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Pulmonary and Intraperitoneal Inflammation Induced by Cellulose Fibres

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The inflammatory effects of respirable cellulose fibres were studied in two short-term animal models: intraperitoneal injection in mice, and inhalation in rats.

The mouse peritoneal cavity is particularly sensitive to fibrous compared to non-fibrous particles. Both cellulose fibres and the positive control fibre, crocidolite asbestos, were administered in doses ranging from 10^4 to 10^8 fibres and caused marked, dose-dependent recruitment of inflammatory cells to the mouse peritoneal cavity, which was highest 1 day following injection. Crocidolite was much more active than cellulose, despite the mass dose of cellulose being 66 times greater for an equivalent number of fibres. Crocidolite at the higher doses caused inflammation to persist through 7 days.

For the inhalation study, rats were exposed daily, 5 days per week, to aerosols of cellulose dust for ca. 3 weeks at a concentration of 1000 fibres ml^{-1}. Inhalation exposure induced an early inflammatory response in rat lungs, as determined by bronchoalveolar lavage, which peaked at 1 day following the start of inhalation and thereafter declined, despite a further 13 days of exposure over a period of 18 calendar days. *In vitro* production of the pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α) by lavaged alveolar macrophages was markedly depressed by the end of the exposure period in cellulose-exposed animals, compared to sham-exposed controls, and this effect was still present in rats that had been allowed to recover for 28 days beyond the end of exposure.

We conclude that the cellulose material studied is less inflammogenic than crocidolite and that the extent of the inflammatory response within the lung appears to reduce with continued exposure over a 14-day period. Copyright © 2000 John Wiley & Sons, Ltd.

INTRODUCTION

There has been considerable research into the pulmonary toxic properties of mineral and other inorganic fibres. It is well established that their toxic effects are related to their fibrous morphology and that durability is also an important determinant of hazard.[1–3] Very little is known about the possible toxicity associated with exposure to respirable organic fibres, such as the natural polymer cellulose.[4]

The structural polysaccharide cellulose makes up approximately half of the cell wall material of wood and other plant material. Cellulose fibres find uses in many man-made products, such as cotton textiles, paper and cardboard, as a substitute for asbestos to give structural strength to cement products, and as insulation for buildings. The manufacture and use of these products can produce airborne cellulose fibres.

Most of the cellulose fibre destined for manufactured products is derived from wood. Wood dust, particularly from hardwoods, is a recognized carcinogen causing nasal cancer among hardwood furniture workers in Europe,[5–7] but with less strong evidence in North America.[7,8] The identity of the carcinogen(s) in wood dust has not been identified. In addition to polymeric components such as cellulose and lignin, wood also contains many relatively lower molecular weight organic compounds with demonstrated biological activity, such as terpenes, phenols, quinones and stilbenes.[7] For example, a methanol extract of beech wood has been demonstrated to be carcinogenic to mouse skin.[9] Some diterpene resin acids (mainly from softwoods), such as abietic acid, and a lignan (plicatic acid) from Western Red cedar, cause asthma,[10,11] possibly through (haptenic) immune responses to conjugates of the acids with human proteins.[11,12] The more volatile monoterpenes include compounds that are also sensitizing agents,[13,14] and compounds that can both promote[15] and prevent carcinogenesis.[16]

A number of studies have examined the health effects associated with working in the paper and pulp industries. Total dust levels in paper mills, especially in those making soft paper, have been as high as 50 mg m^{-3} in the past.[17] Fibre concentrations in one study were in the range 0.2–1.6 fibres ml^{-1}.[17] An excess frequency of cancers of the respiratory tract has been found in Finnish paper and pulp mill workers that could not be explained by smoking habits.[18] This and other epidemiological studies in the paper and pulp industry have also shown increased incidences of tumours in other organs, such as stomach, colon, bladder and lymphatic and haematopoietic systems.[19]

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Some studies have reported increased respiratory symptoms and lowered respiratory function in paper mill workers,[20,21] whereas other studies found no such changes.[22,23] The agents responsible for the epidemiological findings have not been identified, but workers in these industries can be exposed to a variety of chemicals, such as bleaches, sulphites, sulphur dioxide and chlorinated hydrocarbons, in addition to cellulose-containing dusts.[19]

Cotton dust also contains a small and variable fraction of respirable cellulose fibres and particles of cellulose, and is associated with the human disease byssinosis. Studies with cotton dust have focused on non-cellulose components of the dust, such as tannins derived from the bracts of the cotton plant, and endotoxin and proteases derived from the microbial flora on the plants and cotton fibres. Indeed, cellulose dust has been regarded as a material of low toxicity, and in the UK, the maximum exposure limit for total inhalable cellulose dust is higher than those for wood dust and cotton dust (10, 5 and 2.5 mg m^{-3}, respectively). Aerosolized cellulose was used as an 'inert' control for cotton dust in an inhalation study in guinea pigs,[24] which showed that, compared to cotton dust, cellulose had little acute ventilatory effect; however, the study provided no longer term or morphological data. In a review of animal ingestion studies, Anderson *et al*.[25] concluded that, when ingested, purified cellulose had no adverse effects with respect to spontaneous disease and neoplasia, nor to growth, reproduction and tumour promotion.

The potential hazard associated with cellulose inhalation was highlighted by Davis,[4] who noted the absence of rigorous toxicity testing. Studies on the pulmonary toxicity of fibres that have been processed from wood are limited in number. Several studies have shown pathological changes (such as granuloma, alveolitis, epithelial hyperplasia and fibrosis) in the lung following instillation or inhalation of cellulose fibres.[26–31] Cellulose fibres have been shown to be toxic *in vitro* to mouse macrophages, and caused them to release greater amounts of inflammatory mediators than asbestos, glass fibre or rock wool.[32] Overall, there is good reason to seek further information about the potential pathogenic effects of cellulose fibre, and we have included it in a series of investigations that we have been applying to fibres in the Colt Fibre Research Programme.[33–38]

This paper describes the results of some-short term experiments in rats and mice with a high-purity fibrous cellulose. The aim of these experiments was to investigate the inflammatory effects of inhaled cellulose fibres in the rat lung and of injected cellulose fibres in the mouse peritoneal cavity. The protocol for the inhalation experiment followed that used in previous studies with man-made mineral fibres and amosite asbestos, and involved exposure for 7 h day^{-1}, 5 days per week, for periods ranging from 1 day to nearly 3 weeks.[34,35,39] The mouse peritoneal assay has been used by us to study the inflammatory effects of a wide range of fibrous dusts.[40–43] Respirable crocidolite asbestos was used as a positive control in this assay.

