4.6(1.8)	3.2(1.2)	4.1(1.2)
	Aluminium	2.1(1.3)	2.6(1.4)	2.5(1.2)
Granulocytes	Water	0.6(2.4)	0.7(1.3)	2.0(1.8)
	Iron (Fe^{2+})	1.4(1.7)	0.8(2.4)	1.8(1.6)
	Iron (Fe^{3+})	0.9(1.7)	0.8(2.4)	1.4(1.3)
	Aluminium	0.3(1.8)	0.4(1.1)	0.6(1.7)

a. Results are given as the geometric means with the geometric standard deviations in parentheses. Note that to calculate confidence intervals geometric means should be multiplied/divided by the standard deviation raised to the relevant power (1.96 for 95% confidence interval).

Table 4.10. The proportion of neutrophils, as a percentage of total cells, recovered in lavage 7, 32, or 60 days following instillation of LU dust or control dusts.

Treatment	Dose	Mean percent of neutrophils day 7	day 32	day 60
LU	1 mg	0.50	0.40	0.80
Iron	1 mg	2.00	0.60	0.60
Iron + 7.8% quartz	1 mg	7.50	3.00	6.80
High quartz	1 mg	43.75	44.80	40.60
Control		0.50	0.60	0.80
Low quartz	0.078 mg	5.25	1.25	not done
LU	10 mg	15.75	10.25	12.75
Iron	10 mg	9.75	0.50	4.25
Iron + 7.8% quartz	10 mg	31.25	26.25	26.00
High quartz	10 mg	48.50	45.00	49.00
Control		0.25	0.50	0.25
Low quartz	0.78 mg	37.00	43.00	not done
LU	50 mg	10.00	13.00	25.50
Iron	50 mg	14.50	9.00	8.50
Iron + 7.8% quartz	50 mg	18.00	20.00	42.00
High quartz	50 mg	51.50	64.00	62.50
Control		0.00	0.50	0.00
Low quartz	3.9 mg	64.00	53.50	not done

Table 4.11 Lung weights following the instillation of LU dust and other quartz-containing dusts.

Treatment	Dose	Lung weight (mean (SEM)) day 7	day 32
LU	1 mg	2.39(.46) g	2.09(.12) g
Iron + 7.8% quartz	1 mg	2.19(.25)	2.19(.13)
High quartz	1 mg	2.30(.29)	2.40(.10)
Low quartz	0.078 mg	not done	1.72(.07)
LU	10 mg	2.13(.11)	2.23(.07)
Iron + 7.8% quartz	10 mg	2.28(.31)	2.20(.14)
High quartz	10 mg	2.50(.09)	3.23(.10) a
Low quartz	0.78 mg	not done	2.09(.10)
LU	50 mg	2.32(.26)	2.80(.62)
Iron + 7.8% quartz	50 mg	2.23(.11)	2.38(.20)
High quartz	50 mg	4.50(.37) b	7.42(.45) b
Low quartz	3.9 mg	not done	2.40(.07)

a) Lungs in the high quartz (10 mg) group were significantly heavier than lungs treated with 10 mg of LU dust or the iron/quartz mixture, or with 0.78 mg quartz ($p = 0.001$).

b) Lungs treated with 50 mg (high) quartz were significantly heavier than lungs treated with 50 mg of LU dust or the iron/quartz mixture at both 7 ($p = 0.015$) and 32 days ($p = 0.008$).

Table 4.12: TNF production in the absence of LPS

Treatment	TNF concentration (units/ml) geometric mean (range) 1 mg	10 mg	50 mg
None	9 units/ml (8-10)	-	-
LU	14 (10-20)	13 (9-18)	11 (7-19)
Iron	13 (9-18)	13 (9-18)	25 (15-42)
Iron + 7.8% quartz	18 (13-25)	14 (10-19)	25 (15-43)
High quartz	21 (15-29)	14 (10-20)	23 (14-38)
Low quartz	17 (10-29)	20 (12-35)	24 (13-42)

Table 4.13: TNF production in the presence of LPS

Treatment	TNF concentration (units/ml) geometric mean (range) 1 mg [a]	10 mg	50 mg
None [b]	61 units/ml (50-75)	-	-
LU	121 (72-205)	339 (192-601)	366 (159-841)
Iron	92 (55-156)	166 (94-294)	384 (167-883)
Iron + 7.8% quartz [c]	191 (113-322)	447 (253-792)	1331 (543-3261)
High quartz [d]	837 (495-1414)	237 (134-420)	394 (171-906)
Low quartz	140 (58-340)	976 (402-2373)	562 (231-1366)

a: There is a significant dose effect between 1 mg and 10 mg, but not between 10 mg and 50 mg.

b: The control results were significantly lower than those of other treatments.

c: Iron/quartz results were significantly higher than LU or iron alone.

d: For the high quartz results there was a significantly different pattern with dose; the greatest TNF production was with the 1 mg dose.

e: Note that TNF concentration did not vary with time.

Table 4.14. Weights of total dust and quartz recovered from lungs 7 days and 32 days following instillation of 1, 10, or 50 mg LU dust, quartz/iron, or quartz alone.

Treatment	Inoculum		Dust retained [a] day 7	Dust retained [a] day32	Quartz retained day 7	Quartz retained day 32
LU dust	1 mg	MEAN	0.913 [b]	0.532	0.059	0.024
		SEM	0.093	0.069	0.009	0.005
		N=	4	5	4	5
7.8% Q in iron			0.783	0.596	0.062	0.029
			0.204	0.082	0.019	0.002
			4	5	4	4
High quartz			0.793	0.648	0.594	0.414
			0.285	0.047	0.173	0.060
			3	5	3	5
LU dust	10 mg		3.640	4.880	0.297	0.323
			1.17	1.220	0.060	0.026
			4	4	4	4
7.8% Q in iron			4.435	6.943	0.319	0.323
			0.901	0.821	0.075	0.055
			4	4	4	4
High quartz			5.757	5.280	4.610	4.587
			0.837	0.510	1.00	0.753
			4	4	4	4
LU dust	50 mg		23.100	19.370	2.040	1.679
			12.600	6.420	1.09	0.472
			2	2	2	2
7.8% Q in iron			24.400	24.740	1.217	1.174
			16.600	2.180	0.389	0.190
			2	2	2	2
High quartz			32.315	35.715	31.400	31.812
	0.435		0.275	0.705	0.826	
			2	2	2	2

a. Lungs had been lavaged before being sent for dust and quartz analysis.

b. Each cell in the table contains the mean (top), standard error of the mean (SEM; middle) and the number of lungs (N; bottom).

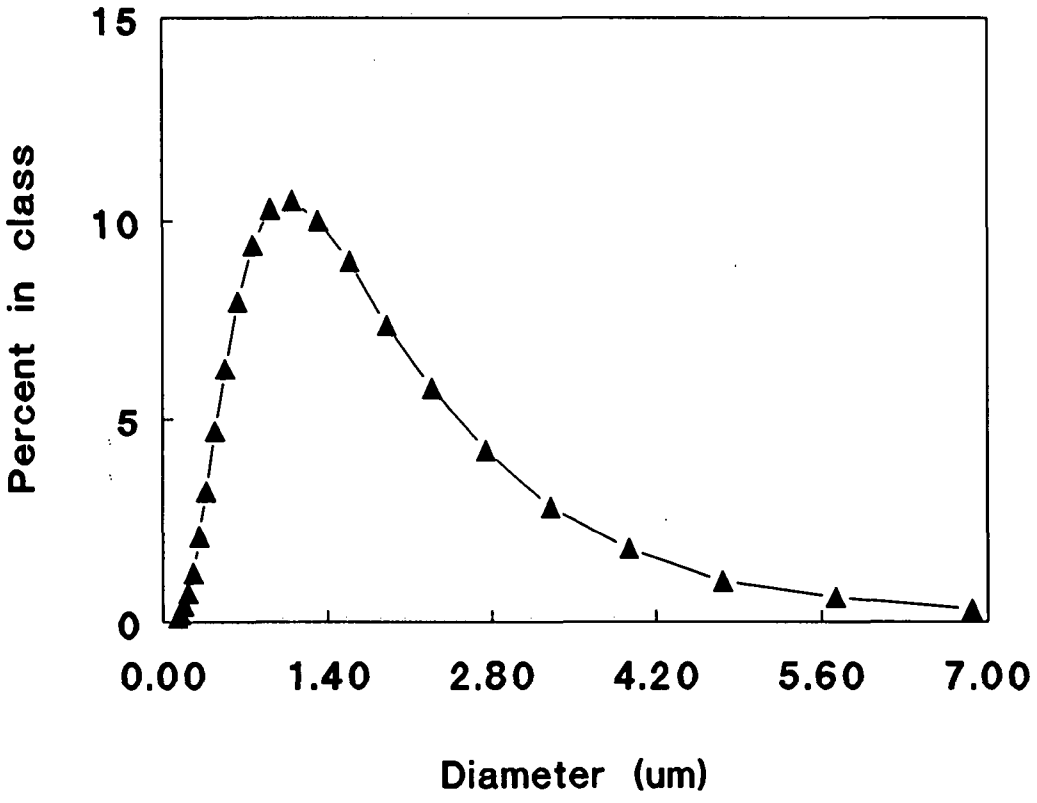

Figure 4.1. Size distribution of particles in LU dust measured on a Zeta-sizer 4 machine.

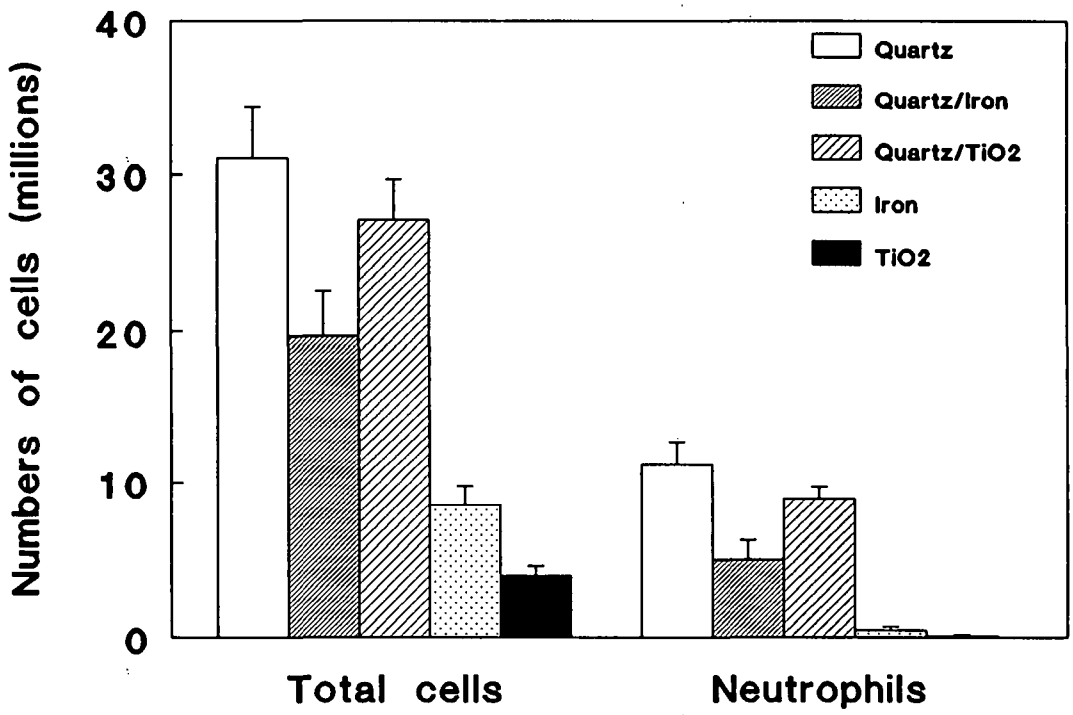

Figure 4.2. Mean (SEM) total cell and neutrophil numbers in lavage 7 days following instillation of 1mg quartz, TiO2, or iron, or 1mg quartz mixed with 1mg particulate iron or TiO2.

