Depletion of Glutathione and Ascorbate in Lung Lining Fluid by Respirable Fibres

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Objective—The use of synthetic vitreous fibres has increased along with a decline in the utilisation of asbestos. There remains concern that these synthetic fibres pose a health risk to workers because of the generation of respirable fibres which can enter the lung and cause adverse health effects. An improved understanding of the mechanism of fibre pathogenicity should allow more rational short-term testing regimes for new fibres as they are developed. We hypothesised that carcinogenic fibres have greater free radical activity compared with non-carcinogenic fibres and that they contribute to disease by causing oxidative stress in the lung. We examined a panel of respirable fibres, designated as being carcinogenic or non-carcinogenic based on previous animal studies for ability to deplete antioxidants from lung lining fluid.

Methods—On the basis of inhalation studies, a panel of fibres was divided into three carcinogenic fibres—amosite asbestos, silicon carbide, and refractory ceramic fibre 1 (RCF1) and three non-carcinogenic fibres—man-made vitreous fibre 10 (a glass fibre MMVF10), Code 100/475 glass fibre, and refractory ceramic fibre 4 (RCF4). We measured the levels of glutathione (GSH) and ascorbate, two antioxidants present in lung lining fluid (LLF) after fibre treatment. All of the experiments were carried out at equal fibre number.

Results—Fibres had the ability to deplete both GSH and ascorbate from both LLF and pure solutions, an effect which was fibre number dependent. The greatest depletion of antioxidants was observed with the two non-carcinogenic glass fibres, and this effect was observed when A549 lung epithelial cells were treated with fibres.

Conclusions—Our results show that antioxidant depletion in cell free solution and lung lining fluid solely is not a simple indicator of the ability of fibres to cause lung pathology and that other biological events in the lung are involved.

Keywords: lung lining fluid; antioxidants; man-made mineral fibres; asbestos

INTRODUCTION

Exposure to asbestos causes increased risk of pulmonary fibrosis, bronchial carcinoma and pleural mesothelioma and the mechanisms underlying these diseases are only partially understood (Donaldson et al., 1993; Kamp et al., 1992; Kane, 1996). The use of asbestos in industrial and domestic applications has declined and this has been matched by increased use of synthetic or man-made fibrous materials, in particular mineral wool and fibres which retain their properties at higher temperatures—high performance fibres, such as ceramic fibres. These fibres are capable of becoming airborne as respirable fibres and cause similar diseases to asbestos in rats (Bunn et al., 1993; Mast et al., 1995). New fibre types, and variations of existing fibre types are likely to occur in attempts to improve performance. A number of test protocols have been advanced to
test these new fibres and a number of in vitro tests for predicting toxicity have been suggested (McClellan et al., 1992; Cullen et al., 1997).

It has been postulated that the surface free radical activity of asbestos fibres is important in initiating the responses that lead to lung disease (Kamp et al., 1992) and free radical activity is measurable at the surface of asbestos fibres (Lund and Aust, 1991; Gilmour et al., 1997; Fubini et al., 1995). The man-made fibres have less free radical activity (Gilmour et al., 1995) but some refractory ceramic fibres can be as potent as asbestos fibres in this regard (Brown et al., 1998a) but this depends on the conditions of the assay as we demonstrated with the plasmid scission and salicylic acid assays for detection of fibre-derived free radicals (Brown et al., 1998a). Free radicals can interact with lung cells and may cause damage (Kamp et al., 1992) or cell stimulation (Donaldson, 1995; Rahman and MacNee, 1998).

In particular, iron present at the fibre surface may be involved in the initiation of some of the reactions which produce free radicals via Fenton chemistry (Gilmour et al., 1997; Fubini et al., 1995). Many genes coding for pro-inflammatory molecules, such as cytokines, and adhesion receptors, are controlled by cis-activating regulators of transcription which are activated by oxidative stress, the best known of which is NF-κB (Rahman and MacNee, 1998). Oxidative stress derived from fibre surfaces may therefore be pro-inflammatory and eventually pathogenic because of its role in signal transduction for transcription of these pro-inflammatory genes.