EXPERIMENTAL

Animals

Specific-pathogen-free, outbred, male Wistar rats, about 12 weeks old at entry to the study, were used in the inhalation experiments. The intraperitoneal inflammation assay was carried out in specific-pathogen-free 8–10-week-old male C57Bl/6 mice. All animals were obtained from Charles River UK Ltd. Throughout the study, all animals were supplied with Edinburgh community tapwater and pelleted standard maintenance diet *ad libitum*.

Materials

The cellulose fibre was a thermally processed wood pulp supplied by Laxa Bruks AB of Sweden (Inorphil Thermocell mechanically processed woodpulp), normally used as a bitumen stabilizer in road construction. It was supplied as a high-purity cellulose material containing no anti-rot or fire-retardant impregnating substances and $<0.1\%$ of a surfactant (principally dialkyl dimethyl ammonium chloride).

Generation of cellulose fibre aerosol and collection of a respirable fraction

Cellulose dust clouds were generated from Inorphil Thermocell Mechanical Wood Pulp using a Jet-O-Mizer mill (Fluid Energy Aljet, Plumsteadville, PA, USA) according to the manufacturer's instructions. Briefly, the wood pulp was passed from a hopper via a screw feed into the mill, where high-velocity jets of air broke up the material. The cellulose dust was then passed in a stream of compressed air into the exposure chamber via a 1-l cyclone.

For the intraperitoneal injection experiments, a respirable fraction of the generated aerosol was collected on filters using an elutriator attached to a high-volume pump (200 l min^{-1}). This dust from the filters was pooled and mixed before use.

Characterization of respirable fibre

Fibre size distribution and number to mass ratio The respirable cellulose fibres were counted and sized by scanning electron microscopy (SEM) and the data used to calculate the fibre number to mass ratio for use in determining dose in the intraperitoneal injection experiments. A total of six SEM samples were prepared from aqueous suspensions of three precisely weighed samples of the respirable fibre. The length and diameter of fibres was measured at a magnification of ×10K following a modified version of the WHO method.[44] For each sample at least 100 fibres longer than 0.4 μm and 15 fibres longer than 20 μm were sized, thus giving a total (over six samples) of at least 600 fibres longer than 0.4 μm and 90 fibres longer than 20 μm. The same procedure was used to count and size the positive control fibre, crocidolite asbestos. In addition, the number of non-fibrous particles was recorded.

Cellulose purity It was expected that the wood pulp, being a natural product, would be likely to be contaminated by bacteria or fungi and could contain substances such as endotoxin. The respirable cellulose was microbiologically screened to identify the nature and extent of any bacterial or fungal contamination. The amount of endotoxin present was determined by washing samples of both the bulk cellulose and the respirable fraction in sterile, endotoxin-free water for 2 h at room temperature (2 mg dust ml^{-1}). The fluid was then recovered and endotoxin measured using a 'Coatest' kit (Chromogenix AB, Molndal, Sweden). The purity of the respirable cellulose was checked further by undertaking X-ray diffraction analysis of ashed samples to determine whether any crystalline mineral phases were present.

Intraperitoneal injection study in mice

Test fibres Respirable cellulose fibres were collected on filters from aerosols generated as described above. For comparison, a sample of respirable crocidolite asbestos was obtained from the Thermal Insulator Manufacturing Association (TIMA) fibre repository. The numbers of fibres longer than 5 μm per unit mass were determined by scanning electron microscopy. Thus, masses of 0.1767 mg and 11.587 mg were required to give 10^8 fibres of crocidolite and cellulose respectively. In terms of mass dose, the amount of cellulose injected for the 10^8 group was 66 times greater than the mass injected to give 10^8 crocidolite fibres. This was because the cellulose contained more thick fibres. In terms of volumetric dose, the amount of cellulose was approximately 150 times greater than that of crocidolite because of its lower density.

Fibre injection and peritoneal lavage Groups of six male C57Bl/6 mice of 10–12 weeks of age were injected intraperitoneally with various doses (10^8, 10^7, 10^6, 10^5 and 10^4 fibres) of respirable cellulose suspended in 0.5 ml of phosphate-buffered saline (PBS). Positive controls were provided by injection of mice with three doses (10^8, 10^6 and 10^4 fibres) of crocidolite, and negative controls by injecting 0.5 ml of PBS alone. Fibre suspensions were sonicated prior to injection, to aid dispersion.

One, 3 and 7 days after injection, groups of two mice were sacrificed using CO_2 and the peritoneal cavities lavaged with 3 × 2 ml volumes of sterile PBS containing 10 U ml^{-1} heparin. Cells in the lavage fluid were recovered by centrifugation and resuspension in RPMI-1640 medium (Sigma Chemical Co., Poole, UK). Total and differential counts were carried out on the recovered cells. The experiment was repeated three times to give a total of six mice per treatment per time point. Because of injuries sustained during sporadic fighting in the first experiment, some animals were excluded from the study. Additional animals were included in subsequent experiments to restore the total of six mice per treatment per time point.

Inhalation exposure

Rats were exposed by whole-body inhalation in chambers with ca. 1.3 m^3 internal volume, as described by Davis *et al.*[1], to cellulose fibre at a target concentration of 1000 (WHO) fibres ml^{-1} (by phase contrast optical microscopy, PCOM)[45] for 7 h day^{-1}. The airborne mass concentration of respirable dust was measured daily using a Casella MRE 113A dust sampler, and fibre concentrations were determined using PCOM for samples taken at 1-h intervals. Four dosing regimens were used: 1, 3, 8 and 14 days of actual exposure over a 3-week calendar period. The 8-day and 14-day regimen included one and two non-inhalation weekend breaks, respectively. Six rats per time point were used for the cellulose group and three rats per time point were used for control sham-exposed animals. An additional group of six cellulose-exposed rats were maintained, without further inhalation exposure, for a period of 28 days to determine whether there was persistence of inflammation.

Animals were sacrificed with a single intraperitoneal injection (70 mg kg^{-1} body weight) of sodium pentobarbitone (Nembutal; Sanofi Limited, Watford, UK), 18 h after the completion of each exposure period.