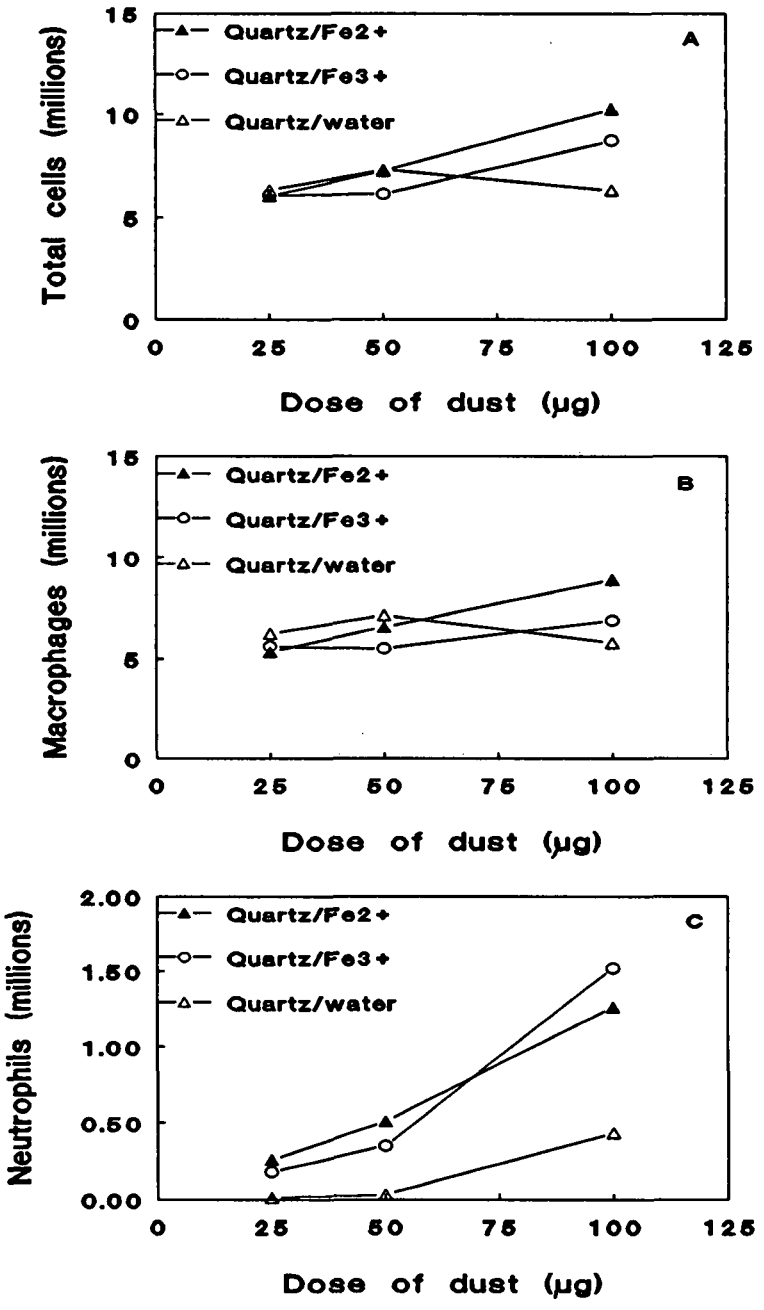

Figure 4.3. The effect of coating quartz with ferrous or ferric iron on the numbers of leukocytes recovered 32 days post-instillation. Geometric means of (A) total cells, (B) macrophages, and (C) neutrophils.

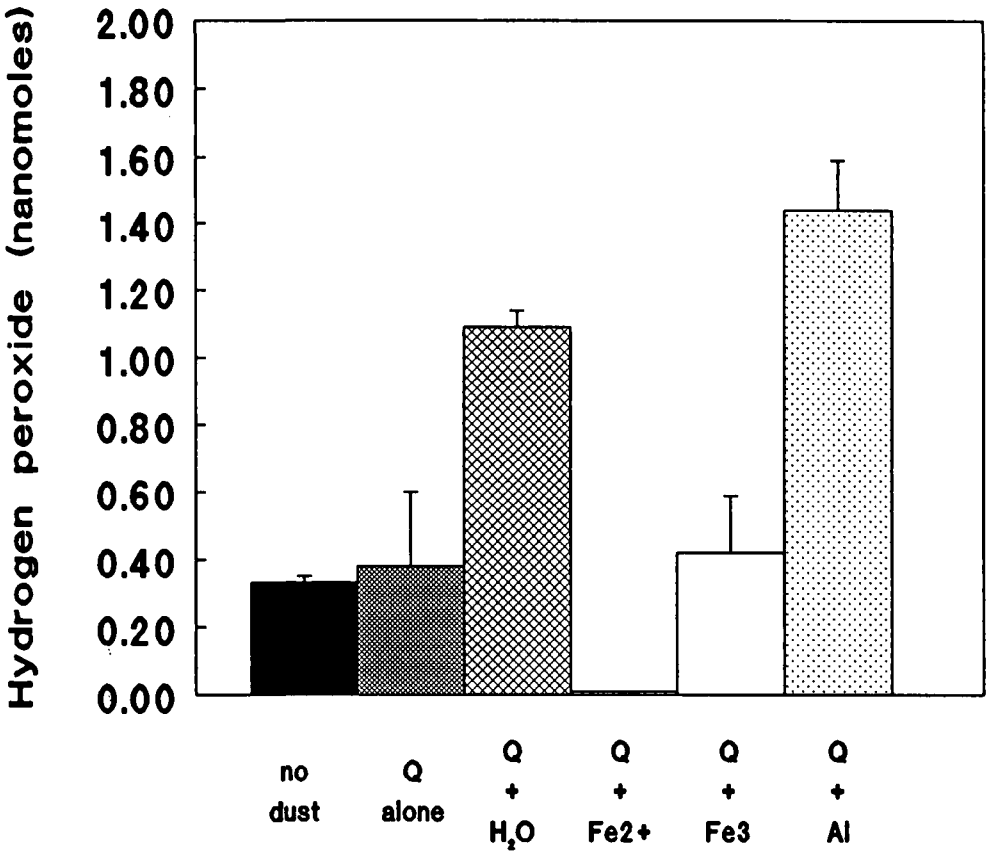

Figure 4.4. Stimulation of macrophage hydrogen peroxide production in vitro: the effect of coating quartz with ferrous or ferric iron, or aluminium. Mean and SEM.

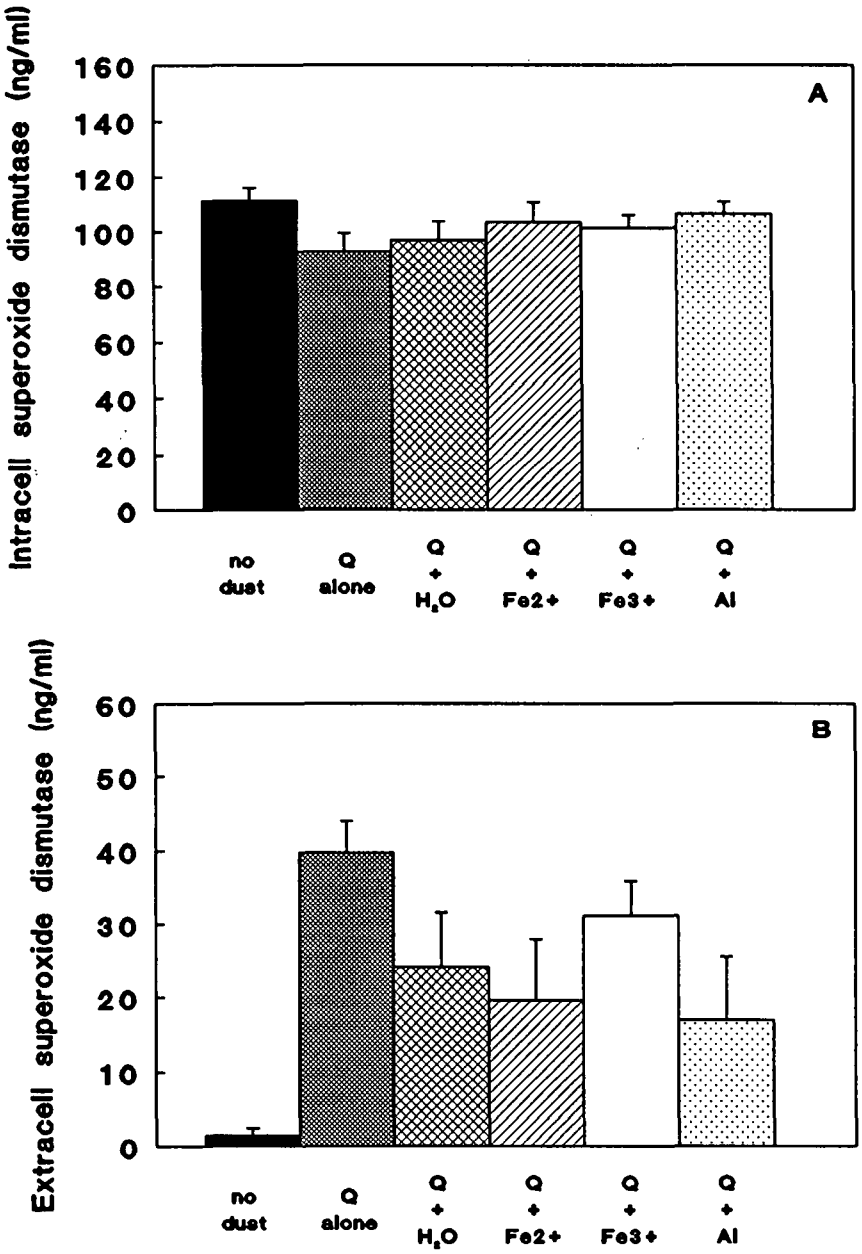

Figure 4.5. Intracellular (A) and extracellular (B) concentrations of macrophage superoxide dismutase following exposure in vitro to quartz or quartz coated with iron or aluminium.

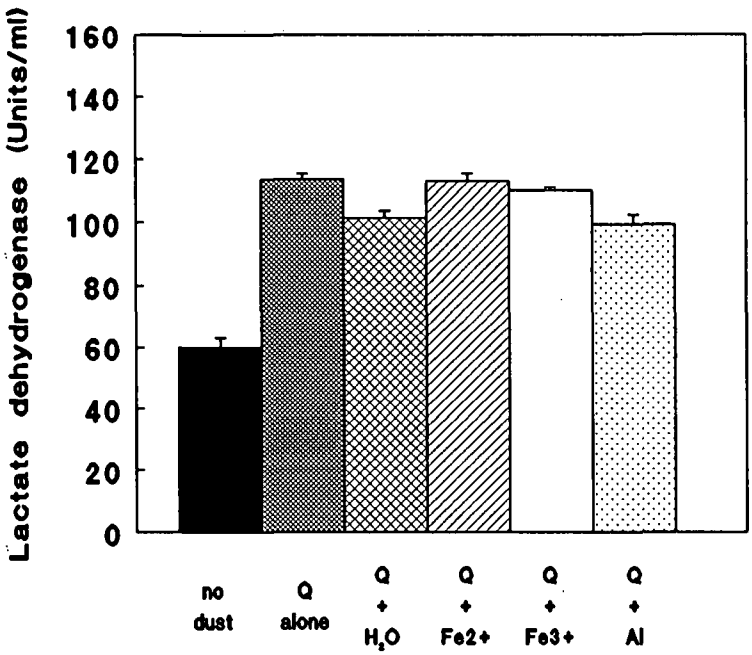

Figure 4.6. Release of lactate dehydrogenase (mean and SEM) from alveolar macrophages treated in vitro with quartz or quartz pre-treated with ferrous or ferric iron or aluminium.

