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Application to the University of Edinburgh

for the award of the degree of DSc

(Two volumes)

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1) Abstract

The applicant's research career has been focused almost exclusively on the response of the lung to inhaled particles and how these responses relate to the development of pathological change in the lung. This DSc submission contains the 105 published papers that comprise the formal publication output of this research. The papers are grouped into the 7 areas that the papers cover, preceded by an overview of the research.

Section 1 Experimental pathology studies with rats, including lung dose determination studies. These studies on the pathological responses of rats to following inhalation of fibres or particles and were aimed at determining the types of pathological response and the important characteristics of fibres and particles that dictate their pathogenicity. Additionally the rat peritoneal cavity was used as a model site for studying the development of mesotheliomas in response to fibres.

Section 2 Effects of particles on cells *in vitro*. These studies were carried out in order to dissect the cellular events that lead to pathogenic change in response to particles and utilised macrophages, epithelial cells and mesothelial cells. Studies took the general form of exposing cells in culture to fibres and particles and measuring endpoints that relate to pathogenicity, e.g. pro-inflammatory mediators.

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Section 3 Studies on inflammatory leukocytes elicited by particles. Study of the characteristics of the inflammatory cells elicited by pathogenic particles are the logical consequence of the studies in Section 3. The general design of these experiments was exposure of rat and mouse lung or peritoneal cavity followed by harvest of the inflammatory cells for examination of various relevant end-points.

Section 4 The effects of particles on the immune system. It has been suggested that particles may enhance the immune response, which could promote inflammation and pathological change. The studies in this section describe the impact of particles on the immune system.

Section 5 Studies on mesothelial cells and asbestos. Because of the paucity of information on the mechanism whereby asbestos and other fibres cause the unusual pleural cancer mesothelioma, studies were carried out using various models for this disease.

Section 6 Neutrophils and oxidative stress in the lung. Recently the applicant has diversified to studying cigarette smoke and other oxidant-mediated lung injury. In addition, studies have shown that particles have free radical activity and this may have substantial impact on the understanding of the mechanism of particle-mediated lung disease.

Section 7 Reviews, development and methodology papers. This section includes review articles, book chapters and methodology papers that have been produced in the course of the main body of research.

2) Declaration

a) In fulfilment of Section 1.4.4(a) of the Regulations for Higher Degrees, the candidate declares that he previously submitted a thesis to The University of Edinburgh in fulfilment of a PhD degree:-

Donaldson K. The effects of asbestos injection on the peritoneal macrophage population of the mouse. PhD Thesis. Edinburgh: The University, 1982;

the publications marked [H] in Section 4 arose from the work carried out for this thesis.

b) In fulfilment of Section 1.4.4 (b) the list in Section 4 defines the contribution made by the candidate to each of the publications. This is indicated by a letter in square

brackets which follows the reference and denotes one of the following levels of involvement:-

A) Minor - part of a team carrying out autopsies and monitoring exposure and associated work.
B) Minor - planning of work and carrying out injection and autopsy
C) Minor/major - ideas, planning and autopsies; involved in the write-up.
D) Major - collaboration with researchers in another discipline or Institute.
E) Major -conceived by me and funding secured by me; carried out under my supervision; written by me.
F) Major - my idea executed by a technician; written up by me.
G) Major - funding secured by me; carried out under my supervision; written by my PhD student or research assistant assisted by me.
H) Major -part of PhD work; written up by me.
I) Major - funding secured by me; carried out under my supervision; written by visiting scientist with assistance from me.
J) Major- joint review or hypothesis paper written equally
K) Major - funding secured collaboratively with Doctor William MacNee; carried out under our supervision.
L) Major - Idea conceived by collaborator and carried out by me.

3) Commentary

The applicant has carried out research that has been focused almost entirely on the response of the lung to inhaled particles with a small body of research on allied topics. The commentary summarises this research output within the 7 sections; within each section, the papers are in order of publication date. The numbers refer to the papers as they appear in the list in Section 4 below .

Section 1 Experimental pathology studies

Initially the candidate was part of a team carrying out experimental pathology studies in rats exposed to fibres in a programme carried out in the Institute of Occupational Medicine, Edinburgh. These studies utilised two models. The first model involved the generation of defined clouds of fibres or particles at pre-determined airborne mass concentrations and placing the rats in the clouds for 7 hours a day, 5 days a week for up to 2 years. Rats were then followed for the remainder of their lifetime to assess development of pathology in the lung and in other sites. In the second model, rats were instilled intraperitoneally with asbestos fibres of different types and sizes and the mesothelioma response was quantified. This latter model was utilised because of the low pleural mesothelioma response found in rats inhaling asbestos fibres. Clearly the

peritoneal mesothelioma model requires careful interpretation in terms of its relation to pleural mesothelioma.