Bronchoalveolar lavage

Following sacrifice, the thoracic cavity was opened, the trachea were cannulated and the lungs were removed and sequentially lavaged with four 8-ml aliquots of saline at 37°C. Cells were recovered from lavage fluid by centrifugation, pooled from the four aliquots and resuspended in F-10 medium (Gibco, Inchinnan, UK) containing 0.2% bovine serum albumin (Sigma Chemical Co., Poole, UK). Total cell counts were made, and cytocentrifuge smears were prepared and stained with Diffquick (Merz Dade, Switzerland) to obtain differential cell counts.

Ex vivo tumour necrosis factor production by alveolar macrophages

Lavaged macrophages were cultured at 1×10^6 $well^{-1}$ in 24-well plates with and without the addition of 10 μg ml^{-1} lipopolysaccharide (LPS) (from *E. coli* 0111B4; Sigma Chemical Co., Poole, UK). After 24 h, supernatants were harvested, centrifuged free of cells and debris and stored at −70°C until required for assay. Tumour necrosis factor alpha (TNF-α) was measured in the supernatants using an ELISA kit according to the manufacturer's recommendations (DuoSet Kit: Genzyme, Cambridge, MA, USA) and is described briefly as follows. Wells of ELISA-grade microtitre plates (Nunc Maxisorp; Gibco, Inchinnan, UK) were coated overnight with capture antibody—hamster anti-mouse TNF-α—diluted to 6 μg ml^{-1}. Following thorough washing with PBS containing 0.05% Tween 20 to remove unbound reagents, wells were treated with blocking buffer (Dynex Technologies, Billingshurst, UK), which was decanted after 2 h. Test supernatants and TNF standards (in the range 1.23–900 pg ml^{-1}) were added to the wells, the plates were incubated for 1 h at 37°C and then the wells were washed thoroughly with PBS–Tween. The detection antibody—horseradish peroxidase-conjugated goat anti-mouse TNF-α—was then added at 3 μg ml^{-1}) and the plates incubated for 1 h at 37°C. The wells were washed and tetramethylbenzidine dihydrochloride (TMB) substrate ($0.1\ \mathrm{mg\ ml^{-1}}$) was added. The reaction was stopped by adding 2 N sulphuric acid and the optical density was measured on a plate-reader (MRX; Dynex Technologies, Billingshurst, UK) at 450 nm. Results were expressed as pg TNF-α 10^{-6} macrophages using the standard curve prepared with recombinant mouse TNF-α. The use of mouse TNF-α is justified because of the known strong cross-reactivity between mouse and rat TNF-α.

Histopathology

Rat lungs from additional animals in the 14-day inhalation and 28-day recovery groups were inflation-fixed with 10% formalin in PBS at a pressure of 30 cm of water and later embedded in paraffin. Peripheral sections and deep sections were cut and stained with haematoxylin and eosin (H&E) for light microscopy. Histology slides were examined by an experienced experimental animal pathologist.

Statistical analysis

All variables were analysed on the natural log scale. Zero values in the percentages of the various cell types, calculated from the differential counts, were replaced by 0.2% before total numbers of cells were calculated. The cell numbers from the bronchoalveolar lavage and the TNF data were cross-classified by treatment (cellulose versus control) and exposure regime, giving a balanced two-way design that was analysed by analysis of variance (ANOVA) with least-squares estimation of a very few missing values (one of three control rats at the 28-day recovery point).

The data from the intraperitoneal injection assay in mice had three time points crossed with three or five doses, plus a control, in three experimental runs. The late replacement of injured animals (as described in the above section on fibre injection and peritoneal lavage) made the design unbalanced, so the analysis had to be fitted as a random effects model, using the REML estimation method.

Results of all analyses yielded tables of predicted means and standard errors, which are presented graphically. All analyses were carried out in Genstat.[46]

RESULTS

Characterization of respirable cellulose

Optical microscopy suggested that each milligram of respirable cellulose contained ca. 10^6 optically visible fibres longer than 5 μm. Analysis by SEM indicated that there were 8.6×10^6 WHO fibres $\mathrm{mg^{-1}}$ (i.e. fibres longer than 5 μm). About three-quarters of the fibres sized were greater than 5 μm in length (Table 1) and half of the fibres had a branched 'hairy' appearance.

Bivariate size distributions for cellulose and crocidolite are shown in Table 1. With respect to fibre length, there was a great deal of similarity between the two materials, the main differences being the greater number of long fibres (>15 μm) in the respirable cellulose sample (24.3% compared to 11.7% for crocidolite) and fewer cellulose fibres in the 1–5 μm category (44.8% compared to 58.5%). Cellulose fibres tended to be thicker than crocidolite fibres, especially for the long fibres.

Table 1. Bivariate size distributions from scanning electron microscopy of respirable fibre samples of cellulose and crocidolite, showing the actual number of fibres counted per size category

Fibre	Diameter category (μm)	Length category (μm)						
		≤0.9	1–5	5–10	10–15	15–20	>20	All
Cellulose	≤0.1	3	4	0	0	0	0	7
	0.2	2	90	14	3	1	2	112
	0.3	1	85	25	6	3	5	125
	0.4–0.7	0	125	70	19	13	23	250
	0.8–1.5	0	11	48	14	19	47	139
	>1.5	0	0	3	9	6	52	70
	All	6	315	160	51	42	129	703
	All %	0.9	44.8	22.8	7.3	6.0	18.3	
Crocidolite	≤0.1	6	137	37	19	7	14	220
	0.2	7	318	101	36	24	30	516
	0.3	0	57	29	13	4	12	115
	0.4–0.7	0	19	16	5	7	7	54
	0.8–1.5	0	0	0	1	0	0	1
	>1.5	0	0	0	0	0	1	1
	All	13	531	183	74	42	64	907
	All %	1.4	58.5	20.2	8.2	4.6	7.1	