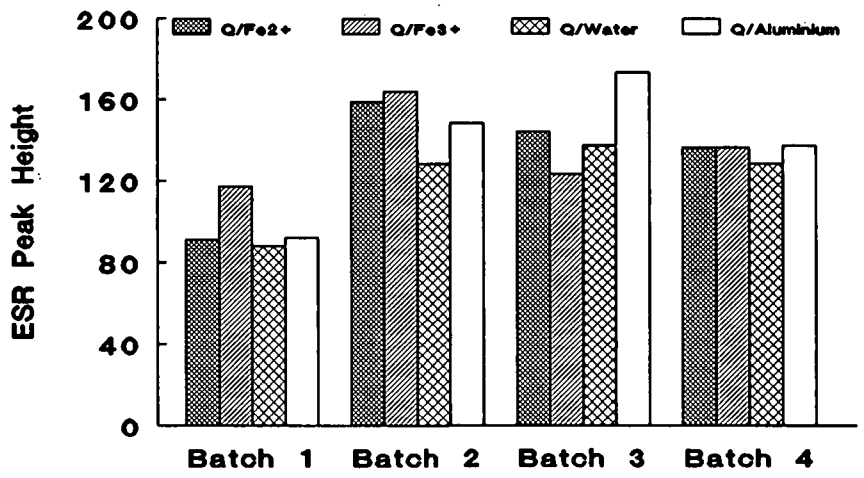

Figure 4.7. Radical activity measured by electron spin resonance on the surface of quartz particles: effect of coating quartz with iron or aluminium.

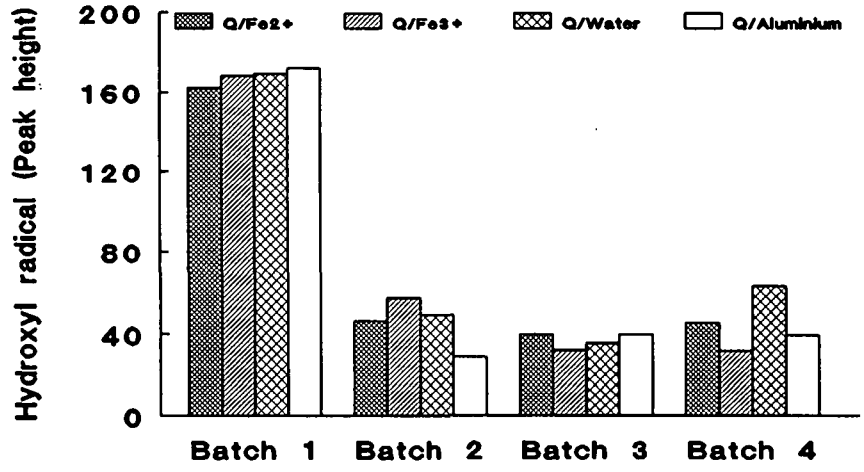

Figure 4.8. Production of hydroxyl radicals by quartz using electron spin resonance: effect of coating quartz with iron or aluminium.

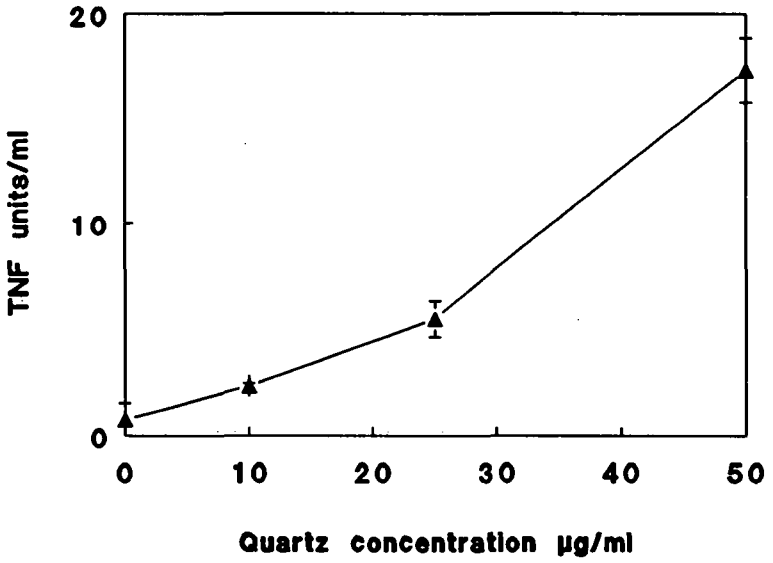

Figure 4.9. In vitro stimulation of alveolar macrophage production of tumour necrosis factor by quartz.

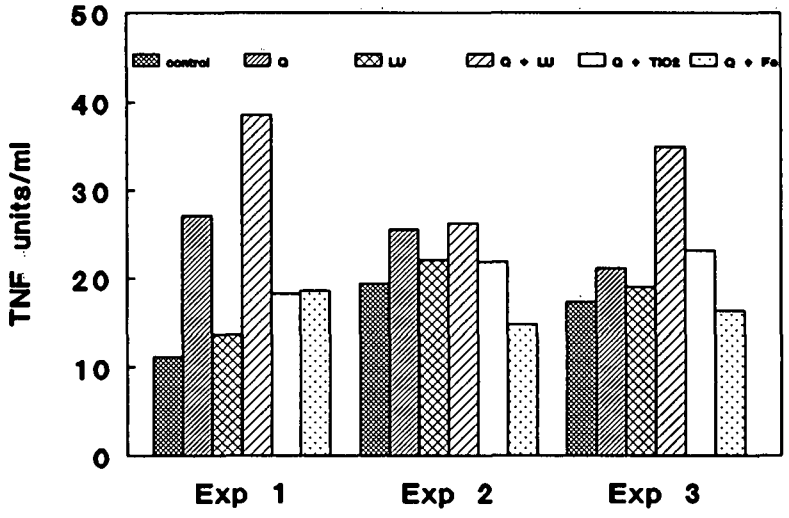

Figure 4.10. In vitro production of TNF by alveolar macrophages exposed to LU dust, quartz, or combinations of quartz with LU dust, iron, or TiO2.

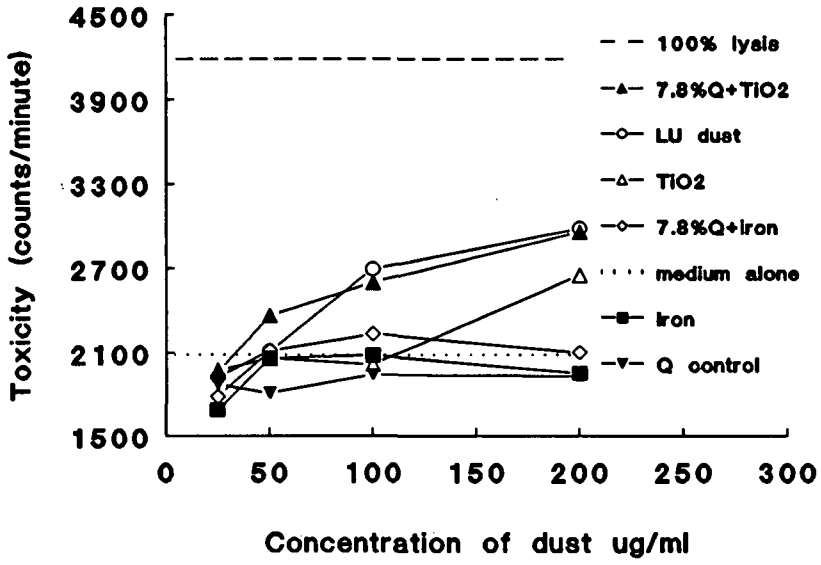

Figure 4.11. Toxicity of various dusts for macrophages as the release of (51)chromium. Geometric means of radioactive counts pooled from 5 experiments.

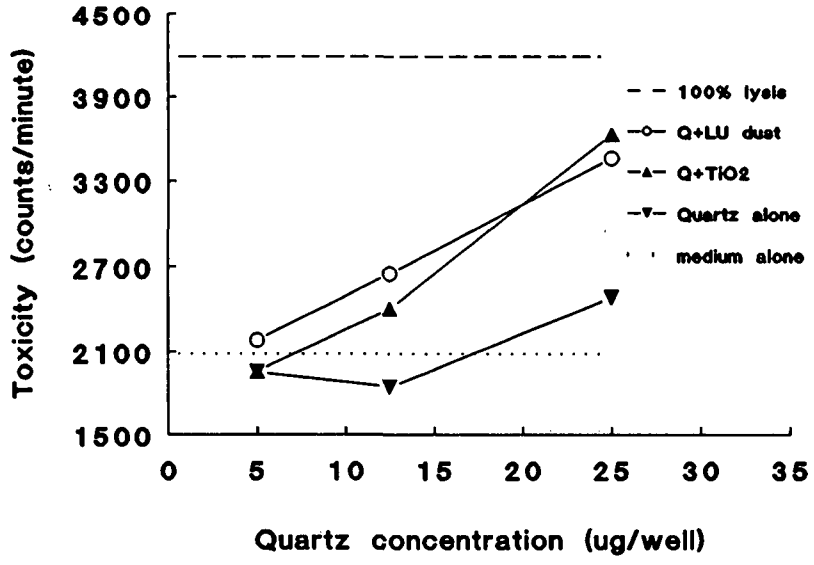

Figure 4.12. Toxicity to macrophages of 1:1 mixtures of quartz and LU dust or of quartz and TiO2. Geometric means of radioactive counts pooled from 5 experiments.

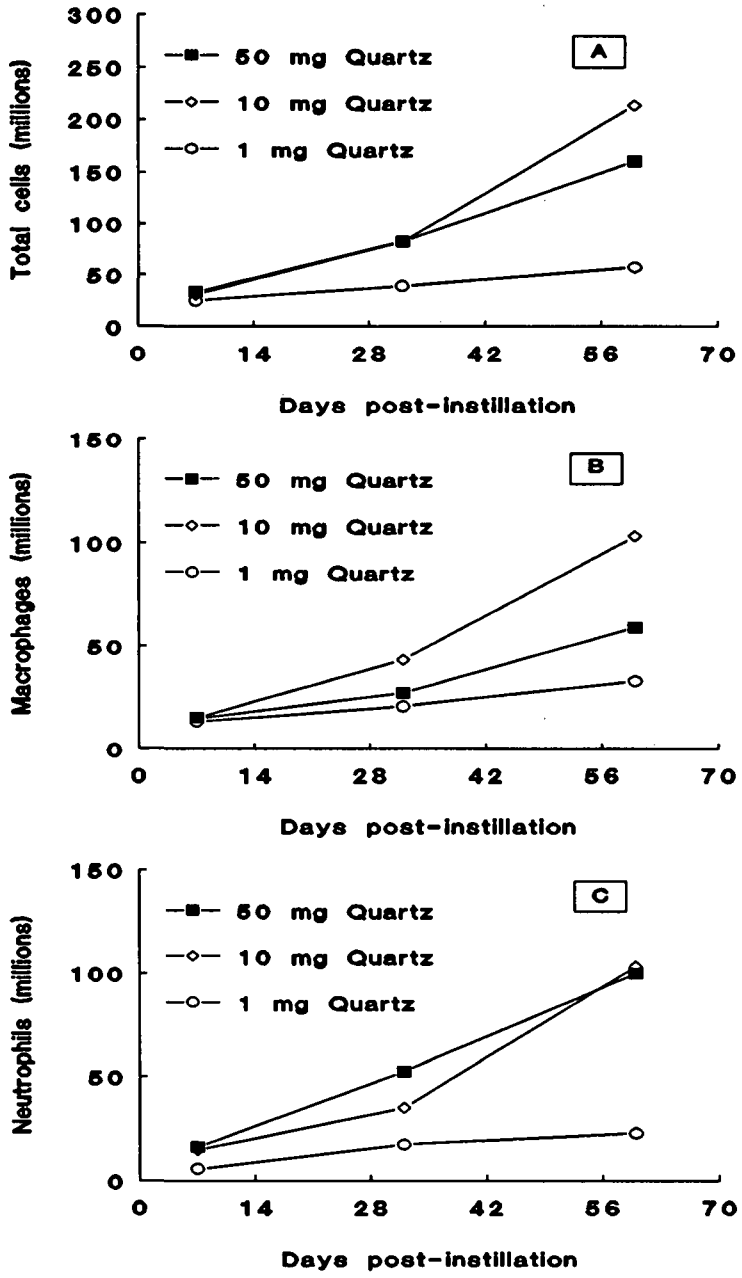

Figure 4.13. Mean numbers of (A) total cells, (B) macrophages, and (C) neutrophils in lavage 7, 32, or 60 days post-instillation of 1, 10, or 50 mg quartz (high dose quartz).