These studies provided information on the characteristics of fibres that imbued them with carcinogenicity [1,4,] and alerted the fact that man-made fibres, the important asbestos contaminant tremolite and a novel asbestos formulation could also be carcinogenic in the intra-peritoneal mesothelioma model.[5,6,7] Calcium silicate insulation material was found to be non-pathogenic.[9] An important result from these studies was the demonstration that long fibres were much more pathogenic than short fibres [8]. This pointed out an important flaw in the UICC standard samples of amphibole asebstos, i.e. they were relatively short and so were misleading as to pathogenicity when compared to workplace -derived fibres [2] . In fact, subsequent research has re-confirmed the important role that fibre length plays in fibre pathogenicity. Similarly, an early aim of these studies was to determine whether there might be an 'overload' of the lung's defences at high airborne mass concentration [3] and in this early study although we derived some evidence for an overload effect, there was no statistical significance in the increase in pathology at high exposure compared to the same overall dose spread out over time.

Dose is an important part of the toxicology equation and there were constant attempts to determine the effective dose in the lung [12] later refined to modelling studies where the relationship of dose to response was expressed mathematically [10,11] . In the latest studies we have been quantifying cellular proliferation as the earliest pathological effect in fibre-exposed lungs [13], with the aim of deriving an early marker for long-term pathological effects of inhaled particles for the purposes of testing. Early indications are that sustained proliferation following a short-term exposure may indeed be a useful early marker of potential to cause pathological change in the long term.

Section 2 Effects of particles on cells *in vitro*

Following on from his initial experiences in the pathology studies described above, the applicant became interested in the mechanism of pathological change at the cellular level. Because of the central importance of the macrophage in defence, inflammation and immunity, initial studies addressed the effect of particles on the macrophage. These *in vitro* studies have continued to form a major part of the applicant's research output.

Macrophages release two products that are capable of causing direct tissue damage, oxidants and proteases, and these have been implicated in the pathological change that results from particle exposure. Macrophages also release cytokines that are capable of promoting inflammation and proliferation and have other effects that could lead to pathological change.

Initial studies revealed the potential of macrophages to release of superoxide anion and hydrogen peroxide on exposure to particles.[14,15,17,32] In later experiments opsonisation with immunoglobulin was found to be a major modifying factor in determining the extent of release of superoxide anion by alveolar macrophages exposed to fibre. [24,25] Macrophages were also capable of releasing large amounts of pro-inflammatory fibrinolytic activity on contact with pathogenic particles.[16] Macrophages were also shown to release cytokine on contact with particles and fibres[24] and that long fibres of asbestos were very active in this respect [18,19,28], which may, in part, explain their pathogenicity. Recent work with organic dust from the air of wool-mills demonstrated that, while the particles were not very toxic,[23] endotoxin in the dust particles could cause up-regulation of adhesion molecules on the surface of macrophages, which could contribute to pathogenicity[21].

Other cell types studies have been epithelial cells and fibroblasts. Epithelial cells are considered to be central cells in maintaining tissue balance as well as contributing to inflammation via release of mediators. We have demonstrated that long asbestos fibres have the potential to cause loss of epithelial adhesion[20] as well as causing chromosomal aberrations in fibroblasts, the later being of potential importance in the carcinogenic effects of the long fibres.[22,27]

Section 3 Studies on inflammatory leukocytes elicited by particles

Whilst studies on normal macrophages, as described above, are useful in dissecting the early events in lung response to fibres it was clear from early work that the deposition of particles in tissue results in inflammation and that the pathological change typical of particle exposure, fibrosis and cancer, are likely to arise from the activities of these inflammatory cells. We therefore studied populations of leukocytes elicited by particles with regard to release of mediators in order to try and understand the process of lung pathology following deposition of particles.

Early work with asbestos demonstrated the presence of inflammation following deposition of particles in the mouse peritoneal cavity [29] and that the macrophages were not activated the cytotoxic state [29,30] but release an immunosuppressive molecule(s) possibly prostaglandin [63] as well as oxidants [32] and that these cells were slightly more susceptible to injury by further exposure to particles [31]. Subsequently peritoneal inflammatory response was used as a short term test to investigate the properties of particles that imbue them with toxicity. [35,36,40,57,58,60]

Although the mouse peritoneal cavity has yielded important information as to the characteristics of particles that cause inflammation the differences between the peritoneal cavity and the lung led us to investigate the inflammatory cell populations elicited by particles in the lung. Inhalation exposure of rats to quartz [34], coalmine dust [49] and asbestos fibres[34,61] all showed inflammatory cells in the bronchoalveolar lavage. However, titanium dioxide, a dust of low biological activity *in vitro* had very little effect, except at 'overload', when inflammation arose.[34] The inflammatory cells elicited by coalmine dust inhalation had enhanced proteolytic activity [44] which could be important in causing tissue re-modelling leading to fibrosis. Inhalation of quartz, coalmine dust and asbestos ,all dusts capable of causing pneumoconiosis, were found to result in macrophages in the BAL that showed inhibited ability to move whereas titanium dioxide had no such effect. [46,50] This suggests that loss of macrophage ability to migrate and clear particles from the lung could be an important factor in allowing dose of particles to build up to a toxic level in pneumoconiosis.