The cellulose samples were produced by elutriation with a sampler designed to correspond to the human respirable range. Therefore, injection tests with these samples are relevant to fibres that might deposit in human lungs. However, the differences in diameter prompt consideration of whether the comparison of the cellulose and crocidolite by inhalation in the rat might be affected by differences in aerodynamic size distribution of airborne fibres. The aerodynamic diameter is proportional to the physical diameter and the square root of density: it also increases slowly with aspect ratio (length/diameter). The density of cellulose is between 1.3 and $1.6\ \mathrm{g\ cm^{-3}}$, compared to $3.5\ \mathrm{g\ cm^{-3}}$ for crocidolite. Thus a 20-μm-long cellulose fibre of 1 μm diameter and density $1.5\ \mathrm{g\ cm^{-3}}$ would have the same aerodynamic diameter (d_{ae}) as a crocidolite fibre of 0.65 μm with the same aspect ratio, i.e. with a length of 13 μm. Fibres with the same d_{ae} have the same gravitational settlement speed. Thus, cellulose fibres with geometric diameter <1 μm are in the aerodynamic size range of crocidolite fibres, with geometric diameter <0.65 μm, which is approximately the upper end of the crocidolite diameter distribution. Cellulose fibres with physical diameter <2 μm also have values of d_{ae} below the estimated upper limit of ca. 5 μm for rat respirable.[47] Thus, the bulk of the cellulose fibres were within the respirable range for rats. Fibre penetration to the alveolar lung is also dependent on interception, which is affected by both length and shape. For example, Middleton *et al.*[48,49] reported lower alveolar deposition fractions for chrysotile than for amphibole asbestos.

The microbiological screening of the respirable cellulose found very low numbers (<140 mg^{-1}) of pasteurization-resistant bacteria to be present. Endotoxin was detected in the cellulose but at the low level of 0.012 ng endotoxin mg^{-1} cellulose. The mineral content of the ashed cellulose samples was 1.1%.

Inflammation in the mouse peritoneal cavity

There were differences in the numbers of the various types of cell recovered from the mice peritoneal cavities, which were related to dose, time and fibre type.

Total cells Crocidolite at the 10^8 and 10^6 doses caused a marked increase in total cell numbers in comparison to PBS (Fig. 1). Cell numbers reduced with time but remained higher at day 7 for animals exposed to crocidolite than for the PBS group. The reduction in numbers with time was greater for the 10^8 dose than for the 10^6 dose. A similar pattern of dose and time effects was seen with cellulose, except that with the 10^8 dose the levels of cell recruitment were lower for cellulose and, interestingly, there was no difference in the number of all cells between the 10^7 and 10^8 cellulose doses at any of the time points (Fig. 1). For the 10^4 dose there was little difference from the PBS control for both crocidolite and cellulose.

Granulocytes Numbers of granulocytes (predominantly neutrophils, with some eosinophils and basophils) showed significant dose and time differences (Fig. 2). Crocidolite at the 10^8 fibre dose was significantly more active than cellulose at 10^8 fibres at all time points. In contrast to the total cell data there were more granulocytes recovered from animals injected with 10^8 cellulose fibres than those injected with 10^7 fibres at each time point. Analysis of the percentages of granulocytes demonstrated a similar pattern to that of total granulocyte numbers, except that between 10^7 and 10^8 cellulose fibres the percentage increase in granulocytes appeared larger because of the constant level of total cells. Because of the similarity in trend, the percentage data have not been shown. The inflammatory response to cellulose, for all doses, had resolved by day 7 whereas for crocidolite at 10^6 and 10^8 granulocyte numbers were still raised compared to controls. The lowest (10^4) doses of crocidolite and cellulose produced no more granulocyte recruitment than PBS.

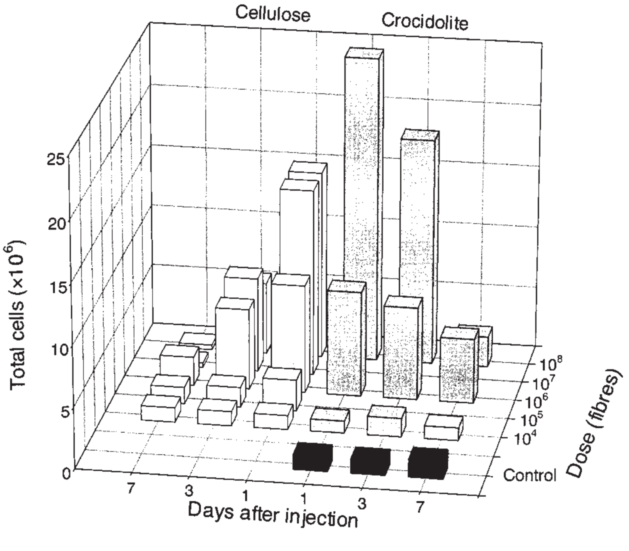

Figure 1. Mean total numbers of cells recovered by peritoneal lavage 1, 3 or 7 days following intraperitoneal injection in mice of 10^8, 10^7, 10^6, 10^5 or 10^4 respirable cellulose fibres, 10^8, 10^6 or 10^4 respirable crocidolite fibres or saline.

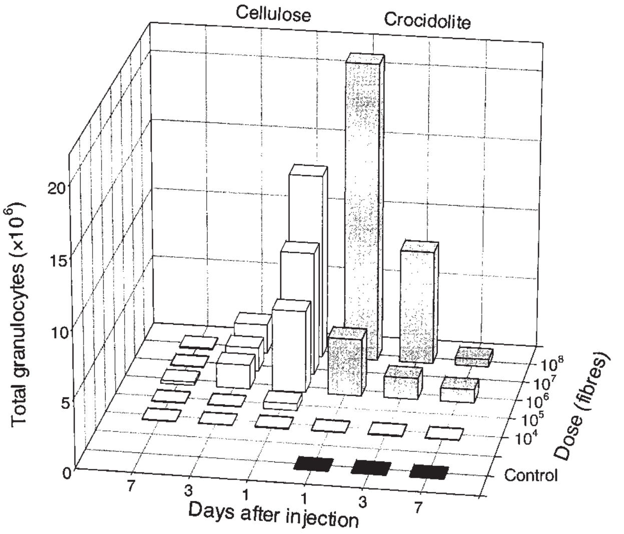

Figure 2. Mean numbers of granulocytes recovered by peritoneal lavage 1, 3 or 7 days following intraperitoneal injection in mice of 10^8, 10^7, 10^6, 10^5 or 10^4 respirable cellulose fibres, 10^8, 10^6 or 10^4 respirable crocidolite fibres or saline.

