Injurious effects of wool and grain dusts on alveolar epithelial cells and macrophages in vitro

David M Brown, Kenneth Donaldson

Abstract

Epidemiological studies of workers in wool textile mills have shown a direct relation between the concentration of wool dust in the air and respiratory symptoms. Injurious effects of wool dust on the bronchial epithelium could be important in causing inflammation and irritation. A pulmonary epithelial cell line in vitro was therefore used to study the toxic effects of wool dust. Cells of the A549 epithelial cell line were labelled with ^{51}Cr and treated with whole wool dusts and extracts of wool, after which injury was assessed. Also, the effects of grain dust, which also causes a form of airway obstruction, were studied. The epithelial injury was assessed by measuring ^{51}Cr release from cells as an indication of lysis, and by monitoring cells which had detached from the substratum. No significant injury to A549 cells was caused by culture with any of the dusts collected from the air but surface "ledge" dust caused significant lysis at some doses. Quartz, used as a toxic control dust, caused significant lysis at the highest concentration of 100 $\mu\text{g}/\text{well}$. To determine whether any injurious material was soluble the dusts were incubated in saline and extracts collected. No extracts caused significant injury to epithelial cells. A similar lack of toxicity was found when ^{51}Cr labelled control alveolar macrophages were targets for injury. Significant release of radiolabel was evident when macrophages were exposed to quartz at concentrations of 10 and 20 $\mu\text{g}/\text{well}$, there being no significant injury with either wool or grain dusts. These data suggest that neither wool nor grain dust produce direct injury to epithelial cells, and further studies are necessary to explain inflammation leading to respiratory symptoms in wool and grain workers.

A survey of workers in wool textile mills in the north of England has previously been carried out by the Institute of Occupational Medicine, Edinburgh.¹⁻³ The study showed a clear relation between increased airborne mass concentrations of wool dust and increased prevalence of respiratory symptoms¹; radiographic examination of the workforce showed no evidence of pulmonary fibrosis. These findings were suggestive of irritation or inflammation of the airways.

Epithelial cells are one cell type potentially likely to be injured by deposited dust,^{4,5} and we had previously shown that dust from ledges in British wool mills had the ability to cause injury to lung epithelial cells in vitro.² In the present study, inspirable fractions of wool dust were collected from the air of two wool mills and tested in the same assay. Also, as exposure to grain dust causes a form of airway irritation,⁶ dust collected by sieving grain was also used in the assays. Alveolar macrophages also encounter depositing dusts, so these cells, obtained from rats, were used in an in vitro assay to determine the injurious potential of the dusts.

Materials and methods

RATS

Male Wistar derived rats of the HAN strain from the Institute of Occupational Medicine breeding unit were used throughout.

DUST COLLECTION

Wool dusts

Dusts designated S (start) and M (middle) of the wool processing procedure were collected from two mills. A series of six Institute of Occupational Medicine static inspirable dust samplers were placed at each site in the dustiest zones. Samplers were operated for a full work shift and the dust was collected on Gelman GLA filters with a 5 μm pore size (Gelman Hawksley, Northampton). Dust was removed from filters with a soft brush. Dust from each mill was pooled into a tube, weighed, mechanically rotated for 24 hours to ensure mixing, and stored at -20°C until required. A single sample of surface "ledge" dust was obtained by sweeping ledges and surfaces in wool mill S.

Grain dusts

Samples were collected from ledges of a barn where wheat and barley were stored. Material was placed in a 200 μm mesh size sieve that had a 45 μm mesh underneath, and shaken mechanically for 30 minutes. Grain dust collected at the end of this process was therefore less than 45 μm diameter and this fraction was used in all subsequent assays.

PREPARATION OF WOOL AND GRAIN DUST EXTRACTS

Extracts of inspirable fractions of wool dust and sieved grain dust were prepared by rotating samples at 1 mg/ml in serumless Newman and Tytell (N and T) medium (Gibco, Paisley) for 24 hours at room temperature. The suspensions were centrifuged at 3000 rpm for 15 minutes to sediment particulate material and the supernatant was filtered through 0.22 μm filters. The extracts were used immediately in the epithelial injury assay, undiluted or diluted 1:1, 1:5, and 1:10 with N and T medium that had been rotated for 24 hours at room temperature.