Inflammatory leukocytes from quartz-inflamed lung were found to be able to cause protease-mediated injury to epithelial cells and extra-cellular matrix components, another mechanism whereby lung injury could be mediated. [35,38,39,40,42,47,48,52,53,54]

Coating with aluminium lactate dramatically attenuated the pathogenicity of quartz, revealing the importance of the quartz surface in mediating its pathogencity.[43] Organic dust collected from the air of wool mills caused nflammation principally by activating complement and casing immunostimulation[51,62] Short-term inhalation exposure to asbestos was found to be more inflammogenic that an equal number of glass fibres[55]

Section 4 The effects of particles on the immune system

Because of the role of the macrophage in the initiation of immune response as well as inflammation, research also addressed the potential effects of pneumoconiotic particles

on aspects of the immune response. Early studies in the mouse peritoneal cavity showed that the inflammatory macrophages released immunosuppressive activity [64] and this could be related to the systemic immnosuppression seen in mice injected intra-peritoneally with asbestos.[63,65,69] Immunosuppression could be important in carcinogenesis through its impact on the anti-tumour immune defences.

In contrast to the suppression seen with asbestos, quartz instillation into the lung causes activation of alveolar macrophages, with release of immunostimulatory cytokines.[66,67,68] More recently the importance of persistent immunostimulation in the lung with a hapten confirms that this can lead to fibrosis.[70] Although a definite role for classical immune responses to inorganic particles has not been demonstrated the link that the macrophage forges between the inflammatory and immune responses makes this an important potential area of future research.

Section 5 Studies onthe pleura and asbestos

Asbestos exposure is associated with a range of pleural pathologies including pleural plaque, fibrosis, effusion and mesothelioma. Since serosal cells are particularly threatened by coagulation, fibrinolysis is important in the pleura as shown by the plasminogen activator activity demonstrated in normal pleural leukocytes.[73] An inhibitor of plasminogen activator which was secreted by the normal pleural leukocytes in small amounts, was produced in increased quantities by pleural leukocytes from asbestos-exposed lungs[74] although there was no evidence of a fibre-specific modulation of mesothelial cell fibrinolytic activity.[72] Thus the presence of fibres in the lung could be pro-inflammatory to the pleura via an effect on the pleural leukocytes in enhancing fibrin formation at the pleural surface and this could contribute to pleural pathology. In addition to these fibrinolytic effects, the instillation of asbestos also resulted in alterations in the cytokine profile of the pleural leukocytes.[75,77] but there was little evidence that, even under conditions of severe lung inflammation, there was substantial transport of particles from the bronchoalveolar space to the pleural space.[76] The activated pleural leukocytes present in the pleural space of fibre-exposed rats were found to able to cause detachment injury to mesothelial cells in culture,[78] indicating that protease-mediated injury to the mesothelial cells and loss of integrity of the mesothelial barrier could be another mechanism whereby inflammation could be enhanced in the pleura by fibres.

Section 6 Neutrophils and oxidative stress in the lung

This research was aimed at dissecting the contribution of a) direct particle and oxidant-derived factors and b) inflammatory neutrophil-derived oxidants, to oxidative stress and inflammatory damage in acute and chronic lung inflammation. Early studies examined the proteolytic activity of the neutrophils[80,83,84] but we rapidly concluded that the oxidative injury to the epithelium was central to the early response and focused on this area. The thiol antioxidant glutathione (GSH) has been a topic of interest from the point of view of its ubiquitousness and its role in the antioxidant defences of the epithelium. We have demonstrated that extracellular GSH and its analogue n-acetyl cysteine (nAC) has little effect on the production of oxidants by leukocytes [79] but that it can protect against damage to epithelial cells by oxidants such as cigarette smoke.[81,85,87] In an attempt to explore the role of neutrophils and the site of any neutrophil-mediated injury, we have developed a model of neutrophil sequestration in the rat lung,[82,88] in combination with assays of epithelial permeability and damage *in vivo* and *in vitro*.[89] These studies have highlighted the importance of GSH in maintaining epithelial integrity and importance of the cytokine TNF in causing depletion of GSH and increased epithelial permeability.[89] Recently we have focused on the direct free radical activity at the surface of fibres and demonstrated that asbestos and other man-made fibres have the ability to generate hydroxyl radical at their surface [90,91], which could be important in causing cell injury and activation. A rat ozone inhalation study funded by the Health Effects Institute, Boston, showed that ozone caused inflammation that showed adaptation with ongoing exposure.[86]