Inhalation experiment

Dust cloud characteristics The mean respirable cellulose fibre concentration (across the 14 days of inhalation exposure) by PCOM was 1004 fibres ml^{-1}, with a standard error of 13.6. The daily mean concentration of respirable cellulose dust as mass was 73 mg m^{-3}, with a standard error of 1.8.

Cellular recruitment to the lung Exposure to cellulose fibre did produce an inflammatory reaction, as shown by the significantly raised numbers of neutrophils and other granulocytes in lavage. The mean total numbers of granulocytes (predominantly neutrophils) recovered in lavage are shown in Fig. 3. The error bars were calculated at one standard error on the log scale, and are thus equivalent to multiplying or dividing the geometric mean by the geometric standard error. Note that lavage of normal (control) rats provides very few granulocytes, typically <1%, and the levels in the treated animals are very much greater than this background level. Both the percentage and the number of neutrophils were greatest at the first time point (1 day of exposure) and thereafter declined.

Lymphocyte numbers from cellulose-treated animals were raised in lavage, peaking at the 3-day time point (Fig. 4).

Tumour necrosis factor alpha

Changes in the *in vitro* production of TNF-α by lavaged alveolar macrophages at various periods from

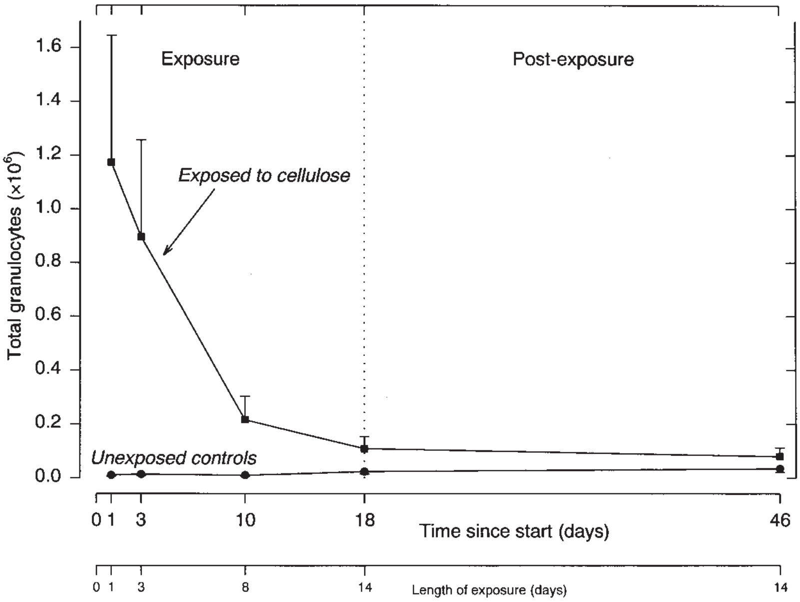

Figure 3. Mean numbers of granulocytes recovered by bronchoalveolar lavage 18 h following various periods of inhalation exposure of rats to cellulose fibre aerosols (1000 fibres ml^{-1}). Vertical bars represent the standard error of the mean. The horizontal axes show the elapsed time from the start of the inhalation and the number of days of actual exposure. Exposure was for 5 days per week.

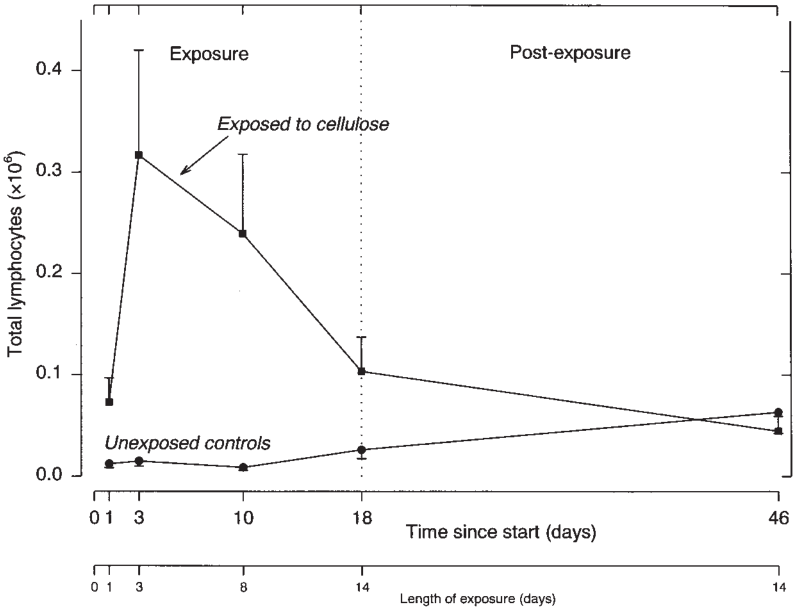

Figure 4. Mean numbers of lymphocytes recovered by bronchoalveolar lavage 18 h following various periods of inhalation exposure of rats to cellulose fibre aerosols (1000 fibres ml^{-1}). Vertical bars represent the standard error of the mean. The horizontal axes show the elapsed time from the start of the inhalation and the number of days of actual exposure. Exposure was for 5 days per week.

the start of inhalation are shown in Fig. 5a and the effect of adding LPS to the cultures is shown in Fig. 5b. In the absence of LPS there was no difference in TNF-α levels between the cellulose and control groups through the first three time points, but after 14 days of inhalation there was a reduction in the cellulose group that became even more marked at the 28-day recovery point. The addition of LPS to the macrophages enhanced production of TNF-α for both control and cellulose macrophages. The effect was most marked for the cellulose group at the first three time points, and thereafter there was a decline in TNF-α for the cellulose macrophages.