The surfactant system forms a protective barrier in protecting the lung from oxidative and other damage (Slade et al., 1993). Antioxidant components of this complex mixture which have previously been shown to play a major role include glutathione (GSH) and ascorbate, both of which have been measured in the bronchoalveolar lavage fluid (BAL) of humans (Cantin et al., 1989; Bui et al., 1992) and rats (Jenkinson et al., 1988; Skoza et al., 1983; Willis and Kratzing, 1974). Depletion of the antioxidant defence screen by pollutants can result in oxidative stress to the underlying epithelial cells (Kelly et al., 1996).

In this study, we examined the effects of pathogenic or non-pathogenic fibres for their ability to deplete GSH and ascorbate from rat lung lining fluid and lung epithelial cells in vitro.

### MATERIALS AND METHODS

#### Preparation of lung lining fluid (LLF)

Female Wistar rats aged 3 months were killed with a single injection of Nembutal, the thoracic cavity exposed and the lungs cannulated with a blunt needle. Lungs were removed en bloc and lavaged with 7 × 2 ml volumes of sterile saline at room temperature. After each instillation, the lungs were gently massaged and the lavageate withdrawn and pooled into a single tube. Tubes were spun at 1200 rpm for 5 min to pellet cells and the supernatant transferred to a separate tube. The supernatant (lung lining fluid) was used in the subsequent treatments.

#### Fibres

The fibres used in this study were long fibre amosite asbestos, silicon carbide fibre (Advanced Composite Materials Corporation), Refractory Ceramic Fibres from the TIMA repository and man-made vitreous fibres and Code 100/475 glass fibres, originally made by Johns Manville. Fibre numbers were based on phase contrast optical microscopy counts of each fibre type. The size distribution of the fibres is shown in Table 1.

#### Fibre treatment

Conditions were set up to give the same fibre number per treatment. Fibres were resuspended at 8.24 × 10^6 fibres ml⁻¹ LLF and briefly sonicated to disperse the fibres. One millilitre of each fibre suspension was added to each well of a 24-well plate and incubated at 37°C for 4 h with intermittent mixing. After incubation, the fibre suspensions were transferred to sterile 1.5-ml eppendorf tubes and centrifuged at 2500 rpm for 5 min to pellet the fibres. The supernatants were transferred to separate tubes for use in the various assays and for GSH extraction. In some experiments, fibres were suspended at different fibre number in either lung lining fluid or stock solutions of ascorbate (Sigma, Poole, Dorset) (1 mM) or GSH (reduced form) (Sigma) (12.5 μM) and incubated at 37°C for 4 h.

<table>
<thead>
<tr>
<th>Composition</th>
<th>&gt; 10 μm</th>
<th>&gt; 20 μm</th>
<th></th>
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<tbody>
<tr>
<td>LFA</td>
<td>64.75</td>
<td>35.25</td>
<td></td>
</tr>
<tr>
<td>SiC</td>
<td>60.86</td>
<td>27.6</td>
<td></td>
</tr>
<tr>
<td>RCF1</td>
<td>77.36</td>
<td>45.27</td>
<td></td>
</tr>
<tr>
<td>RCF4</td>
<td>59.35</td>
<td>17.96</td>
<td></td>
</tr>
<tr>
<td>MMVF10</td>
<td>85.24</td>
<td>67.17</td>
<td></td>
</tr>
<tr>
<td>Code 100/475</td>
<td>50.00</td>
<td>19.32</td>
<td>Glass fibre</td>
</tr>
</tbody>
</table>

Table 1. Percentage size distribution of the fibres used in the study
Oxidant generating system

In order to examine the effect of artificially produced oxygen radicals on the antioxidant profile of the LLF, the following system based on the method of Simeonova and Luster (1995), was used. In this system, Fenton chemistry generates powerful oxidising agents, including superoxide and hydroxyl radicals. Briefly, stock solutions of ferric sulphate (0.4 mM) and hydrogen peroxide (10 mM) were prepared in Chelex-treated water and 50 μl of each added to 900 μl LLF or pure solutions of GSH and ascorbate. Some treatments consisted of various dilutions of the ferric sulphate/hydrogen peroxide mixture in Chelex-treated water. The solutions were incubated at 37°C for 4 h and the extraction procedure for GSH/GSSG followed.