Section 7 Reviews, development and methodology papers

In the course of the research alluded to above there have been several methodological developments associated with assessing macrophage activity. [92,93,94,96,98,100] and also comparing the different methods of detecting/predicting the pathogenic effects of particles.[95,97] Most recently a hypothesis regarding the toxicity of environmental air pollution particles was derived [102] and a number of invited reviews of the field of particles and the lung have been produced. [101,103,104,105]

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VARIATIONS IN THE CARCINOGENICITY OF MINERAL FIBRES

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Abstract—An intraperitoneal injection assay system in laboratory rats was used to examine the relative carcinogenicity of five asbestiform dusts of chrysotile origin and two of amosite origin. Twenty-five milligram suspensions of dust were injected into each of 32 rats for each treatment and the animals subsequently monitored for the development of tumours. The intraperitoneal route was shown to provide a useful assay of the tumour-producing potential of particulate materials, with an increased sensitivity when compared with intrapleural inoculation methods. The results showed that the chrysotile samples tended to be more carcinogenic than the amosite preparations and some of the possible reasons for this are discussed. It was concluded, from estimations of particle size using the scanning electron microscope, that relative fibre length did not provide a useful measure of the carcinogenicity of the seven dusts, owing in part to problems arising from the preparation of realistic, representative, samples for microscopy.

INTRODUCTION

THE ASSOCIATION between asbestos exposure and primary mesothelioma of the pleura and peritoneum was first demonstrated by WAGNER *et al.* (1960) amongst crocidolite miners and millers of South Africa. Several epidemiological studies have since shown that a history of exposure to asbestos is associated with an increased risk of development of mesothelial tumours many years later (SELIKOFF *et al.*, 1970; NEWHOUSE, 1973; MCDONALD and LIDDELL, 1979). All the three main commercial types of asbestos (chrysotile, crocidolite, amosite) have now been implicated and there is some evidence that crocidolite is particularly active.

The experimental production of mesothelioma in animals following asbestos inhalation has proved to be somewhat difficult with only occasional mesotheliomas developing after prolonged exposure (REEVES *et al.*, 1974; WAGNER *et al.*, 1974; DAVIS *et al.*, 1978), although, once again, all the three main types of asbestos have been implicated. WAGNER (1962) showed that mesothelial tumours could be simply produced in appreciable numbers in rats by intrapleural inoculation with suspensions of asbestos dust. Whilst this method may be criticized for failing to provide an adequate mimic of the biological protective clearance mechanisms associated with the normal (pulmonary) portal of entry of fibre into the body, it has proved to be a useful carcinogenicity bioassay of particulate pollutants. The work has since been extended by many workers using either intrapleural injection techniques (SMITH *et al.*, 1965; WAGNER and BERRY, 1969; WAGNER *et al.*, 1970) or a direct intrapleural implantation technique (STANTON and WRENCH, 1972), to provide comparisons of the biological activity of fibrous particulates.

In our laboratory industrial mineral dusts are tested for their harmful effects and we routinely undertake both inhalation studies to examine the dusts' potential to produce

pulmonary fibrosis and tumours and injection studies to explore their potential for mesothelioma production. The present study is part of a programme designed to examine and compare the biological effects of the standard UICC asbestos samples with dusts collected from other sources, including experimental and industrial applications. We report here the results of the first of the injection assays, in which the carcinogenicity of seven different types of asbestiform material have been examined using laboratory rats. We have chosen to use the intraperitoneal injection route, since previous experience in our laboratory has indicated that this may be a more sensitive method of bioassay than intrapleural inoculation. Some comparison will be made with the results of *in vitro* tests using the same dusts (WRIGHT *et al.*, 1980). The first of the parallel inhalation studies has been reported elsewhere: UICC dust samples (DAVIS *et al.*, 1978); factory dust samples (DAVIS *et al.*, 1980).

METHODS

A total of 232 male AF/HAN random-bred spf Wistar laboratory rats was used, 8–10 weeks old at the time of injection, housed four/cage with *ad libitum* access to standard pelleted laboratory diet (BP Nutrition Ltd) and tap water. They were stratified by age and then randomly allocated into seven treatment groups of approx. 32 animals each, as shown in Table 1. An additional four animals were added to two of the chrysotile-treated groups to replace those dying from acute peritonitis in the first few days following injection. The seven dusts are described in summary form in Table 1. All the samples are described as 'elutriated' to signify that they were collected from airborne asbestos clouds generated as part of the inhalation studies (BECKETT, 1975) by using an absolute filter assembly in the ducting between the dust generator and the animal exposure chamber. This was undertaken in an attempt to provide a closer comparison of the results of inhalation and injection experiments by using dusts prepared in a similar manner. Representative samples of the various dusts were prepared on Nuclepore membrane filters (BECKETT, 1973), both from the airborne clouds and from the liquid injection suspensions, for estimations of the particle size distributions using a scanning electron microscope (Cambridge Instruments S.600) and the criteria of SCHNEIDER (1979). Thus an aspect ratio of 3:1 was used and all particles that conformed to or exceeded this were assessed. For practical purposes, constraints imposed by the working resolution of the scanning electron microscope