Histopathology

Additional groups of six rats were sacrificed for histopathology at the 14-day inhalation point and the 28-day recovery point. After 14 days of inhalation, lesions were present at the bifurcations of the terminal and respiratory bronchioles in the lungs of all six rats examined. At these sites and in adjacent alveoli there were congregations of macrophages, and epithelial cells had become rounded. Some small solid lesions were present, formed by the interstitialization of macrophages and other inflammatory cells, mainly fibroblasts. The largest interstitial cellular aggregates had the

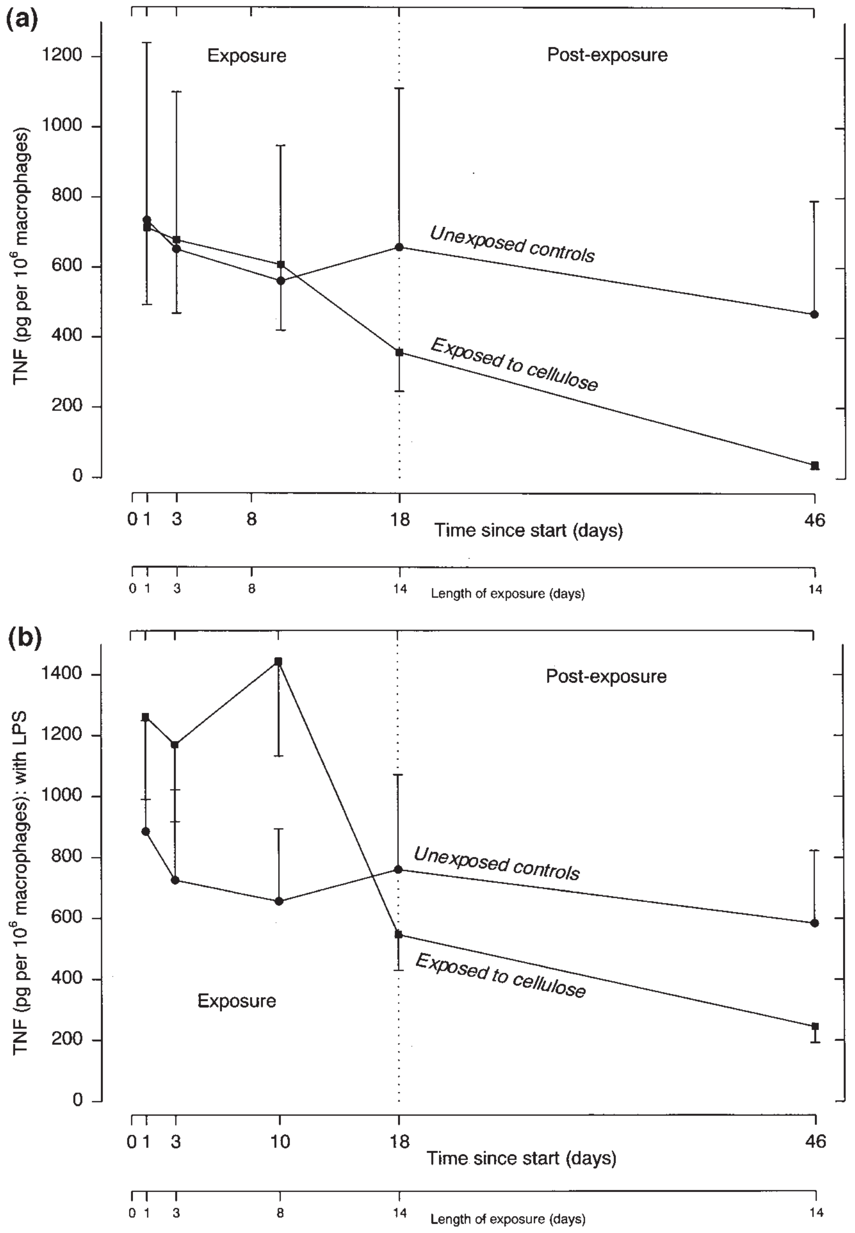

Figure 5. *In vitro* production of tumour necrosis factor alpha (TNF-α) by alveolar macrophages recovered by bronchoalveolar lavage of rat lungs exposed to aerosols of cellulose fibre (1000 fibres ml^{-1}): (a) mean TNF-α concentration per 10^6 macrophages cultured in the absence of lipopolysaccharide (LPS); (b) mean TNF-α concentration per 10^6 macrophages cultured with LPS present. Vertical bars represent the standard error of the mean. The horizontal axes show the elapsed time from the start of the inhalation and the number of days of actual exposure. Exposure was for 5 days per week.

appearance of microgranulomas, although this term strictly should be used only where some evidence of fibrosis (reticulin) is present. The presence of cellulose fibres within macrophages could not be confirmed by optical microscopy, probably because of the low refractive index of the test material. In the hilar lymph nodes there was no cellular evidence of transport of cellulose to those nodes.

There was no evidence of progression of the lesions during the 28-day recovery period. In fact, there appeared to be less reaction at the bifurcation sites after the recovery period. This was mainly due to a reduction in the numbers of macrophages close to the airway bifurcations, but the interstitial ‘microgranulomas’ also appeared less pronounced.

DISCUSSION

Intraperitoneal inflammation assay

The mouse intraperitoneal assay allows investigation of the relative toxicity of different fibrous materials to mesothelial tissue, a target for fibre-induced neoplasia (mesothelioma). The basis of the sensitivity of the mouse i.p. test is thought to be the toxic effect of the long fibres on mesothelial cells. Mesothelial cells appear to be 10-fold more sensitive to fibres than bronchial epithelial cells and 200-fold more sensitive than fibroblasts.[50] The inflammatory response is dependent on the number of injected fibres, rather than injected mass,[41,42] and non-fibrous toxic dusts such as quartz and coalmine dust are much less active than fibrous dusts.[40] Donaldson *et al.*[41] used samples of amosite asbestos of differing mean fibre lengths, to demonstrate that the response is also dependent on fibre length. A later study of 13 fibre types injected at a dose of 8.2×10^7 fibres (by PCOM), however, indicated that further discrimination beyond fibres longer than 20 μm was not possible for those fibres.[43]

The mouse peritoneal injection experiments described here confirm the relationship between fibre number and inflammatory response. They also indicate that cellulose was less inflammogenic than crocidolite, despite a much greater dose by mass or by volume. We infer from our previous work[41,42] that the contribution of the significant mass of non-fibrous cellulose material to the observed inflammatory responses is markedly less than that of the fibrous component. For example, intraperitoneal injection of up to 2.5 mg of the non-toxic, non-fibrous dust, titanium dioxide, resulted in significantly less inflammation than equivalent mass doses of amosite asbestos.[41] In two other reports, we found that crocidolite from the TIMA fibre repository was the most active material out of a large panel of fibre types, in terms of granulocyte recruitment to the mouse peritoneal cavity.[42,43] However, optical counting (PCOM) of fibres in the Donaldson *et al.* experiment may have given an underestimate of fibre number for crocidolite because of its many fine fibres.[42,43] Such underestimation did not occur in the current study because both cellulose and crocidolite were counted by SEM.