PREPARATION OF ENDOTOXIN

Three lipopolysaccharide *E. coli* serotypes (0127:B8 (A), 011:B4 (B), and 055:B5 (C); Sigma, Poole, Dorset) were diluted to 1 mg/ml in PBS (Gibco, Paisley) and stored at -70°C in 200 μl aliquots until required. These were diluted in N and T medium to give a range of concentrations from 100 to 1 $\mu\text{g}/\text{well}$ (500 μg to 5 $\mu\text{g}/\text{ml}$), which were then tested for their ability to cause epithelial injury.

ALVEOLAR EPITHELIAL CELL LINE

A549 cells derived from a human lung carcinoma⁷ were maintained in routine culture in minimum essential medium plus 10% heat inactivated foetal calf serum (complete medium); (Gibco, Paisley). These cells retain the main morphological features of alveolar type 2 cells, having prominent lamellar bodies and the ability to secrete surface active material.

PREPARATION OF DUSTS FOR THE EPITHELIAL INJURY ASSAY

Suspensions of wool and grain dust were prepared in N and T medium to give concentrations of 5 μg , 50 μg , and 500 $\mu\text{g}/\text{ml}$. The samples were sonicated for two minutes to disperse the dust and 200 μl of each suspension were added to triplicate groups of wells containing the previously ^{51}Cr labelled A549 cells to give final concentrations of 1, 10, and 100 $\mu\text{g}/\text{well}$. The standard mineral dusts, titanium dioxide (TiO_2 ; rutile; Tioxide UK Ltd) and DQ_{12} quartz were included as controls.

EPITHELIAL INJURY ASSAY

A549 cells were removed from continuous culture with 0.1% trypsin/EDTA solution (Gibco, Paisley)

and resuspended in complete medium at a concentration of 2.5×10^5 cells/ml, containing ^{51}Cr (Amersham, Buckinghamshire) at an activity of 370 KBq/ml. Two hundred microlitres of the labelled cell suspension were plated into Linbro microtitre plate wells (Flow Labs, Hertfordshire) and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 for four hours. Monolayers were washed twice with PBS and 200 μl of the previously prepared dusts, extracts, and endotoxins were added to triplicate groups of wells at the appropriate concentration. Plates were incubated at 37°C in 5% CO_2 for four hours. Apart from control wells containing medium alone, a group was exposed to 0.1% Triton-X (Sigma, Poole) to produce total lysis reflecting total uptake of radiolabel. After four hours of incubation, the amount of ^{51}Cr released from the A549 cells was measured by aspirating 50 μl of supernatant from each well and measuring in a γ -counter. The result obtained was multiplied by four to give total counts released attributable to cell lysis. Cells that were injured and had become detached from the plate were measured by removing the remaining supernatant and washing the wells with $2 \times 200 \mu\text{l}$ aliquots of phosphate buffered saline (PBS). These fractions were pooled and counted by γ -counter. The 150 μl of supernatant containing counts due to cell lysis were subtracted from the total pooled counts to give counts due to detached cells alone.

PREPARATION OF DUSTS FOR ALVEOLAR MACROPHAGE INJURY

Dusts were prepared at concentrations of 5 μg , 50 μg , and 100 $\mu\text{g}/\text{ml}$ in F-10 medium (Gibco, Paisley) containing 2% bovine serum albumin (BSA) fraction V; (Sigma, Poole). Control dusts TiO_2 and DQ_{12} quartz were included.

ALVEOLAR MACROPHAGE INJURY ASSAY

Control male rats were killed by an overdose of nembutal administered intraperitoneally. Lungs were dissected from the thoracic cavity and sequentially lavaged with four 8 ml aliquots of sterile saline at 37°C . Cells (1.5×10^6) were resuspended in 100 μl PBS containing 7.5 MBq ^{51}Cr and incubated in a water bath at 37°C for 20 minutes, washed twice with PBS, and resuspended at 2.5×10^5 cells/ml in F-10 medium (Gibco, Paisley) containing 2% BSA. Two hundred microlitres of cell suspension were added to Linbro microtitre plate wells and incubated at 37°C in 5% CO_2 for one hour. The medium was replaced with 200 μl of previously prepared dust suspensions in triplicate groups, giving final concentrations of 1, 10, and 20 $\mu\text{g}/\text{well}$. Plates were incubated for 24 hours at 37°C in 5% CO_2 . After centrifuging for five minutes at 1000 rpm 150 μl of supernatant was removed from each well and counted in a γ -counter.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Experiments were carried out three times on separate occasions. All assays were performed in microtitre plates and had three replicate wells for each condition plus controls. Data for all experiments were analysed using two way analysis of variance in the Minitab statistical package from which means and standard deviations were obtained. Differences between means were statistically evaluated using Student's *t* test.