TABLE 1. EXPERIMENTAL LAYOUT

Dust sample (25 mg injected i/p in 2.0 ml PBS)	No. of animals injected
Elutriated UICC amosite	32
Elutriated factory amosite	32
Elutriated UICC chrysotile 'A'	36
Elutriated factory chrysotile	32
Elutriated heated chrysotile	32
Elutriated parent chrysotile	36
Elutriated wet-dispersed chrysotile	32

meant that there was a minimum fibre diameter slightly less than 0.2 μm and therefore, by inference, a minimum fibre length of approximately 0.6 μm. There were no effective maximum limits to the fibre dimensions. The data is expressed graphically as cumulative fibre length distributions from which the relative proportion of fibres exceeding certain stated lengths may be obtained.

Table 1 shows that elutriated samples of UICC amosite and chrysotile 'A' have been used, together with two 'factory' samples that were originally obtained from the exhaust filter units at asbestos factories. These factory samples were found to contain other undefined non-asbestos, largely non-fibrous, impurities. The contamination was considerable: the chrysotile sample contained only 60% chrysotile by i.r. estimation, and the amosite sample was found to contain 90% amosite. The non-asbestos component was not further characterized, but analysis did confirm that there was no cross-contamination of each sample with other asbestos types. The other asbestiform samples tested for carcinogenicity were: a chrysotile preparation heated up to 850°C; an unheated 'parent' commercial Canadian chrysotile sample, and an experimental preparation of 'wet dispersed' chrysotile produced during the development of anionic surfactant textiles (HERON and HUGGETT, 1971).

After the elutriated samples of each dust had been collected, they were dry heat sterilized at 60°C for 30 min, mixed with sterile Dulbeccos PBS (Biocult) and injected intraperitoneally into rats, each animal receiving 25 mg of dust suspended without ultrasonication in 2.0 ml PBS under light ether anaesthesia. All animals were examined daily and were killed when distressed or moribund. Representative histological tissue samples were taken from the majority of mesothelial tumours and from any other abnormalities noted at autopsy.

The results were analysed using estimates of the survival function based on the product limit (KAPLAN and MEIER, 1958) method of calculation. This is similar to the actuarial life table methods used by other workers (BERRY and WAGNER, 1969), except that it is based on individual survival times, thus providing the maximum amount of information from the relatively small experimental groups. The method permits a survival function to be calculated for each individual death, based upon the proportion of individuals within the treatment group surviving at the time of each death. The mortality experience of each treatment group was divided into two components, one related to mesothelioma and one related to other causes of death, and the survival functions calculated for each death within each group. A BMD program was used for the computations (BMDP 1L, revised November 1979, originally developed at the Health Services Computing Facility, University of California, under NIH Grant RR3).

RESULTS

A total of 11 deaths from acute peritonitis occurred amongst the experimental groups in the first 10 days following injection. Of these, five occurred with the elutriated UICC chrysotile 'A' sample and three with the elutriated 'parent' chrysotile sample. An extra four animals were added to each of these two treatment groups, making a total of 221 animals available for analysis, distributed as shown in Table 2. The number of peritoneal tumours produced in each treatment group is also shown in Table 2, and it can be seen that the dose level of 25 mg of dust caused the production of mesothelial tumours in almost all animals in six treatments out of seven.

TABLE 2. DETAILS OF THE NUMBERS OF MESOTHELIOMAS PRODUCED AMONGST THE TREATED ANIMALS

Treatment	No. of animals injected	No. of animals after 14 days*	No. of peritoneal mesotheliomas	Percentage with peritoneal mesothelioma
Elutriated UICC amosite	32	32	30	94
Elutriated factory amosite	32	31	29	94
Elutriated UICC chrysotile 'A'	36	31	30	97
Elutriated factory chrysotile	32	30	29	97
Elutriated heated chrysotile	32	32	13	41
Elutriated parent chrysotile	36	33	33	100
Elutriated wet-dispersed chrysotile	32	32	32	100

* i.e. the number of animals available for long-term follow-up.