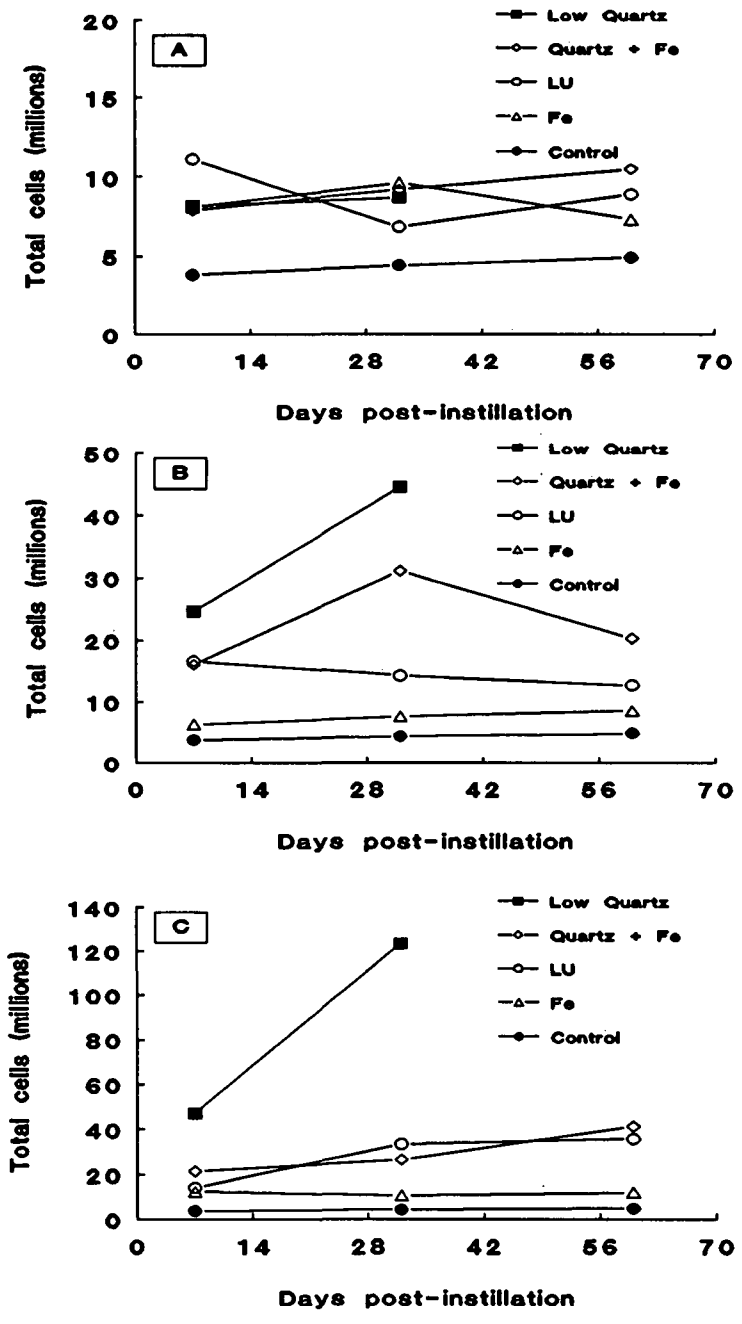

Figure 4.14. Mean numbers of total cells in lavage 7, 32, or 60 days post-instillation of LU dust or control dusts at doses of (A) 1, (B) 10, or (C) 50 mg.

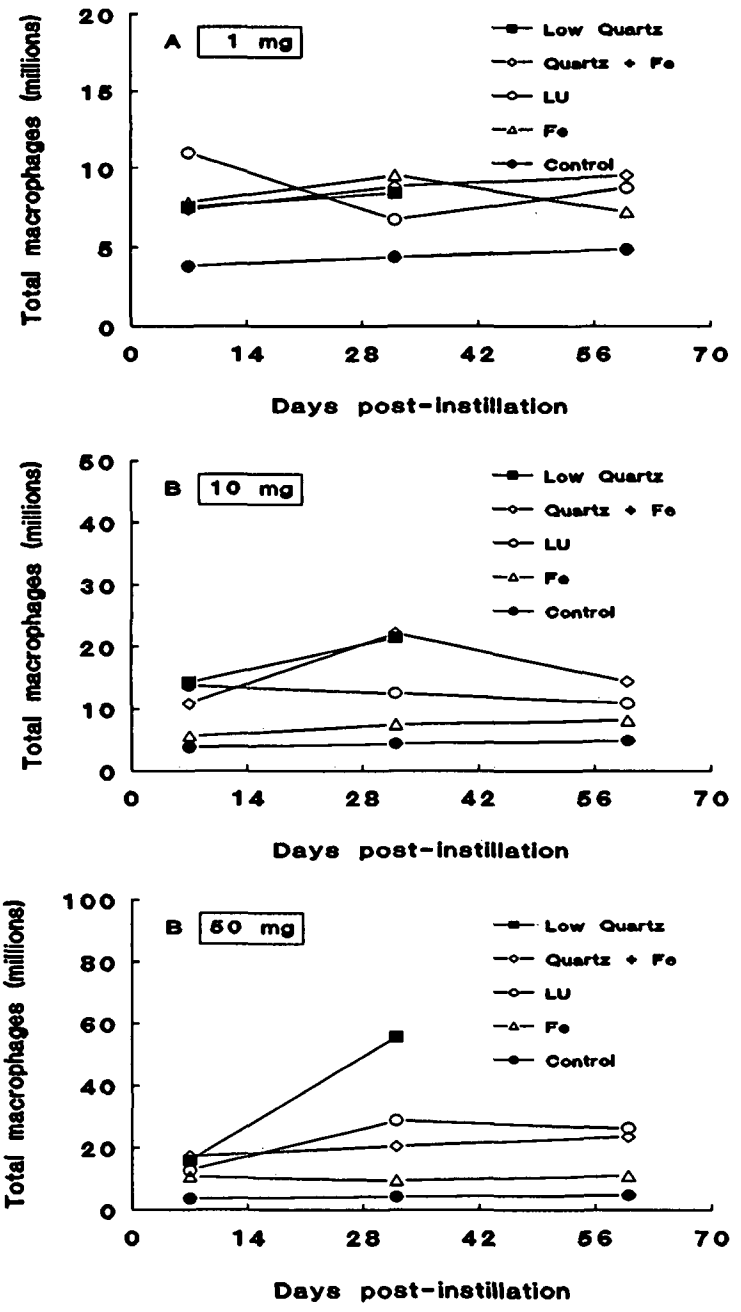

Figure 4.15. Geometric mean numbers of macrophages in lavage 7, 32, or 60 days post-instillation of LU dust or control dusts at doses of (A) 1, (B) 10 or (C) 50 mg.

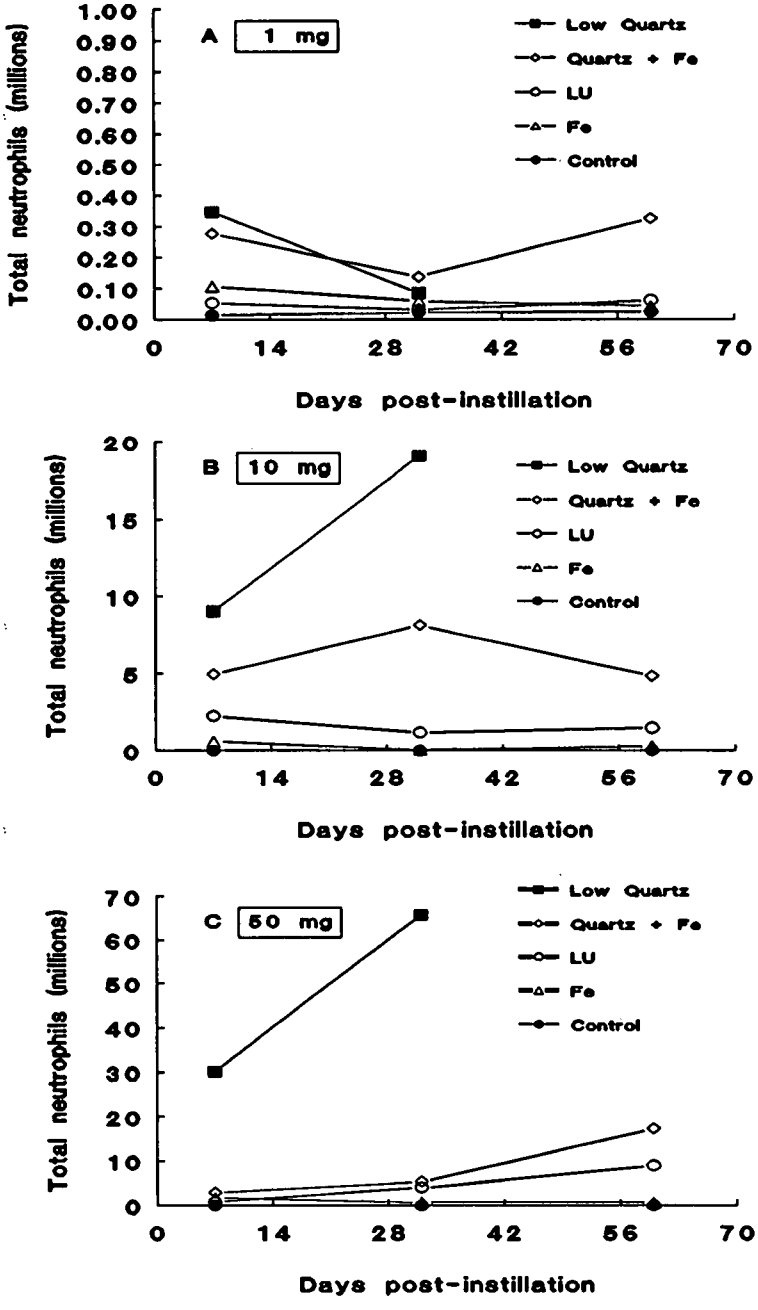

Figure 4.16. Mean numbers of neutrophils in lavage 7, 32, or 60 days post-instillation of LU dust or control dusts at doses of (A) 1, (B) 10, or (C) 50 mg.