Results

INJURIOUS EFFECT OF WOOL DUST ON A549 CELLS

Two types of wool dust samples were used in this study: (1) wool dust samples collected in an "inspirable" dust sampler from wool mills S and M, and (2) a single "ledge" dust sample collected by sweeping surfaces in wool mill S. The only significant lytic effect was shown by the ledge dust and by the positive

control dust, quartz (figs 1 and 2). Neither ledge dust nor the inspirable samples caused detachment injury at any dose. Titanium dioxide dust was neither lytic nor able to cause detachment at the doses used.

EFFECT OF AQUEOUS WOOL DUST EXTRACTS ON A549 CELLS

Figure 3 summarises the injury to A549 cells after treatment with S and M wool dust extracts. Neither dust extract caused significant lytic effects either diluted or undiluted. As in the experiments with whole wool dusts, no detachment injury was produced as a result of treatment with wool extracts.

EFFECT AND COMPARISON OF ENDOTOXINS ON A549 CELLS

Figure 4 illustrates the lytic and detachment injury to A549 cells by various types of endotoxin prepared at a range of concentrations. No dose response to any of the endotoxins, and no significant difference between medium alone and endotoxin at any concentration ($p > 0.05$) were found except in the case of endotoxin

Figure 1 Effect of wool dust S on A549 epithelial cells with respect to A, lytic injury and B, detachment injury. Injury assessed by measuring ⁵¹Cr released from labelled cells and represented by means (SEM) of triplicate experiments (***p* < 0.01; ****p* < 0.001 compared with medium control).

Figure 2 Effect of wool dust M on A549 epithelial cells with respect to A, lytic injury and B, detachment injury. Injury assessed by measuring ⁵¹Cr released from labelled cells and represented by means (SEM) of triplicate experiments (***p* < 0.001 compared with medium control).

Figure 3 Effect of extracts of wool dusts S and M on A549 epithelial cells with respect to A, lytic injury and B, detachment injury. Extracts were used undiluted and diluted at various ratios with medium. Injury assessed by measuring ^{51}Cr released from labelled cells and represented by means (SEM) of triplicate experiments (no significant differences were found compared with medium control).

type C, which showed significant lysis at the highest dose of 10 $\mu\text{g}/\text{ml}$ ($p < 0.05$). Detachment of A549 cells increased above background after treatment with all three endotoxins, although this was not statistically significant.

EFFECT OF GRAIN DUST ON A549 CELLS

Figure 5 shows the effect of grain dust treatment on A549 cells. In keeping with previous experiments, significant lysis only occurred with the quartz control at the highest dose of 100 $\mu\text{g}/\text{well}$ ($p < 0.002$). Grain dust and TiO_2 produced no significant lysis at any concentration. Similarly, detachment injury was not present and was slightly reduced compared with the background control.

EFFECT OF GRAIN DUST EXTRACT ON A549 CELLS

Figure 6 shows that grain dust extract has the ability

to cause lysis in labelled A549 cells although a dose dependent effect was not clearly indicated when extract was diluted at various ratios with control medium. The data suggest that diluting the extract to 1:1, 1:5, and 1:10 with medium actually increased the amount of lysis compared with medium alone but these effects were not statistically significant. Undiluted extract caused reduced numbers of cells to detach from the wells compared with the medium control. Dilution of the extract with medium produced increasing detachment of cells at lower concentrations of extract. This effect appeared to be dose dependent, although the differences were not statistically significant at any concentration. A similar effect was noted for detachment by wool S (fig 3).

CYTOTOXIC EFFECTS OF WOOL DUST ON ALVEOLAR MACROPHAGES

Figure 7 summarises the cytotoxic effects of both wool dusts on control alveolar macrophages. No statistically significant cytotoxicity to alveolar

Figure 4 Effect of endotoxins A, B, and C on A549 epithelial cells with respect to A, lytic injury and B, detachment injury. Injury assessed by measuring ^{51}Cr released from labelled cells and represented by means (SEM) of triplicate experiments (* $p < 0.05$ compared with medium control).

Figure 5 Effect of grain dust on A549 epithelial cells with respect to A, lytic injury and B, detachment injury. Injury assessed by measuring ⁵¹Cr released from labelled cells and represented by means (SEM) of triplicate experiments (***p < 0.002 compared with medium control).

macrophages was noted for any of the dusts compared with the medium control. Quartz, at 10 µg dust/well, produced significant cytotoxicity (p < 0.001) at the two higher doses.