The structure of the peritoneal mesothelioma produced in experimental animals by the injection of asbestos has been described in detail by DAVIS (1974a). The majority of the peritoneal tumours produced in the present study were of the classical advanced multinodular type with copious straw-colour or blood-stained ascites, numerous free floating nodules, and widespread nodular growth over the visceral and parietal surfaces. A small proportion (11 out of a total of 196) of the tumours were macroscopically predominantly plaque-like, with sheets of thickened cellular tumour masses covering both parietal and visceral surfaces. The variable histological appearance of peritoneal mesothelioma previously reported for both humans and animals (ENTICKNAP and SMITHER, 1964; DAVIS, 1974b) was confirmed in the present study, with both epithelial and connective tissue elements visible in the earlier stages and a more pronounced fibrosarcomatous form being more common in advanced tumours.

Table 3 records both the time taken for the first tumour to become apparent and the mean mesothelioma survival time (i.e. the mean time from injection to death for mesothelioma-bearing animals) for each treatment group. It can be seen that the first mesothelioma developed within 178 days of injection (with the wet-dispersed chrysotile preparation) and that there is a considerable range of values between the seven different treatments, with the heated chrysotile sample producing the first mesothelioma only after 621 days. Similarly there are large differences between the mean mesothelioma survival times of the seven treatments, with the wet-dispersed chrysotile treatment having the shortest time and the heated chrysotile the longest.

Figure 1 presents the information in graphical form, with the cumulative proportion surviving plotted against the time (in days) from injection. This method of presentation means that the survival curves for the most carcinogenic dusts are those closest to the ordinate. There is a clear separation between the curves for five out of the seven treatment groups, although there is little difference between the results for UICC

TABLE 3. COMPARISON OF THE TIME TAKEN FOR THE DEVELOPMENT OF THE FIRST TUMOUR AND OF THE MEAN MESOTHELIOMA SURVIVAL TIME FOR EACH TREATMENT

Treatment	Time taken for first mesothelioma (days)	Mean mesothelioma survival time ±S.E. (days)
Elutriated UICC amosite	292	505±21
Elutriated factory amosite	377	566±20
Elutriated UICC chrysotile 'A'	279	400±24
Elutriated factory chrysotile	245	373±18
Elutriated heated chrysotile	621	840±31
Elutriated parent chrysotile	307	438±13
Elutriated wet-dispersed chrysotile	178	312±24

chrysotile and parent chrysotile. This separation of the curves provides an indication that there are differences in the carcinogenic potential of the dusts, even though there is little difference between the total numbers of tumours produced by all the dust samples except heated chrysotile.

Table 4 summarizes the information by listing the various treatments in descending order of the carcinogenicity (as described by their survival functions from Fig. 1) and it can be seen that the carcinogenicity of the various dust suspensions may be ranked in a similar descending order using either of the other indices of activity—mean mesothelioma survival time or the time to onset of the first tumour. The results of the *in vitro* cell viability assays of these same dust samples reported previously in the form of a cytotoxicity index (WRIGHT *et al.*, 1980) are also included for comparison. Information

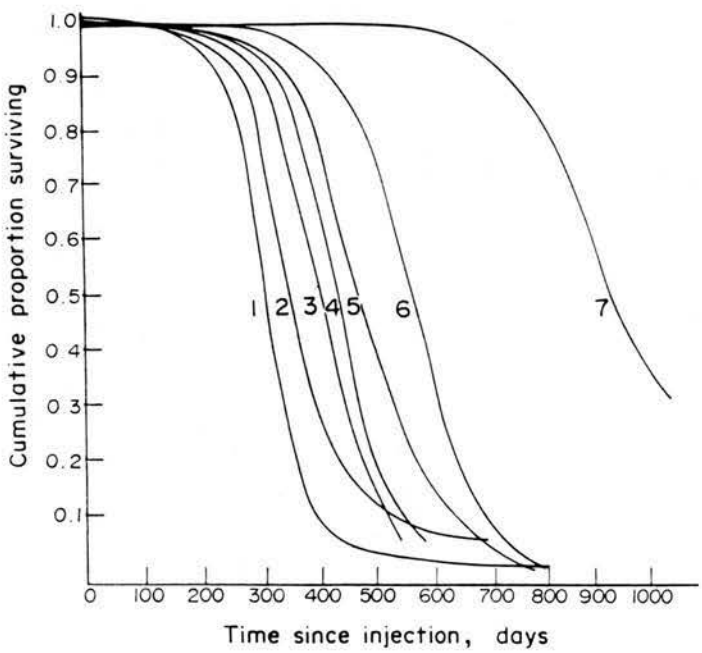


FIG. 1. Plot of cumulative survival of tumour bearing animals against time, showing the relative carcinogenicities of the seven elutriated dusts.

Key: 1 = Wet-dispersed chrysotile, 2 = Factory chrysotile, 3 = UICC chrysotile 'A', 4 = Parent chrysotile, 5 = UICC amosite, 6 = Factory amosite, 7 = Heated chrysotile.