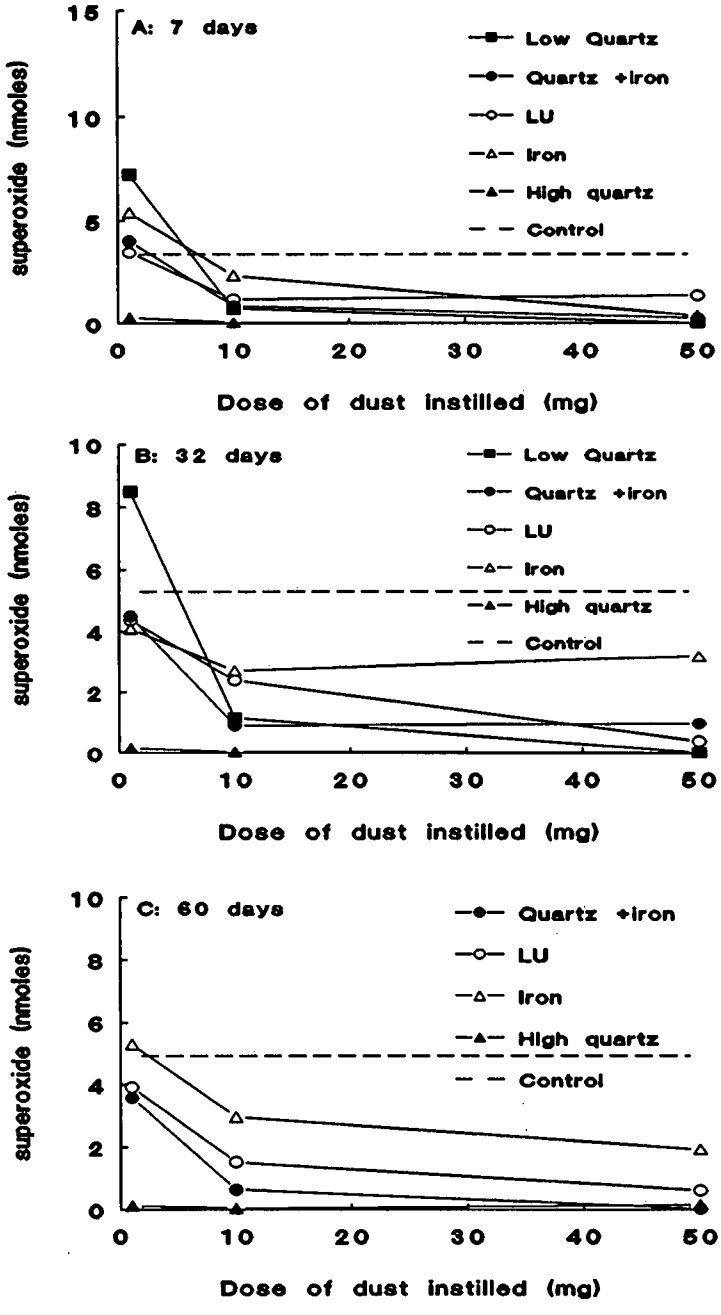

Figure 4.17. Spontaneous production of superoxide in vitro by leukocytes in lavage (A) 7, (B) 32, or (C) 60 days post-instillation of LU or control dusts.

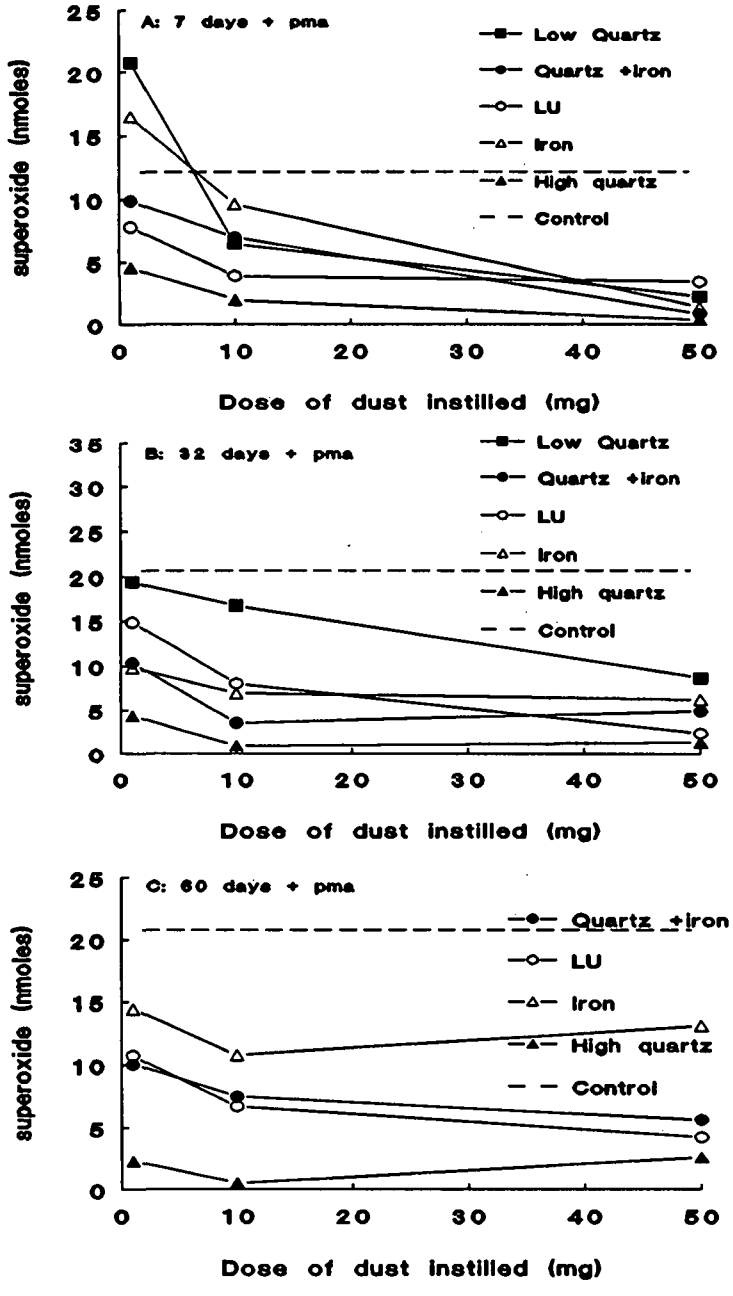

Figure 4.18. PMA-stimulated superoxide production in vitro by leukocytes in lavage (A) 7, (B) 32, or (C) 60 days post-instillation of LU or control dusts.

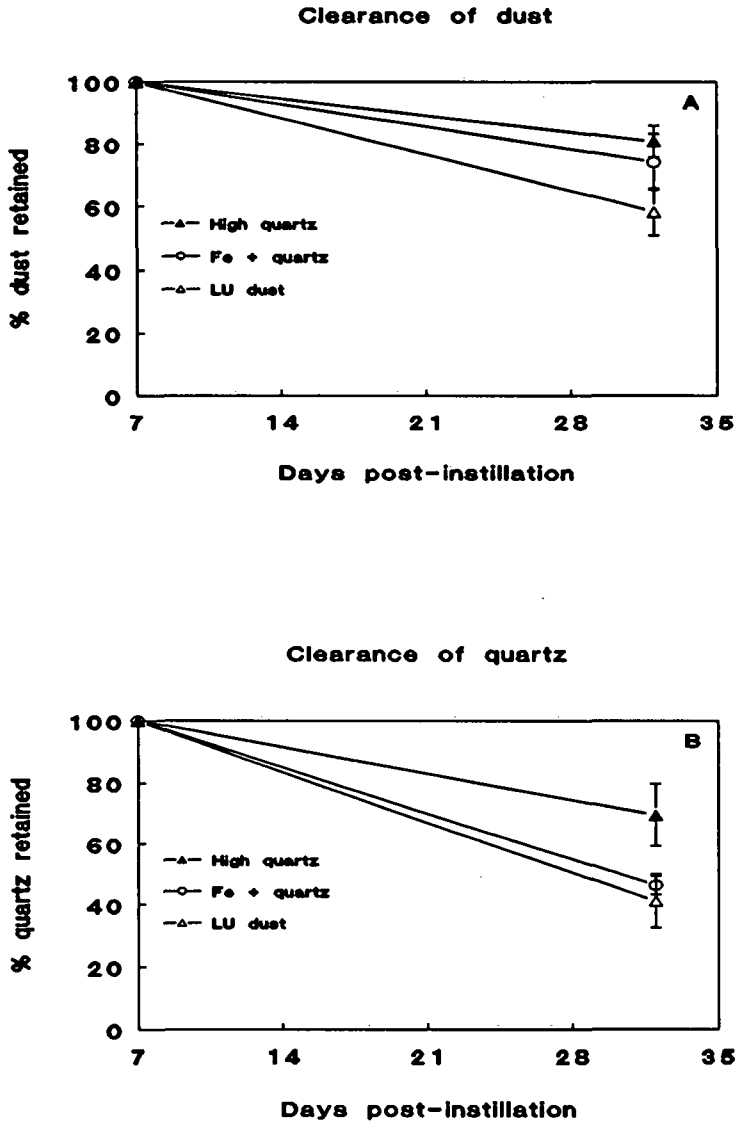

Figure 4.19. Total dust (graph A) or quartz (graph B) retained in lungs at day 32, as a percentage of the dust or quartz present at day 7, following the instillation of a 1 mg dose.

APPENDIX 1

In vitro cytotoxicity of London Underground dust, quartz, and other dusts for alveolar macrophages

Table A1.1 Cytotoxicity of LU dust, quartz, and other dusts; pooled means of log-transformed data (counts/minute) from 5 experiments.

		Concentration of dust (μg/ml)			
Treatment	None	25	50	100	200
None	7.64				
LU		7.57	7.66	7.90	8.00
Quartz in iron		7.49	7.66	7.71	7.65
Quartz in TiO_2		7.58	7.77	7.86	7.99
Low Quartz[a]		7.54	7.50	7.57	7.57
Iron		7.44	7.63	7.64	7.58
TiO_2		7.59	7.63	7.61	7.88
1:1 Quartz/LU		7.69	7.88	8.15	
1:1 Quartz/TiO_2		7.58	7.79	8.20	
High Quartz[b]		7.58	7.52	7.82	
100% lysis	8.34				

a. The concentrations for the "low" quartz controls were 2, 4, 8, and 16 μg/ml.

b. The concentrations for the "high" quartz controls, for the 1:1 mixtures, were 5, 12.5, and 25 μg/ml.

Table A1.2 In vitro cytotoxicity of LU dust, quartz and other dusts: Accumulated analysis of variance

Change	d.f.	s.s.	m.s.	v.r.
+ expt	4	72.22	18.0549	2135.90
+ treat	34	23.17	0.6815	80.62
+ expt * treat	136	16.96	0.1247	14.75
Residual	350	2.96	0.0085	
Total	524	115.31	0.22	

APPENDIX 2

Statistical analysis of bronchoalveolar lavage cell data and in vitro superoxide and tumour necrosis production obtained in experiments where LU dust was instilled into rat lungs

A2.1 Protocol

Various quantities of LU dusts and a number of control dusts (1, 10, or 50 mg) were instilled into rat lungs followed by lavage 7, 32, or 60 days later. Cells recovered in lavage were identified, counted and cultured. Tumour necrosis factor and superoxide anion were then measured in the culture fluids. As our sample of LU dust contained 7.8% quartz and a significant quantity of iron, we prepared a control dust consisting of 7.8% DQ12 quartz mixed with particulate iron. Other control dusts included particulate iron alone, DQ12 quartz alone (High dose quartz; equivalent to 100% quartz) and lower doses of DQ12 quartz alone (Low dose quartz; equivalent to 7.8% quartz). Untreated animals were used as an additional level of control.

A2.2 Cell data analysis

Data consisted of three responses; the total number of cells, the number of macrophages, and the number of neutrophils recovered by bronchoalveolar lavage. Preliminary analysis of the data showed it to have a skewed distribution and thus the data were transformed to the log scale for further analysis. Before transformation zero values were replaced by a small positive value (0.3) to avoid their exclusion from the analysis. Results from control, untreated animals were halved as they corresponded to pooled cells from two animals and these data were given a weighting of two in the analysis to allow for the fact that they would be more accurate than other results. Data associated with exposure to high dose quartz were excluded from the main analysis since the responses were so much greater than the other treatments.

In addition to the main effects of interest, dust, dose, and time lapsed since instillation, the analysis accounted for the effects of differences between experiments. Analysis of each response was carried out using GENSTAT by fitting a multiple linear regression model which accomodates the unbalanced nature of the experimental data. Models were built up sequentially allowing an ongoing assessment of the effects of different factors culminating in an analysis of variance table. Goodness-of-fit measures indicated the relative importance of the different factors. Regression coefficients facilitated the detailed comparisons between factor levels. Residual plots were examined to assess the appropriateness of the fitted models.