CYTOTOXIC EFFECTS OF GRAIN DUST ON ALVEOLAR MACROPHAGES

Figure 8 shows the cytotoxic effect of grain dust on alveolar macrophages. As for the previous experiment with wool dust, grain dust had no cytotoxic effect on control alveolar macrophages.

Discussion

We have previously reported that the frequency of respiratory symptoms in wool textile mills is associated with exposure to dust.¹⁻³ In the absence of evidence of emphysema in the lungs of wool workers, the symptoms of chronic bronchitis, breathlessness, and wheeze may result from direct injury to epithelial cells of the lung in concert with the generation of an inflammatory response. A similar result is seen in

Figure 6 Effect of grain dust extracts on A549 epithelial cells with respect to A, lytic injury and B, detachment injury. Extracts were used undiluted and diluted at various ratios with medium. Injury assessed by measuring ⁵¹Cr released from labelled cells and represented by means (SEM) of triplicate experiments (no significant differences were found compared with medium control).

grain handlers where acute inflammatory reactions can develop, leading to chronic bronchitis with or without airways obstruction, after prolonged exposure to the dust.⁸ The A549 alveolar epithelial cell line has provided a model for the in vitro study of lung injury by various agents and has been used here in an attempt to examine some of the mechanisms by which wool and grain dusts may cause lung injury and account for symptoms present in some members of the workforce in wool textile mills and in grain workers.

Airborne wool dust has been shown to generate an acute inflammatory response when instilled intratracheally into the lungs of rats.⁹ Neutrophils peaked one day after instillation and thereafter decreased to background levels by three days. By contrast, aggregates of mononuclear cells present in the bronchoalveolar lavage reached a maximum by seven days, a timescale indicative of an immune response. Also, ledge dust collected from surfaces in the wool

Figure 7 Cytotoxic effect of wool dusts S and M on ^{51}Cr labelled control alveolar macrophages. Injury assessed by monitoring ^{51}Cr released into the medium. Data represent means (SEM) of triplicate experiments (*** $p < 0.001$ compared with medium control).

Figure 8 Cytotoxic effect of grain dust on ^{51}Cr labelled control alveolar macrophages. Injury assessed by monitoring ^{51}Cr released into the medium. Data represent means (SEM) of triplicate experiments (*** $p < 0.001$ compared with medium control).

mills was found to be cytotoxic to epithelial cells *in vitro*.⁹ The present study was aimed at determining whether the inflammation caused by deposition of wool dust or grain dust could be caused by toxic effects to cells in the airspaces of the lung.

The data suggest that no appreciable injury is caused to alveolar epithelial cells of the lung, as

measured by direct lysis and detachment of cells from the surface of microtitre plates, caused by whole wool or grain dusts. A similar lack of toxicity was seen when extracts of dusts and pure endotoxins were assayed. The results do not, however, exclude a low level of injury and it is important to appreciate that the assay used will not detect subtle types of injury.

It has been proposed that recurrent damage to the bronchoepithelium by exposure to grain dust can increase the sensitivity of irritant receptors,¹⁰ and result in increased cough and production of mucus due to goblet cell hyperplasia. Bacterial endotoxins contained in the dust are thought to play a part in epithelial injury but this has not been generally shown in our assay. We must, therefore, look for causes other than simple direct epithelial injury to explain the inflammation leading to airway obstruction in wool and grain workers.

Wool and grain dust can cause recruitment of inflammatory cells into the lungs after intratracheal instillation.² These activated cells may have the ability to cause tissue injury and degradation of elements of the alveolar septum, by release of proteolytic enzymes.^{11,12} Proteolysis results in production of cell fragments and degradation products both of which have been shown to be chemotactic for inflammatory cells. Leucocytes recruited in response to these generated chemotaxins may further increase epithelial permeability by releasing inflammatory mediators such as metabolites of arachidonic acid, producing oedematous changes in the airways. Chemotaxins such as leukotriene B₄ produced by activated alveolar macrophages and epithelial cells and complement components produced by A549 cells have been found after treatment with various agents.^{13,14} In our assay system, a large scale direct injury to epithelial cells and alveolar macrophages was not shown. The activation of alveolar macrophages by phagocytosis of wool fibres or stimulation of epithelial cells by leached products may, however, be suitable triggers for the release of inflammatory mediators such as tumour necrosis factor or interleukin-1. This may be a sufficient stimulus to recruit inflammatory cells into the lung and produce the symptoms of airways obstruction seen in the workforce; such a possibility is currently being investigated.

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