TABLE 4. SUMMARY OF THE INDICES OF CARCINOGENICITY, WITH A COMPARISON WITH *IN VITRO* VIABILITY ASSAY

Treatment (Ranked by survival function)*	Time for first mesothelioma (days)	Mean mesothelioma survival time (days)	Percentage viability at 48 h č 10 μg/ml
Elutriated wet-dispersed chrysotile	178	312	12.9
Elutriated factory chrysotile	245	373	71.7
Elutriated UICC chrysotile	279	400	58.2
Elutriated parent chrysotile	307	438	72.5
Elutriated UICC amosite	292	505	79.3
Elutriated factory amosite	377	566	80.4
Elutriated heated chrysotile	621	840	81.1

* i.e. in order of descending carcinogenicity (taken from Fig. 1).

from only one dose (10 μg/ml) after 48 h incubation is shown, this giving the best fit with the *in vivo* data. There is broad agreement between the injection and the cytotoxicity tests, with the most carcinogenic dust (the wet-dispersed chrysotile preparation) also proving to be the most toxic *in vitro*.

The information available on the fibre dimensions is shown in Figs. 2 and 3. Figure 2 consists of relative fibre length distributions of five of the dust preparations obtained by sampling the airborne clouds at the time of collection. It subsequently became possible to measure the relative fibre size distributions of the various dust samples following a liquid preparation procedure that more closely reproduced the dispersion state of the samples at the time of injection, and the results of these estimations are presented in Fig. 3. Figures 2 and 3 present results for only five of the dust samples, since satisfactory fibre length distributions could not be produced for the other two. The procedure of heating chrysotile to 850°C resulted in considerable destruction of the fibrous chrysotile morphology and, although a small proportion of the particulate material was fibrous when examined under the SEM and a length distribution could therefore be produced, it would not be a representative assessment of the character of the sample. At the other extreme, the wet dispersed chrysotile sample produced aggregations of very long thin fibres of such tangled complexity that measurement of length distributions were not possible. A further caveat is necessary before the relative fibre length distributions are examined closely: it must be remembered that both of the factory-derived samples contained a substantial non-fibrous component (40% in the case of the factory chrysotile sample) that is not accounted for in Figs. 2 and 3.

The fibre length distributions given in Fig. 3 represent the best available data on the dust samples actually injected into the rats. Since the samples of both chrysotile and

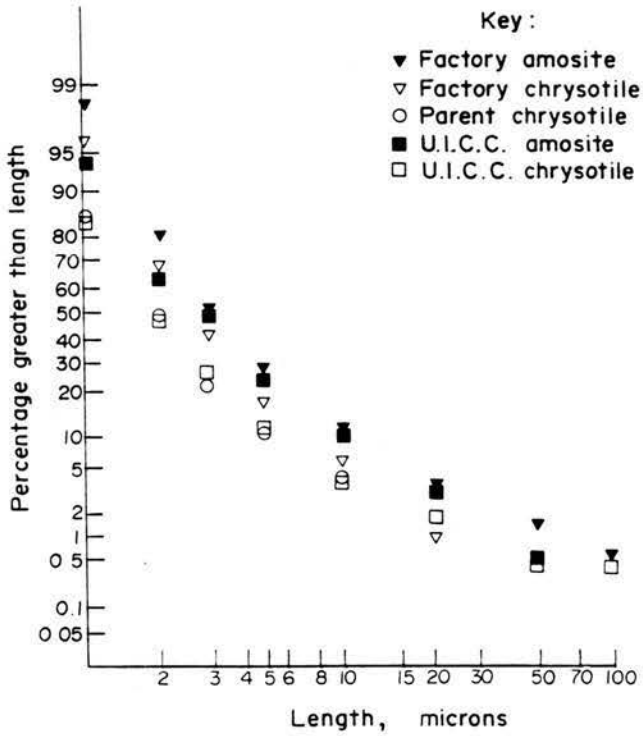


FIG. 2. Relative fibre length distributions of elutriated asbestos samples, prepared from airborne clouds, and estimated using a scanning electron microscope.