A particular ordering was adopted when adding factors to the model, and this is apparent from the analysis of variance (ANOVA) tables presented. This ordering deals with the structure of the data and was a consequence of the experimental design. "Time" was added first to the model as it is at the highest level in the data, i.e. the other factors vary within time. Next "Experiment" was added since "Dust Type" varies within "Experiment". "Dose" varied both within and between "Experiment" and in this analysis "Dose" was added after "Experiment".

Results of the ANOVA showed that the main sources of variation in the total number of cells, total macrophages and total neutrophils (after allowing for variation between experiments) were dust type and dose. Compared with the main effects, any effect of time was small. There was a significant interaction between dust type and dose, that is, the effect of dose was not the

same for all dust types. These effects can be seen in the accumulated ANOVA tables (Tables A2.1, A2.3, A2.5). Geometric means, with ranges, for these data classified by dust type, dose, and time are presented in Table A2.2 for total cells, Table A2.4 for macrophages, and Table A2.6 for neutrophils. Further interpretation of these analyses is provided in the results section of this report.

A2.3 Superoxide production by cells recovered in lavage

Measurements of superoxide generated with and without the membrane stimulant PMA present were analysed using analysis of variance in GENSTAT in a similar way to that described above for cell data. Results for each animal were in triplicate and thus the mean of the three results was used as the response variable. Before analysis data was log-transformed and any zero values were first replaced by a small positive value (0.03) to avoid their exclusion from the analysis.

The results with and without PMA were analysed separately. Because results from the high quartz group were much lower than from the other treatments the analysis was run with and without this data included. In addition, all the results for low quartz at 50 mg were zero and these were excluded. Results from untreated controls were given a weighting of 2 in regression modelling since they were derived from cells pooled from two animals and would be more accurate than other readings.

In the absence of PMA, the main sources of variation in the superoxide readings were due to dust type and dose, after differences between experiment had been allowed for. The effect of dose also differed significantly with dust type. A small interaction was evident between dust type, dose, and time. The accumulated analysis of variance is given in Table A2.7 and geometric means, with ranges, for the data, classified by dust type, dose, and time, are presented in Table A2.8. The main findings of the analysis have been described in the results section of the report.

In the presence of PMA, superoxide production was much higher but the ANOVA still showed significant effects of dust type and dose, after allowance for experimental differences. There was, in this case, no interaction between dust type and dose (Table A2.9) suggesting a similar effect of dose for each dust group (Table A2.10). The effect of dust type was, on the whole, less dominant than for the spontaneous (no PMA) group.

A significant time and dose interaction was evident but this term was distorted by having "Experiment" in the model; time varies within experiment and there may be little remaining information on it after the "Experiment" term is fitted. The interaction proved not to be significant when "Experiment" was withheld from the model. The main findings of the analysis have been described in the results section of the report.

Table A2.1 Accumulated analysis of variance table from fitting regression model to the logarithm of total number of cells. Shown are the terms added to the model and the associated degrees of freedom, sums of squares, mean squares and variance ratios.

Term added to model	d.f.	s.s.	m.s.	v.r.	
+ Time	2	1.5758	0.7879	# 0.80	
+ Experiment	33	32.4229	0.9825	8.66	***
+ Control-vs-rest	1	56.5015	56.5015	498.11	***
+ Time x Control-vs-rest	2	0.9159	0.4579	4.04	*
+ Dust-type	3	11.9531	3.9844	35.13	***
+ Dose	2	15.8298	7.9149	69.78	***
+ Dust-type x Dose	6	9.6804	1.6134	14.22	***
+ Time x Dose	4	1.2433	0.3108	2.74	*
+ Time x Dust-type	5	0.7325	0.1465	1.29	
+ Time x Dust-type x Dose	10	2.3469	0.2347	2.07	*
Residual	86	9.7551	0.1134		
Total	154	142.9572	0.9283		

* $p<0.05$ ** $p<0.01$ *** $p<0.001$

the variance ratio for this term uses the mean square for Experiment as the denominator

Table A2.2 Total cells (millions) by dust type, dose and time. Table cells contain geometric mean, minimum and maximum.

time	dust	dose C	1	10	50	Margin
7 days	Control	3.818	*	*	*	3.818
		1.70	*	*	*	1.70
		5.96	*	*	*	5.96
	LU	*	11.125	16.652	14.114	13.710
		*	10.65	8.02	9.60	8.02
		*	11.61	26.75	20.75	26.75
	Fe	*	8.068	6.373	12.578	8.024
		*	6.90	2.37	9.82	2.37
		*	10.11	9.90	16.11	16.11
	Q/Fe	*	7.877	16.110	21.540	12.824
		*	5.16	5.77	16.50	5.16
		*	15.06	24.62	28.12	28.12
	Q	*	24.850	30.661	33.433	28.681
		*	18.10	14.30	18.00	14.30
		*	42.60	62.75	62.10	62.75
	LowQ	*	8.180	24.605	47.117	21.167
		*	6.00	20.50	40.40	6.00
		*	11.25	30.50	50.40	50.40
	Margin	3.818	10.752	16.677	25.635	11.954
		1.70	5.16	2.37	9.60	1.70
		5.96	42.60	62.75	62.10	62.75
32 days	Control	4.415	*	*	*	4.415
		3.00	*	*	*	3.00
		6.52	*	*	*	6.52
	LU	*	6.836	14.276	33.427	11.924
		*	4.05	12.75	15.10	4.05
		*	9.81	15.85	74.00	74.00
	Fe	*	9.650	7.600	10.681	9.012
		*	8.16	5.85	9.75	5.85
		*	11.40	9.60	11.70	11.70
	Q/Fe	*	9.201	31.129	26.646	17.389
		*	7.56	16.25	19.85	7.56
		*	10.05	93.00	35.77	93.00
	Q	*	39.305	82.052	82.613	58.794
		*	25.00	46.00	70.00	25.00
		*	55.65	136.50	97.50	136.50
	LowQ	*	8.700	44.663	123.358	36.326
		*	5.70	25.50	108.00	5.70
		*	10.95	68.50	154.00	154.00
	Margin	4.415	11.711	26.213	47.821	15.72
		3.00	4.05	5.85	9.75	3.00
		6.52	55.65	136.50	154.00	154.00

Table A2.2 contd./

60 days Control	4.934	*	*	*	4.934
	1.75	*	*	*	1.75
	13.87	*	*	*	13.87
LU	*	8.899	12.763	35.896	13.074
	*	7.50	9.60	32.62	7.50
	*	10.80	16.85	39.50	39.50
Fe	*	7.307	8.573	12.045	8.481
	*	4.65	7.72	9.00	4.65
	*	9.45	9.51	16.12	16.12
Q/Fe	*	10.491	20.247	41.350	15.653
	*	6.51	14.00	41.35	6.51
	*	29.75	28.00	41.35	41.35
Q	*	57.318	213.403	159.931	111.409
	*	35.75	193.00	126.00	35.75
	*	105.40	231.00	203.00	231.00
LowQ	*	*	*	*	*
	*	*	*	*	*
	*	*	*	*	*
Margin	4.934	14.062	26.222	41.088	15.702
	1.75	4.65	7.72	9.00	1.75
	13.87	105.40	231.00	203.00	231.00
Margin Control	4.352	*	*	*	4.352
	1.70	*	*	*	1.70
	13.87	*	*	*	13.87
LU	*	8.632	14.477	25.680	12.856
	*	4.05	8.02	9.60	4.05
	*	11.61	26.75	74.00	74.00
Fe	*	8.302	7.460	11.740	8.511
	*	4.65	2.37	9.00	2.37
	*	11.40	9.90	16.12	16.12
Q/Fe	*	9.223	21.654	26.721	15.236
	*	5.16	5.77	16.50	5.16
	*	29.75	93.00	41.35	93.00
Q	*	39.452	81.275	76.159	58.525
	*	18.10	14.30	18.00	14.30
	*	105.40	231.00	203.00	231.00
LowQ	*	8.436	33.150	76.238	27.729
	*	5.70	20.50	40.40	5.70
	*	11.25	68.50	154.00	154.00
Margin	4.352	12.073	22.306	36.301	14.309
	1.70	4.05	2.37	9.00	1.70
	13.87	105.40	231.00	203.00	231.00

Table A2.3 Accumulated analysis of variance table from fitting regression model to the logarithm of number of macrophages. Shown are the terms added to the model and the associated degrees of freedom, sums of squares, mean squares and variance ratios.

Term added to model	d.f.	s.s.	m.s.	v.r.
+ Time	2	1.6196	0.8098	# 1.33
+ Experiment	33	20.1248	0.6098	5.92 ***
+ Control-vs-rest	1	40.4295	40.4295	392.23 ***
+ Time x Control-vs-rest	2	0.5775	0.2887	2.80 *
+ Dust-type	3	5.7323	1.9108	18.54 ***
+ Dose	2	6.0191	3.0095	29.20 ***
+ Dust-type x Dose	6	4.2283	0.7047	6.84 ***
+ Time x Dose	4	1.6855	0.4214	4.09 **
+ Time x Dust-type	5	0.6900	0.1380	1.34
+ Time x Dust-type x Dose	10	2.1374	0.2137	2.07 *
Residual	86	8.8646	0.1031	
Total	154	92.1083	0.5981	

the variance ratio for this term uses the mean square for Experiment as the denominator

* $p<0.05$ ** $p<0.01$ *** $p<0.001$

Table A2.4 Number of macrophages (millions) by dust type, dose and time. Table cells contain geometric mean, minimum and maximum.

time	dust	dose C	1	10	50	Margin
7 days	Control	3.799	*	*	*	3.799
		1.70	*	*	*	1.70
		5.96	*	*	*	5.96
	LU	*	10.985	13.769	12.652	12.369
		*	10.44	7.62	9.41	7.62
		*	11.38	21.67	17.01	21.67
	Fe	*	7.845	5.619	10.684	7.302
		*	6.69	2.18	8.64	2.18
		*	10.11	8.91	13.21	13.21
	Q/Fe	*	7.378	10.786	17.221	10.175
		*	5.11	4.44	11.22	4.44
		*	13.25	15.02	26.43	26.43
	Q	*	13.293	15.157	15.130	14.377
		*	8.53	6.01	11.52	6.01
		*	24.28	34.51	19.87	34.51
	LowQ	*	7.540	14.201	15.803	11.916
		*	5.70	11.83	12.12	5.70
		*	10.57	18.30	18.85	18.85
	Margin	3.799	9.138	11.243	14.368	9.002
		1.70	5.11	2.18	8.64	1.70
		5.96	24.28	34.51	26.43	34.51
32 days	Control	4.388	*	*	*	4.388
		2.97	*	*	*	2.97
		6.52	*	*	*	6.52
	LU	*	6.767	12.554	28.856	11.028
		*	3.93	11.09	13.89	3.93
		*	9.81	15.12	59.94	59.94
	Fe	*	9.573	7.524	9.534	8.763
		*	8.08	5.79	9.36	5.79
		*	11.29	9.60	9.71	11.29
	Q/Fe	*	8.864	22.216	20.490	14.418
		*	7.56	10.56	16.08	7.56
		*	9.65	66.03	26.11	66.03
	Q	*	20.784	43.404	27.300	28.547
		*	12.25	26.68	18.20	12.25
		*	29.49	102.37	40.95	102.37
	LowQ	*	8.460	21.529	55.618	21.637
		*	5.41	11.98	47.52	5.41
		*	10.73	32.19	80.08	80.08
	Margin	4.388	10.090	18.134	27.943	12.204
		2.97	3.93	5.79	9.36	2.97
		6.52	29.49	102.37	80.08	102.37