Extraction for GSH/GSSG estimations

The method of Akerboom and Sies (1981) was used to extract GSH. The extraction mixture was made up as follows: 50 ml of 70% perchloric acid was diluted to 250 ml with distilled water and stored at 4°C (14% perchloric acid). Ten milliliters of 14% perchloric acid, 5 ml distilled water and 180 μl of 0.5 M EDTA were made up to give working strength extraction mixture. Briefly, 0.5 ml of treated LLF was pipetted into 15-ml conical tubes and 0.5 ml perchloric acid/EDTA mixture added to each tube to give a final concentration of 0.3 mM. The tubes were mixed and incubated on ice for 15 min. The extraction procedure was carried out in a cold room. After incubation, the tubes were spun at 3000 rpm to pellet any precipitate. The supernatant was transferred to clean conical centrifuge tubes and stored at room temperature for 30 min. A series of ascorbate standards were prepared as for the GSH assay using NaOH in place of phosphate buffer, and GSSG standard was prepared as for the GSH assay using NaOH in place of phosphate buffer.

Ascorbic acid assay

The method used was adapted from that described by Shichi et al. (1997). Briefly, 100 μl of prepared sample (Materials and methods section) were mixed with 50 μl of 6% TCA and incubated in eppendorf tubes at room temperature for 15 min to precipitate protein. Tubes were then centrifuged at 13 000 rpm for 5 min and the supernatant pipetted into fresh tubes. The reagent for the assay consisted of 1% bicinchoninic acid (Sigma) dissolved in buffer containing 100 mls distilled Chelexed water, 9.75 g Na₂CO₃ and 1.34 g NaHCO₃ (Reagent A). Reagent B consisted of a solution of 4% aqueous copper sulphate. The working solution was prepared immediately prior to the assay by mixing solutions A and B in a ratio of 50A:1B.

A series of ascorbate standards were prepared containing TCA at the same concentration as the samples in the range of 1–0.0325 mM; 5 μl of each standard or sample were pipetted into triplicate groups of wells on a 96-well plate and 100 μl of working strength reagent added to each well. A blank set of wells consisted of TCA with no ascorbate. The plate was incubated at room temperature for 30 min and read at 550 nm on an automatic plate reader. To test that ascorbate was being measured in this assay, a separate series of wells were set up containing 5 μl standard or sample and 2.5 μl of 1 M NaOH. The tests were incubated at room temperature for 30 min prior to the addition of the assay reagent and incubated for a further 30 min at room temperature. Oxidation of ascorbate occurs at alkaline pH, and in these experiments no absorbance was measured after NaOH treatment.

Statistics

Data were analysed using analysis of variance with Tukey multiple comparison test.
**RESULTS**

**Depletion of GSH from lung lining fluid and from solution**

**Effect of free radical generating system.** We first set out to demonstrate that free radicals do have the ability to deplete the anti-oxidant defences of rat lung lining fluid. After treatment using an exogenous oxidant generating system, the levels of GSH and ascorbate were measured both in LLF and in pure solutions. Data for GSH depletion in LLF and a pure solution are shown in Fig. 1. Chemically produced oxidants significantly depleted GSH in LLF and a dilution effect of the generating system was evident. At all concentration ranges for the generating system, there was significant depletion ($P < 0.05$) of GSH from a pure solution. In LLF, however, GSH was better conserved and although there was depletion at all of the concentrations used, significance was only reached at the two highest concentrations.

Fibres at different doses in this series of experiments fibres were used at the 1× and 5× fibre dose. Increasing the fibre number to five times that used in original experiments produced a greater depletion of LLF GSH (Fig. 2). The largest effect was obtained with the two glass fibres and MMVF10 was most active, almost completely depleting GSH at the 5× concentration. Statistical analysis showed that at higher fibre number, there was a significant decrease in GSH compared with the control for all fibres ($P < 0.05$) with the exception of RCF1. At the lower fibre number, only MMVF10 was significantly different from the control. All fibres were able to deplete GSH from a pure solution at the higher fibre number (Fig. 3). At lower fibre numbers, only Code 100, RCF1 and MMVF10 caused depletion compared with the control, these effects were not significant. At five times normal fibre number, the greatest depletion was observed with Code 100/475 and MMVF10, all fibres were significantly different from the control ($P < 0.05$).