Short-term inhalation

Comparison with trends for other fibres The development of a sustained inflammatory response following inhalation exposure is believed to be indicative of the potential of a dust to cause lung injury and disease.[51] Therefore, it is useful to compare the extent to which the pattern of inflammatory response differs among fibre types examined in a previous short-term inhalation study. One of the main features of the current results is that the initially high level of inflammation (measured in terms of granulocyte recruitment) diminished with continuing exposure to cellulose, and reduced to control levels after the 28-day post-exposure recovery period. The variation over time shows some interesting differences and similarities compared to other studies.

The inflammatory effects of short-term inhalations to a man-made organic fibre, aramid, over periods of 3, 5 and 14 days have been studied by Warheit,[52,53] who reported transient increases in neutrophil numbers in lavage fluid that returned to normal levels within 1 month of the end of exposure. No data were presented for the 1- or 2-day points in the exposure periods and consequently it is not known whether the levels of inflammation would have been higher or lower at these points. However, the persistence of inflammation between days 1 and 7 post-exposure in the first Warheit study[52] indicates that the kinetics of the inflammatory response to aramid are different to that seen with cellulose.

The pattern of initial high recruitment of granulocytes, followed by a fall, in response to inhaled cellulose is similar to that observed with the mineral fibre amosite asbestos at the same exposure concentration of 1000 fibres ml^{-1}, except that with asbestos the granulocytes started to increase again by 14 days of exposure.[35] In contrast, a glass microfibre (code 100/475) that is known to have little potential to cause disease in rats[34] produced a lower but more consistent level of inflammation over 14 days of exposure to 1000 fibres ml^{-1}. Silicon carbide whiskers, which are known to be highly carcinogenic,[34] induced a greater inflammatory response than amosite and this response continued to increase throughout the 14-day inhalation period.[35] In this mineral fibre study there was no 28-day post-exposure recovery period. It should be noted that although the exposure concentrations in terms of fibre number were similar for all of these fibre types (1000 fibres ml^{-1}), the mass concentration for cellulose was much greater than for the mineral fibres (e.g. 73 mg m^{-3} for cellulose compared to ca. 5 mg m^{-3} for amosite). These differences reflect the relatively large proportion of non-fibrous material within the cellulose sample. The relative contributions of fibrous and non-fibrous particles to inflammation within the lung are not known. However, even non-fibrous, low-toxicity dusts such as titanium dioxide will cause inflammation under conditions of overload (see below).[54,55]

It is unclear why the strong initial inflammatory response to cellulose and amosite during the early phases of the inhalation experiments did not continue throughout the exposure period. The insignificant day-to-day variations seen in exposure concentrations within the chambers are insufficient to explain the changes seen in neutrophil numbers. It is possible that after the initial ‘shock’ response to certain dusts the rat lung is able to recover, to some extent, in terms of the levels of inflammatory cells maintained in the alveolar spaces. It is also clear that in the cellulose-treated rats the release of the pro-inflammatory cytokine TNF-α from alveolar macrophages is markedly reduced at the later time points, even in the presence of LPS, which is a known stimulant of TNF production. It would be valuable to know whether inflammation would continue at a lower level in the face of further exposure for, say, 28 days or longer, and whether TNF-α production would continue to be depressed.

Influence of overload Exposure to relatively high concentrations of low-toxicity dusts produces an impairment (overload) of macrophage-based pulmonary clearance of particles.[56,57] Associated with impairment of pulmonary clearance is a recruitment of phagocytes (monocyte/macrophages and neutrophils) into the alveolar region, culminating in inflammation and an increase in the rate of dust translocation to the lymph nodes.[55,58,59] Morrow hypothesized that the observed impairment of alveolar macrophage-mediated clearance is due to dust overloading of individual macrophages, which affects their motility.[59] Overload was hypothesized to be initiated when the particle volume exceeded ca. 60 $\mu\mathrm{m}^3$ per alveolar macrophage, and was complete, with a virtual cessation of clearance, when the particle volume exceeded ca. 600 $\mu\mathrm{m}^3$ per macrophage. The lung burden level from which overload becomes apparent was estimated to be 1 mg for unit-density particles.[60] For non-unit-density particles this is scaled according to the density. Thus, for cellulose of density 1.5 the critical level of lung burden will be ca. 1.5 mg. An approximate estimate of the amount of cellulose depositing in the rat lungs serves to indicate whether the lung burden may have reached a level at which volumetric overload could be the cause of inflammation. From Table 1, $>90\%$ of the fibres have a diameter <1.5 μm and an aspect ratio <20; consequently, they have an aerodynamic diameter <4 μm. Particles with aerodynamic diameter <4 μm have an alveolar deposition fraction in the range 5–10%.[47,61] Thus, assuming an alveolar deposition fraction of ca. 5% for 90% of the cellulose fibres and a breathing rate of 170 ml air inhaled min^{-1}, the 14 days of exposure would produce a lung burden exceeding 3 mg. This approximate estimation of lung burden indicates that by the end of exposure the lung burden would be at the level where overload and the inflammation due to overload would be expected. The low level of inflammation after 14 days of exposure may, therefore, be either residual inflammation from the initial response at day 1 or the start of inflammation provoked by overload. Thus, although inflammation subsided after the initial response to cellulose, it would be expected, from other particle studies,[50,58,59] that inflammation would rise again because of overload if exposure at this level were to be continued.

Influence of cellulose fibre contaminants The small levels of contamination present in the cellulose seem unlikely to be sufficient to explain the inflammatory response seen in both the short-term inhalation experiment in rats and the intraperitoneal experiment in mice. There was no evidence of microbial infection, either from histopathology or from examination of lavage cell suspensions. The cellulose contained very little of the potent inflammogen, endotoxin (0.012 ng mg^{-1}). In a previous study we found that 1 ng of LPS, the main active component of bacterial endotoxin, instilled into rat lungs produced a small increase in neutrophil numbers (6×10^4) 3 days later.[62] It is, therefore, unlikely that the level of endotoxin in cellulose could have accounted for the levels of neutrophil infiltration seen in the inhalation experiment. Gordon and Harkema[63] exposed rats to aerosols of LPS at concentrations of 0.3, 3.1 and 52.4 μg m^{-3} for 3 h day^{-1} for 3 days (i.e. a maximum cumulative exposure at the 0.3 μg m^{-3} concentration of 2.7 μg m^{-3} h). Twenty-four hours following the final exposure, they found an inflammatory cell (neutrophil) response in the centriacinar region of the lungs with the two highest concentrations but not with the 0.3 μg m^{-3} concentration. The authors of that study[63] estimated that the dose of LPS retained in the lungs for the low dose would have been no more than 3 ng. This would be considerably more than the quantity of LPS depositing in lungs in our study, because the aerosol concentration of LPS would have been ca. 0.9 ng m^{-3} (i.e. a maximum cumulative exposure of 0.09 μg m^{-3} h).