Table A2.4 contd./

60 days Control	4.894	*	*	*	4.894
	1.73	*	*	*	1.73
	13.74	*	*	*	13.74
LU	*	8.774	10.917	26.309	11.599
	*	7.42	8.16	25.77	7.42
	*	10.58	12.97	26.86	26.86
Fe	*	7.263	8.098	10.937	8.140
	*	4.60	7.03	8.73	4.60
	*	9.45	9.32	13.70	13.70
Q/Fe	*	9.557	14.440	23.569	12.337
	*	6.31	11.06	23.57	6.31
	*	22.01	19.04	23.57	23.57
Q	*	33.033	103.117	59.088	55.544
	*	19.28	81.06	44.10	19.28
	*	57.97	125.67	79.17	125.67
LowQ	*	*	*	*	*
	*	*	*	*	*
	*	*	*	*	*
Margin	4.894	11.909	19.048	25.396	12.597
	1.73	4.60	7.03	8.73	1.73
	13.74	57.97	125.67	79.17	125.67
Margin Control	4.324	*	*	*	4.324
	1.70	*	*	*	1.70
	13.74	*	*	*	13.74
LU	*	8.527	12.358	21.257	11.631
	*	3.93	7.62	9.41	3.93
	*	11.38	21.67	59.94	59.94
Fe	*	8.194	6.996	10.367	8.070
	*	4.60	2.18	8.64	2.18
	*	11.29	9.60	13.70	13.70
Q/Fe	*	8.640	15.125	19.657	12.253
	*	5.11	4.44	11.22	4.44
	*	22.01	66.03	26.43	66.03
Q	*	21.584	40.784	29.007	28.962
	*	8.53	6.01	11.52	6.01
	*	57.97	125.67	79.17	125.67
LowQ	*	7.987	17.485	29.646	16.057
	*	5.41	11.83	12.12	5.41
	*	10.73	32.19	80.08	80.08
Margin	4.324	10.303	15.504	21.138	11.098
	1.70	3.93	2.18	8.64	1.70
	13.74	57.97	125.67	80.08	125.67

Table A2.5 Accumulated analysis of variance table from fitting regression model to the logarithm of number of neutrophils. Shown are the terms added to the model and the associated degrees of freedom, sums of squares, mean squares and variance ratios.

Term added to model	d.f.	s.s.	m.s.	v.r.
+ Time	2	7.3325	3.6663	# 0.52
+ Experiment	33	231.0111	7.0003	7.38 ***
+ Control-vs-rest	1	506.0237	506.0237	533.68 ***
+ Time x Control-vs-rest	2	11.5674	5.7837	6.10 **
+ Dust-type	3	121.5730	40.5243	42.74 ***
+ Dose	2	223.7131	111.8566	117.97 ***
+ Dust-type x Dose	6	32.5659	5.4277	5.72 ***
+ Time x Dose	4	3.5339	0.8835	0.93
+ Time x Dust-type	5	8.3826	1.6765	1.77
+ Time x Dust-type x Dose	10	18.6467	1.8647	1.97 *
Residual	86	81.5428	0.9482	
Total	154	1245.8928	8.0902	

* $p<0.05$ ** $p<0.01$ *** $p<0.001$

the variance ratio for this term uses the mean square for Experiment as the denominator

Table A2.6 Number of neutrophils (millions) by dust type, dose and time. Table cells contain geometric mean, minimum and maximum.

time	dust	dose	C	1	10	50	Margin
7 days	Control		0.015	*	*	*	0.015
			0.01	*	*	*	0.01
			0.06	*	*	*	0.06
	LU		*	0.054	2.257	0.847	0.416
			*	0.03	0.40	0.19	0.03
			*	0.23	4.81	3.73	4.81
	Fe		*	0.106	0.610	1.770	0.375
			*	0.03	0.17	1.08	0.03
			*	0.40	0.99	2.90	2.90
	Q/Fe		*	0.276	4.936	2.890	1.398
			*	0.02	1.33	1.69	0.02
			*	3.01	9.36	4.95	9.36
	Q		*	5.707	14.717	16.420	10.298
			*	0.73	8.29	6.48	0.73
			*	17.47	25.10	41.61	41.61
	LowQ		*	0.347	9.006	30.188	4.552
			*	0.18	6.56	28.28	0.18
			*	1.21	11.58	33.18	33.18
	Margin		0.015	0.315	3.899	6.338	0.690
			0.01	0.02	0.17	0.19	0.01
			0.06	17.47	25.10	41.61	41.61
32 days	Control		0.023	*	*	*	0.023
			0.01	*	*	*	0.01
			0.05	*	*	*	0.05
	LU		*	0.033	1.203	4.011	0.293
			*	0.02	0.63	1.21	0.02
			*	0.07	3.33	13.32	13.32
	Fe		*	0.060	0.042	0.716	0.082
			*	0.03	0.02	0.29	0.02
			*	0.10	0.08	1.75	1.75
	Q/Fe		*	0.137	8.104	5.303	1.172
			*	0.02	4.87	3.57	0.02
			*	0.80	26.97	7.87	26.97
	Q		*	17.516	35.102	52.347	27.521
			*	12.00	19.32	51.10	12.00
			*	26.16	50.75	53.62	53.62
	LowQ		*	0.085	19.056	65.760	4.735
			*	0.03	9.69	60.48	0.03
			*	0.17	34.93	70.84	70.84
	Margin		0.023	0.217	3.067	12.292	0.619
			0.01	0.02	0.02	0.29	0.01
			0.05	26.16	50.75	70.84	70.84

Table A2.6 contd./

60 days Control	0.026	*	*	*	0.026
	0.01	*	*	*	0.01
	0.06	*	*	*	0.06
LU	*	0.063	1.501	9.010	0.492
	*	0.02	0.85	6.85	0.02
	*	0.17	3.54	11.85	11.85
Fe	*	0.045	0.287	0.660	0.144
	*	0.02	0.10	0.18	0.02
	*	0.09	0.54	2.42	2.42
Q/Fe	*	0.326	4.815	17.367	1.423
	*	0.09	2.16	17.37	0.09
	*	7.14	10.56	17.37	17.37
Q	*	22.937	103.149	99.877	51.776
	*	10.37	80.94	81.90	10.37
	*	47.43	131.67	121.80	131.67
LowQ	*	*	*	*	*
	*	*	*	*	*
	*	*	*	*	*
Margin	0.026	0.382	3.825	9.323	0.660
	0.01	0.02	0.10	0.18	0.01
	0.06	47.43	131.67	121.80	131.67
Margin Control	0.021	*	*	*	0.021
	0.01	*	*	*	0.01
	0.06	*	*	*	0.06
LU	*	0.048	1.597	3.128	0.391
	*	0.02	0.40	0.19	0.02
	*	0.23	4.81	13.32	13.32
Fe	*	0.064	0.194	0.942	0.160
	*	0.02	0.02	0.18	0.02
	*	0.40	0.99	2.90	2.90
Q/Fe	*	0.228	5.775	5.273	1.321
	*	0.02	1.33	1.69	0.02
	*	7.14	26.97	17.37	26.97
Q	*	13.999	37.631	44.114	25.154
	*	0.73	8.29	6.48	0.73
	*	47.43	131.67	121.80	131.67
LowQ	*	0.171	13.100	44.555	4.643
	*	0.03	6.56	28.28	0.03
	*	1.21	34.93	70.84	70.84
Margin	0.021	0.291	3.559	8.936	0.654
	0.01	0.02	0.02	0.18	0.01
	0.06	47.43	131.67	121.80	131.67

Table A2.7 Accumulated analysis of variance table from fitting regression model to the logarithm of superoxide results (no PMA). Shown are the terms added to the model and the associated degrees of freedom, sums of squares, mean squares and variance ratios. (Note: results from LowQ at 50 mg dose, which were all zero, have been excluded.)

Term added to model	d.f.	s.s.	m.s.	v.r.	
+ Time	2	3.2766	1.6383	# 0.54	
+ Experiment	32	97.0550	3.0330	6.28	***
+ Control-vs-rest	1	25.4706	25.4706	52.78	***
+ Time x Control-vs-rest	2	0.3334	0.1667	0.35	
+ Dust-type	3	11.2094	3.7365	7.74	***
+ Dose	2	15.0044	7.5022	15.55	***
+ Dust-type x Dose	5	12.3142	2.4628	5.10	***
+ Time x Dose	4	2.7953	0.6988	1.45	
+ Time x Dust-type	5	2.7494	0.5499	1.14	
+ Time x Dust-type x Dose	9	9.8797	1.0977	2.27	*
Residual	77	37.1610	0.4826		
Total	142	217.2489	1.5299		

* $p<0.05$ ** $p<0.01$ *** $p<0.001$

the variance ratio for this term uses the mean square for Experiment as the denominator

Table A2.8 Superoxide readings (no PMA) by dust type, dose and time. Table cells contain geometric mean, minimum and maximum.

time	dust	C	1	10	50	Margin
7 days	Control	3.3665	*	*	*	3.3665
		0.030	*	*	*	0.030
		11.424	*	*	*	11.424
	LU	*	3.4667	1.1849	1.3683	1.7498
		*	2.356	0.857	1.000	0.857
		*	5.569	1.714	1.928	5.569
	Fe	*	5.3952	2.3457	0.3709	2.0552
		*	3.856	1.714	0.030	0.030
		*	9.853	3.570	0.928	9.853
	Q/Fe	*	4.0304	0.8495	0.2693	1.1058
		*	2.356	0.030	0.143	0.030
		*	5.783	3.998	0.428	5.783
	Q	*	0.2909	0.0582	0.0300	0.0859
		*	0.071	0.030	0.030	0.030
		*	0.928	0.500	0.030	0.928
	LowQ	*	7.2643	0.7269	0.0300	0.5411
		*	3.427	0.030	0.030	0.030
		*	19.135	2.999	0.030	19.135
	Margin	3.3665	2.9290	0.6308	0.1243	0.9394
		0.030	0.071	0.030	0.030	0.030
		11.424	19.135	3.998	1.928	19.135
32 days	Control	5.2934	*	*	*	5.2934
		1.499	*	*	*	1.499
		12.923	*	*	*	12.923
	LU	*	4.3749	2.3854	0.3996	2.2709
		*	1.856	1.856	0.071	0.071
		*	7.568	3.427	2.285	7.568
	Fe	*	4.0889	2.7142	3.1795	3.3653
		*	2.213	1.071	2.285	1.071
		*	8.354	4.070	3.570	8.354
	Q/Fe	*	4.4908	0.9075	0.9685	1.8996
		*	2.428	0.030	0.857	0.030
		*	12.495	1.999	1.142	12.495
	Q	*	0.1641	0.0418	0.0300	0.0733
		*	0.030	0.030	0.030	0.030
		*	1.285	0.143	0.030	1.285
	LowQ	*	8.5079	1.1454	0.0300	0.6637
		*	4.427	0.286	0.030	0.030
		*	15.422	2.499	0.030	15.422
	Margin	5.2934	2.4450	0.7760	0.1793	1.2873
		1.499	0.030	0.030	0.030	0.030
		12.923	15.422	4.070	3.570	15.422

table A2.8 contd.\

		C	1	10	50	Margin
60 days	Control	4.9282	*	*	*	4.9282
		1.000	*	*	*	1.000
		12.352	*	*	*	12.352
	LU	*	3.9191	1.5183	0.6275	1.9897
		*	1.856	0.071	0.286	0.071
		*	10.639	5.284	1.285	10.639
	Fe	*	5.2937	2.9701	1.9259	3.5698
		*	1.999	0.785	1.571	0.785
		*	10.781	6.426	2.356	10.781
	Q/Fe	*	3.5858	0.6476	0.0300	1.1208
		*	0.785	0.030	0.030	0.030
		*	10.496	2.999	0.030	10.496
	Q	*	0.1174	0.0527	0.1611	0.0929
		*	0.030	0.030	0.030	0.030
		*	0.857	0.286	1.928	1.928
	LowQ	*	*	*	*	*
		*	*	*	*	*
		*	*	*	*	*
	Margin	4.9282	1.7192	0.6263	0.3797	1.2988
		1.000	0.030	0.030	0.030	0.030
		12.352	10.781	6.426	2.356	12.352
Margin	Control	4.4896	*	*	*	4.4896
		0.030	*	*	*	0.030
		12.923	*	*	*	12.923
	LU	*	3.9744	1.6251	0.7001	2.0089
		*	1.856	0.071	0.071	0.071
		*	10.639	5.284	2.285	10.639
	Fe	*	4.8142	2.6641	1.3145	2.9781
		*	1.999	0.785	0.030	0.030
		*	10.781	6.426	3.570	10.781
	Q/Fe	*	4.0169	0.7933	0.2897	1.3545
		*	0.785	0.030	0.030	0.030
		*	12.495	3.998	1.142	12.495
	Q	*	0.1646	0.0504	0.0525	0.0835
		*	0.030	0.030	0.030	0.030
		*	1.285	0.500	1.928	1.928
	LowQ	*	7.8616	0.9124	0.0300	0.5993
		*	3.427	0.030	0.030	0.030
		*	19.135	2.999	0.030	19.135
	Margin	4.4896	2.2815	0.6779	0.1843	1.1654
		0.030	0.030	0.030	0.030	0.030
		12.923	19.135	6.426	3.570	19.135

Table A2.9 Accumulated analysis of variance table from fitting regression model to the logarithm of superoxide results (with PMA). Shown are the terms added to the model and the associated degrees of freedom, sums of squares, mean squares and variance ratios.