**Depletion of ascorbate from lung lining fluid and in solution**

**Effect of free radical generating system.** Figure 4 shows the same system with ascorbate as the target antioxidant. Although a clear depletion of ascorbate and an effect of oxidant concentration could be demonstrated, this was not statistically significant for LLF. Using a pure ascorbate solution, a significant effect was noted at the two highest oxidant concentrations ($P < 0.05$).

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*Fig. 1. GSH depletion from a 12.5 μM pure solution and LLF after treatment with various concentrations of a chemically produced oxidant generating system. Normalised data represents the mean and SEM of three separate experiments ($P < 0.05$).*

*Fig. 2. GSH content of lung lining fluid after 4-h treatment with 8.24 × 10^6 and 4.12 × 10^7 fibres ml⁻¹. Normalised data represents the mean and SEM of three separate experiments ($P < 0.05$).*

*Fig. 3. GSH depletion from a 12.5 μM pure solution after 4-h treatment with 8.24 × 10^6 and 4.12 × 10^7 fibres ml⁻¹. Normalised data represents the mean and SEM of three separate experiments ($P < 0.05$).*
Effect of fibres at different doses. The data obtained for the ascorbic acid content of the LLF (Figs 5 and 6) after fibre treatment was similar to the findings for GSH, and only the two glass fibres at the highest dose produced a significant decrease in ascorbate compared with the control. All fibres, except LFA had the ability to deplete a pure ascorbate solution at the highest dose, although only RCF1 had this activity at the lower dose.

DISCUSSION

The ability of fibres to cause lung disease has been associated with fibre dimensions such as length and diameter (Donaldson et al., 1993; Davis and Donaldson, 1986; Stanton et al., 1981). However, numerous studies have demonstrated that asbestos fibres have free radical activity (Lund and Aust, 1991) that cause cell damage (Kamp et al., 1992), activation of transcription factors (Gilmour et al., 1997) and cytokine release (Simeonova and Luster, 1995). Furthermore, in recent in vivo studies, non-pathogenic fibres of similar dimensions to pathogenic fibres have been shown to accumulate in the lung and to persist (Bunn et al., 1993; Davis et al., 1996; Hesterberg et al., 1998). For instance, although RCF1 was pathogenic in inhalation studies and MMVF10 was not (Bunn et al., 1993) they are similarly biopersistent (Hesterberg et al., 1998); thus another explanation for this difference must be sought. Therefore, while dimensions and durability are probably important in pathogenicity, surface reactivity may also be important. The primary aim of this study was to investigate a panel of three pathogenic and three non-pathogenic fibres based on data obtained from animal studies (Bunn et al., 1993; Davis et al., 1996) to determine the effect of respirable fibres on the antioxidants present in lung lining fluid.

Oxidant stress from fibres could activate oxidant stress-responsive transcription factors, such as NF-κB, leading to the expression of pro-inflammatory
genes (Rahman and MacNee, 1998) as we have previously demonstrated (Gilmour et al., 1997). Previous in vitro studies have largely ignored the fact that fibres entering the lung interact with the surface active lining layer, thereby altering the fibres’ surface chemistry and changing the reactivity with other components of the lung. We have demonstrated that the fibre surface chemistry and reactivity are important in events which could lead to pathogenic effects of fibres (Gilmour et al., 1997; Brown et al., 1998a; 1998b). Fibre-derived iron has been implicated in free radical production particularly with asbestos, although MMVF10 fibres release greater amounts of iron than amphibole asbestos under neutral or acid conditions and in the presence or absence of LLF (Fisher et al., 1998) but MMVF10 is not strongly active in producing free radicals (Brown et al., 1998a). This is not related to total iron content of the fibres, since amphibole asbestos has much more total iron, but to biologically active soluble salts of iron. This may be related to the relative importance of free versus surface-bound iron. The balance between free radical production and antioxidant status within the lung is important in normal homeostasis. LFA antioxidant depletion could lead to transmission of oxidative stress to epithelial cells causing increased epithelial permeability (Li et al., 1998) and increased pro-inflammatory cytokine production (Rahman and MacNee, 1998). The present study aimed to examine the antioxidant levels in lung lining fluid and lung epithelial cells after treatment with three known pathogenic and three known non-pathogenic fibres.