The cellulose fibre sample was a commercial product containing a small amount (0.1%) of surfactant, mainly the quaternary ammonium surfactant dialkyl dimethyl ammonium chloride. Many cationic surfactants in solution are mucous membrane irritants at concentrations in the range 0.1–0.5%. However, as discussed above, the maximum amount of dust retained within rat lungs at the end of inhalation exposure would have been ca. 3 mg, of which only 3 μg (based upon the manufacturer's figure of 0.1% by weight) would have been surfactant. Although some effect of surfactant in the inhalation (and in the peritoneal injection) studies cannot be ruled out, the concentrations in lung or peritoneal fluids would have been one or more orders of magnitude $<0.1\%$. Consequently, any contribution of the surfactant to the inflammatory effects must have been extremely minor.

Comparison with adaptation to ozone The attenuation of the inflammatory and TNF responses is reminiscent of the 'adaptation' seen in rats and mice repeatedly exposed to ozone where the first day's exposure leads to an inflammatory response but with reducing neutrophil numbers on subsequent exposure days.[64–67] However, indicators of cell damage and histopathology were shown to continue in some of these studies.[66,67]

Pathology

Several studies have shown pathological changes (such as granuloma, alveolitis, epithelial hyperplasia and fibrosis) in the lung following instillation or inhalation of cellulose fibres. Milton *et al.*[26] found that instilled cellulose caused fibrosing granulomas and patchy thickening of alveolar septa in rats. In a series of more recent instillation studies by another laboratory, purified cellulose for chromatography caused fibrosing alveobronchiolitis with thickened alveolar septae, epithelial injury, foreign body granulation with giant cells and some aspecific sinus histiocytosis in regional lymph nodes.[28,29,31] In an inhalation study, Hadley *et al.*[27] exposed rats to three concentrations (100, 500 and 2000 mg m^{-3}) of aerosols of cellulose building insulation for 28 days and found dose-related pulmonary changes. These included diffuse macrophage infiltration of lung parenchyma, alveolitis, epithelial cell hyperplasia and some granulation tissue.

Our study has confirmed that as little as 14 days of cellulose fibre inhalation can cause lesions in the lungs of rats. These lesions were similar to those reported in other studies listed above and thus cellulose has some potential to damage lung tissue. However, similar reactions have been observed recently in studies in our laboratory with low-toxicity dusts, under overload conditions, and cannot be considered to be typical of fibres only.[70] What makes fibres hazardous is mainly their elongated shape, which makes clearance difficult and causes tissue damage due to incomplete phagocytosis. For chemically durable materials, slow clearance facilitates accumulation in the lung but even with durable fibres full testing for pathogenicity requires prolonged exposure so that dust can build up to toxic levels. The present study was not designed to examine these long-term effects but has shown that the initial rapid tissue response to cellulose is reduced following cessation of exposure. In view of the findings of Muhle *et al.*[30] that cellulose fibres are durable in lung tissue, this needs an explanation. The early response seen in the present study may have been due mainly to fibres deposited in sites from which they can be readily cleared. It would certainly be of great interest to determine if a prolonged recovery period, after 14 days of cellulose inhalation, would allow the lung to return completely to normal. It has been argued that only that portion of inhaled fibres that becomes sequestered in the interstitium is involved in disease production,[68] hence the need for prolonged exposures to permit the necessary accumulation. In these circumstances, recovery from very short exposures might be expected even if the initial tissue response has been vigorous, and our results should be taken as indicating the need for long-term studies rather than suggesting that the reaction of the lung to cellulose fibres will always be transient.

Durability

Fibre durability is believed to be a key determinant of potential hazard.[69] Cellulose has a micellar construction with a crystalline supermolecular unit of bundles of parallel chains (micelles), which is believed to account for its mechanical strength and chemical stability. Many microrganisms and some protozoa and snails can decompose cellulose but its digestion in ruminants is due to the presence of microorganisms in their specialist digestive tracts. The enzymes required for degradation of cellulose are unlikely to be present in the lung and therefore inhaled cellulose is likely to be very durable. This has been confirmed recently by experiments with cellulose fibres instilled into rat lungs.[30] The durability of cellulose in lung tissue contrasts with the relatively short biopersistence of the man-made polymer fibre, para-aramid. The key difference between the two polymers is that the para-aramid structure has some general similarities to a protein and therefore can be degraded by the action of enzymes capable of hydrolysing proteins.[71]

CONCLUSIONS

The intraperitoneal and inhalation experiments described here indicate that our cellulose sample can produce an acute but resolving inflammatory reaction, suggesting that this material is of relatively low toxicity. Other research groups have shown that, in longer term lung instillation studies using relatively high doses, cellulose fibres have the potential to cause inflammation, granuloma and fibrosis.[26,29,30] We are currently exploring the potential of cellulose to cause mesotheliomas in rats following intraperitoneal injection. Such experiments have been heavily criticized because of the unnatural route of administration of the fibre and the large masses of fibre injected relative to animal size. However, these are relatively inexpensive (compared to long-term inhalation) and allow ready comparison with published data for a wide range of fibre types[72] and are currently accepted as part of testing protocols for fibre carcinogenicity within the European Community.[73] Nevertheless, it would be desirable in the longer term to investigate the toxicity of cellulose in a chronic inhalation experiment. Such an experiment would provide an important comparison with the long-term intraperitoneal injection study and contribute to the debate on the relevance of the intraperitoneal test to fibre-induced lung pathology. Furthermore, a chronic inhalation study with a durable fibre such as cellulose, but possibly of low toxicity, would provide a valuable comparison with durable mineral fibres of proven toxicity such as asbestos and silicon carbide, and might challenge current European Community exemption rules on carcinogen labelling of fibres under the EC Directive on the classification, packaging and labelling of dangerous substances.[73]

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