Term added to model	d.f.	s.s.	m.s.	v.r.
+ Time	2	4.6239	2.3120	# 0.64
+ Experiment	32	115.5365	3.6105	10.37 ***
+ Control-vs-rest	1	30.1467	30.1467	86.59 ***
+ Time x Control-vs-rest	2	2.4780	1.2390	3.56 *
+ Dust-type	3	4.0078	1.3359	3.84 *
+ Dose	2	8.6847	4.3424	12.47 ***
+ Dust-type x Dose	6	2.8168	0.4695	1.35
+ Time x Dose	4	4.3359	1.0840	3.11 *
+ Time x Dust-type	5	1.4553	0.2911	0.84
+ Time x Dust-type x Dose	10	6.0824	0.6082	1.75
Residual	83	28.8971	0.3482	
Total	150	209.0651	1.3938	

* $p<0.05$ ** $p<0.01$ *** $p<0.001$

the variance ratio for this term uses the mean square for Experiment as the denominator

Table A2.10 Superoxide readings (with PMA) by dust type, dose and time. Table cells contain geometric mean, minimum and maximum.

time	dust	C	1	10	50	Margin
7 days	Control	12.1595	*	*	*	12.1595
		0.357	*	*	*	0.357
		58.262	*	*	*	58.262
	LU	*	7.8637	3.9109	3.4410	4.7978
		*	3.427	1.000	1.642	1.000
		*	13.709	14.566	6.997	14.566
	Fe	*	16.4605	9.6472	1.3848	7.4888
		*	10.567	1.999	0.785	0.785
		*	35.414	22.134	2.285	35.414
	Q/Fe	*	9.9106	7.0204	0.8454	4.9203
		*	6.712	2.856	0.030	0.030
		*	14.566	22.562	8.711	22.562
	Q	*	4.5892	1.9935	0.3650	1.8050
		*	2.999	0.030	0.030	0.030
		*	7.997	10.710	1.642	10.710
	LowQ	*	20.8100	6.4895	2.2283	6.2556
		*	11.710	4.855	1.000	1.000
		*	35.986	9.139	3.998	35.986
	Margin	12.1595	10.5724	5.0506	1.3929	5.5593
		0.357	2.999	0.030	0.030	0.030
		58.262	35.986	22.562	8.711	58.262
32 days	Control	20.6364	*	*	*	20.6364
		4.427	*	*	*	4.427
		45.125	*	*	*	45.125
	LU	*	14.8723	7.9872	2.3574	8.4871
		*	6.426	4.284	0.571	0.571
		*	28.560	11.353	7.568	28.560
	Fe	*	9.7461	6.8717	6.1165	7.8860
		*	3.713	2.356	4.855	2.356
		*	24.633	15.994	7.426	24.633
	Q/Fe	*	10.3088	3.5390	4.8737	6.0983
		*	3.927	0.785	4.141	0.785
		*	31.059	10.853	5.855	31.059
	Q	*	4.3287	0.9322	1.2976	1.9895
		*	2.213	0.030	0.928	0.030
		*	13.138	7.711	1.785	13.138
	LowQ	*	19.3658	16.7048	8.6191	14.0749
		*	10.567	10.996	6.283	6.283
		*	48.409	28.988	10.567	48.409
	Margin	20.6364	10.1957	4.9675	4.3499	8.1519
		4.427	2.213	0.030	0.571	0.030
		45.125	48.409	28.988	10.567	48.409

table A2.10 contd.\

		C	1	10	50	Margin
60 days	Control	20.7377	*	*	*	20.7377
		3.570	*	*	*	3.570
		44.554	*	*	*	44.554
	LU	*	10.7558	6.6961	4.2344	7.6417
		*	1.785	2.071	2.356	1.785
		*	22.705	15.422	7.997	22.705
	Fe	*	14.4483	10.7640	13.0653	12.7462
		*	3.070	3.641	10.210	3.070
		*	54.907	25.133	16.993	54.907
	Q/Fe	*	10.0590	7.5053	5.6076	8.4392
		*	3.713	1.714	5.212	1.714
		*	32.701	14.851	5.998	32.701
	Q	*	2.2889	0.5221	2.6003	1.4105
		*	0.428	0.030	1.428	0.030
		*	13.709	2.071	4.284	13.709
	LowQ	*	*	*	*	*
		*	*	*	*	*
		*	*	*	*	*
	Margin	20.7377	7.7341	4.2832	5.2908	7.5820
		3.570	0.428	0.030	1.428	0.030
		44.554	54.907	25.133	16.993	54.907
Margin	Control	17.5027	*	*	*	17.5027
		0.357	*	*	*	0.357
		58.262	*	*	*	58.262
	LU	*	11.3340	5.9360	3.2506	6.9290
		*	1.785	1.000	0.571	0.571
		*	28.560	15.422	7.997	28.560
	Fe	*	12.7973	8.9360	4.8010	9.2115
		*	3.070	1.999	0.785	0.785
		*	54.907	25.133	16.993	54.907
	Q/Fe	*	10.1196	5.7131	2.4873	6.3720
		*	3.713	0.785	0.030	0.030
		*	32.701	22.562	8.711	32.701
	Q	*	3.4339	1.0082	1.0719	1.7155
		*	0.428	0.030	0.030	0.030
		*	13.709	10.710	4.284	13.709
	LowQ	*	20.0094	10.8690	4.3825	9.6098
		*	10.567	4.855	1.000	1.000
		*	48.409	28.988	10.567	48.409
	Margin	17.5027	9.3764	4.7900	2.9257	7.0588
		0.357	0.428	0.030	0.030	0.030
		58.262	54.907	28.988	16.993	58.262

APPENDIX 3

A3 Analysis of TNF results

TNF results were transformed to the log scale prior to carrying out multiple linear regression analysis. Note that each culture fluid was assayed twice giving two values for each animal.

A3.1 LPS present in cultures

In this data set there were a few values that were statistical outliers. Two of these outliers were excluded from the analysis, one from a pair of untreated control animals and the other from an animal treated with 1 mg iron/quartz at the 60 day time point. The analysis was also conducted with and without results from an experiment (Experiment 70: 10 mg dust/ 60 days) in which LPS, unusually, had no effect on TNF production. A possible explanation for the low values could be that LPS was omitted from the cultures. The analysis was also carried out with and without results from the high quartz treatment.

Initially a regression analysis was done on all the data, adjusting for animal effects in order to estimate assay effects. Note that treatment and experiment effects, which are between animals, were thus adjusted by implication when estimating assay effects. Assay effects were adjusted by raising, or lowering, the values of an assay by a fixed amount on the log scale to give results comparable to the median assay values. Three assays were used to re-measure supernatants which had very high activity and it could be argued that adjustments for these assays were not fully justified as these assays were used, all or partly, for values which were known to be high.

As each supernatant had been assayed twice the average was taken of the two readings, following adjustment for assay effects, giving one result per animal. Since the values for untreated controls were derived from the pooled macrophage populations from two animals these were given a weight of two, compared to a weight of one for all other values, in the regression analysis.

The effects of time were estimated from information between experiments and so were compared with experiment to experiment variability. Other factors were estimated from information within experiments and are compared with the residual error, i.e. animal to animal variability.

TNF values for the day 32 time point were higher than for days 7 or 60 but were not statistically significant. Time was therefore excluded in the summary tables (4.12 and 4.13) in the results section.

The accumulated analysis of variance, for TNF production in the presence of LPS, and with the results for experiment 70 and high quartz results included, is summarised in Table A3.1. When the the high dose quartz results are included there is an interaction between dust type and dose which is due to the different pattern of results with high quartz. The analysis of variance table with both experiment 70 results and high dose quartz excluded is shown in Table A3.2. Other variants of the analysis not shown, had high quartz included but experiment 70 excluded or vice versa. Only the inclusion, or exclusion, of the high quartz data affected the outcome of the analysis.

Macrophages from untreated animals produced significantly less TNF than those from treated animals. There was a significant effect of dose with TNF higher with 10 mg treatments than with 1 mg treatments. TNF values for macrophages from the LU dust and iron treatments were significantly lower than those from the quartz/iron treated group.

A3.2 TNF production in the absence of LPS

The analysis was repeated for the results generated in the absence of LPS with the exception that experiment 70 results did not need to be excluded and that there were no outliers. No significant differences were found for time, dust type, or dust dose. The inclusion of high quartz results did not alter this outcome. Table A3.3 shows the accumulated analysis of variance. Note that because assays occurred in pairs for the TNF without LPS data, it was not possible to adjust for assays from within animal information. Instead, averages of the unadjusted results were taken for each animal. The effect of assay in these experiments adjusts for the pair of assays used for each animal. As assays differred, mostly, between experiments, the assay to assay mean squares (m.s.; variability) was compared with the experiment to experiment mean square. The resulting variance ratio (F ratio) may have been an underestimate, but the result was still significant.

Table A3.1 TNF production in the presence of LPS. Accumulated analysis of variance, experiment 70 and high quartz included.

Change	d.f.	s.s.	m.s.	v.r.	p
+ time	2	12.79	6.40	2.29	NS
+ experiment	33	91.97	2.79	3.85	0.001
+ control vs rest	1	110.35	110.35	152.46	0.001
+ dust type	4	24.66	6.16	8.52	0.001
+ dose	2	12.88	6.44	8.90	0.001
+ dust type. dose	8	36.91	4.61	6.37	0.001
Residual	135	97.71	0.72		
Total	185	387.26	2.09		

NS : Not significant

Table A3.2 TNF production in the presence of LPS. Accumulated analysis of variance, experiment 70 and high quartz excluded.

Change	d.f.	s.s.	m.s.	v.r.	p
+ time	2	11.07	5.53	1.65	NS
+ experiment	32	107.41	3.36	4.64	0.001
+ control vs rest	1	82.03	82.03	113.51	0.001
+ dust type	3	14.81	4.94	6.83	0.001
+ dose	2	25.06	12.53	17.33	0.001
+ dust type. dose	6	6.52	1.09	1.50	NS
Residual	103	74.44	0.72		
Total	147	321.34	2.16		

NS : Not significant

Table A3.3 TNF production in the absence of LPS. Accumulated analysis of variance, high quartz included.

Change	d.f.	s.s.	m.s.	v.r.	p
+ assay	10	59.24	5.92	4.08	0.01
+ time	2	0.15	0.07	0.05	NS
+ experiment	26	37.77	1.45	5.61	0.001
+ control vs rest	1	17.71	17.71	68.39	0.001
+ dust type	4	2.46	0.62	2.38	NS
+ dose	2	0.92	0.46	1.78	NS
+ dust type. dose	8	2.89	0.36	1.39	NS
Residual	132	34.19	0.26		
Total	185	155.34	0.84		

NS : Not significant

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