We initially confirmed that we could deplete antioxidant levels in LLF and pure solutions of GSH and ascorbate with an oxidant generating system. There was a clear dose effect of oxidant in depleting GSH and ascorbate in both LLF and pure solutions. Incubation of LLF with all the different fibres used in this study resulted in a decrease in GSH content. However, the most significant effect was observed with the two glass fibres, MMVF10 and Code100/475, both designated as being non-pathogenic in animal studies (Davis et al., 1996; Hesterberg et al., 1993). The effects of these materials were fibre number dependent, but all fibres with the exception of RCF1 caused a significant depletion of GSH. These experiments clearly showed that the depletion of GSH in LLF is fibre number dependent. The two glass fibres also significantly depleted the ascorbate levels in LLF.

When the fibre panel was used in experiments where LLF was replaced with pure solutions of either GSH or ascorbate, similar results were obtained. Both of the glass type fibres, MMVF10 and Code 100/475, produced the most significant ($P < 0.05$) decrease in GSH compared with control levels. There was a less striking effect when ascorbate levels were estimated in a pure solution after fibre treatment, where all fibres depleted ascorbate, but the largest effect was with Code 100/475 and was statistically significant. The MMVF10 fibre did deplete ascorbate, but there was no obvious pattern with any of the other fibres. We conclude that there are complex interactions between MMVF10 and the multiple components of the lung lining fluid and that this contributes to high levels of oxidative stress in this milieu and that these are not present in pure ascorbate solution; the former warrants further research.

We have previously reported (Gilmour et al., 1997) a significant decrease in intracellular GSH with MMVF10 treatment of alveolar macrophages. An interesting finding of this study was that MMVF10 had the ability to deplete GSH and ascorbate in solution, since we were unable to detect any hydroxyl radical-generating activity at the surface of this fibre type (Gilmour et al., 1995; Brown et al., 1998a). LFA showed the opposite effect, that is no depletion of antioxidants, but strong free radical activity in both a plasmid assay and a salicylic acid hydroxylate assay (Brown et al., 1998a). Other factors are clearly important for fibres in terms of the expression of free radical activity in different assays and in different milieus. The involvement of transition metals co-ordinated at the fibres surface may be more important than metal released into the surrounding medium (Gilmour et al., 1997; Brown et al., 1998a; Fisher et al., 1998).

Maples and Johnson (1992) also assessed the ability of a range of fibres to cause mesothelioma after intrapleural instillation. A correlation was demonstrated between mesothelioma formation and the ability of fibres to generate hydroxyl radicals in the case of naturally occurring fibres. However, synthetic glass fibres produced high levels of hydroxyl radical but negligible numbers of mesotheliomas and there was no significant correlation; this is consistent with our findings. We have also reported that MMVF10 can release large amounts of iron (Gilmour et al., 1997) but is not pathogenic.

Oxidative stress in lung cells could arise as an indirect effect of depletion of the LLF antioxidants or could be due to direct effects of fibre interactions with cells. The present study has shown that antioxidant depletion in LLF is not a reliable discriminator of fibre pathogenicity based on the six fibre types used here. Clearly, the production of oxidative stress in the lung is a complex process and the simple in vitro tests used here are not predictive of biological consequences that are clearly multifactorial. Indeed the depletion of antioxidants by non-carcinogenic fibres could occur in vivo but these fibres may lack a key feature without which there are no pro-carcinogenic events; by contrast important other effects of the carcinogenic fibres could
lead to genotoxic events. The likely most important result of oxidative stress is the activation of oxidative stress-responsive transcription factors leading to gene expression for cytokines and other key molecules (Rahman and MacNee, 1998). From the data in the present paper, the former seems unlikely. In support of the latter contention, we have shown substantial nuclear translocation of an oxidant stress-responsive transcription factor, NF-kB, in epithelial cells treated with the pathogenic fibres used here and much less of this activity in cells treated with the non-pathogenic fibres (Brown et al., 1999). In vitro tests predicting pathogenicity should continue to benefit from further understanding of the mechanisms of fibre pathogenicity and the involvement of oxidative stress remains a fertile ground for development of further tests.

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