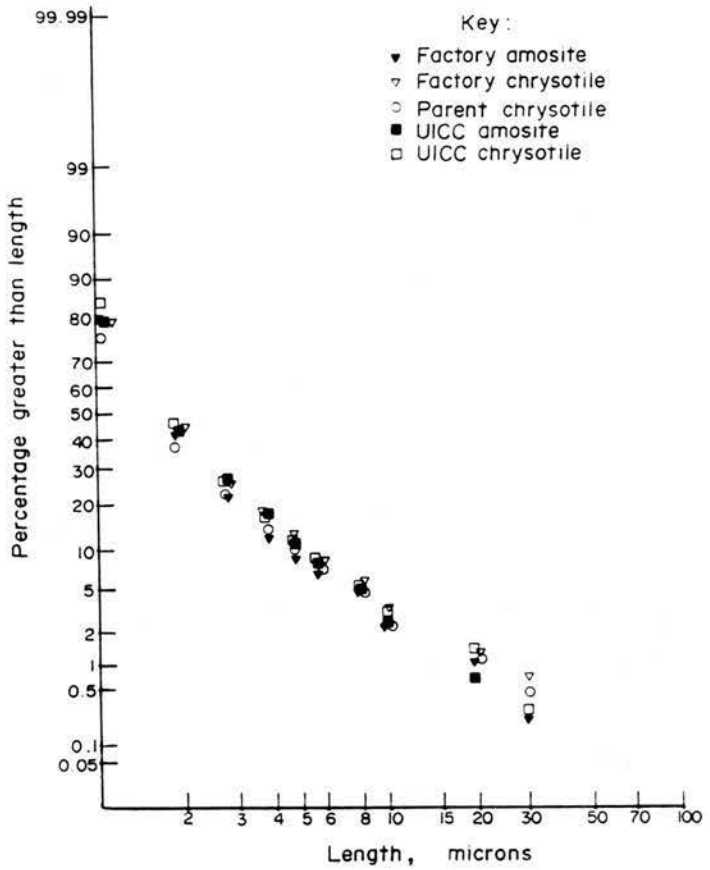


FIG. 3. Relative fibre length distributions of elutriated asbestos samples, prepared from liquid suspensions, and estimated using a scanning electron microscope.

amosite appeared to have a similar relative size distribution in these liquid suspensions and since the chrysotile samples were more carcinogenic than the amosite, the percentage of long fibres in a dust sample would not appear to be the most important factor in determining carcinogenicity.

DISCUSSION

The present work has confirmed that the peritoneal cavity provides a suitable site for the investigation of the development of mesothelioma. The issue of the relative isolation of the peritoneum from the effects of airborne dusts under normal circumstances requires some comment. Having accepted that inoculation methods of investigation of the response of mesothelial tissues to insult are useful (WAGNER *et al.*, 1973), despite their circumvention of pulmonary deposition and clearance mechanisms, the actual choice of the target mesothelium for the measurement of any response is mainly a technical consideration.

It would appear that the peritoneal injection method has several advantages over the pleural site of inoculation. Firstly, the technique is simple, quick, and with less chance of misinjection or inoculation fatality than the intrapleural method. Although LEWIS *et al.* (1966) found that 19.6% of animals inoculated by the intraperitoneal route were misinjected, we found no evidence of misinjection on this scale in the 232 animals

used in the present study. This compares favourably with the experience of various workers using the intrapleural technique.

A second advantage is that the peritoneal site appears to be more sensitive. In our experiments, mesothelial tumours developed in over 90% of animals injected intraperitoneally with 25 mg samples of six out of seven asbestos dusts, with the first tumour detected within 300 days of injection in most treatments. This may be compared with the results of intrapleural injection studies (WAGNER *et al.*, 1973) in which intrapleural injections of 20 mg samples of a range of asbestos types produced mesothelial tumours in up to 60% of treated animals, with the first appearing after about 500 days. One possible reason for this observed enhanced sensitivity of the peritoneal assay could be that at the (presumably) saturation dose level used in the present study, the surface area of mesothelium available for transformation becomes an important factor, and there is a greater mesothelial surface area within the peritoneal cavity than the pleural cavity. In the absence of information on the dose response of injected asbestos in the peritoneal cavity, the dose of 25 mg/rat was chosen for the present investigation, since previous experience has shown that this dose of UICC chrysotile could be expected to produce mesotheliomas. Full dose response studies using the UICC reference samples were initiated soon after, and these are still in progress. The near 100% response encountered in several of the treatment groups in this study provides some problems of interpretation, since it is not possible to discriminate between treatments on the basis of the number of tumours produced. However, the mean tumour survival time provides a useful indication of relative carcinogenicity where a common mechanism of induction may be assumed for several treatments; and analyses of survival have been used in this study to differentiate between different treatments.

A useful corollary of the higher level of response and decrease in the tumour latent period following intraperitoneal injection is that the influence of natural mortality from spontaneous disease is minimized. Experience with the AF/HAN strain of Wistar rat in our laboratory indicates that there is a spontaneous tumour incidence of the order of 30%, mainly occurring within the latter third of the animal's normal life-span. There was no evidence for the existence of a proportion of animals not susceptible to mesothelioma formation as suggested by BERRY and WAGNER (1969) after intrapleural injection. In the present study, the majority of animals had succumbed to mesothelioma within 550 days (i.e. approximately half their normal life-span). The product-limit method of analysis (KAPLAN and MEIER, 1958) used in this study calculates a cumulative survivial function for each treatment group and it therefore takes into account the number of animals surviving at the time of death of any individual animal. The method provides for the exclusion or censoring of those animals dying from causes other than mesothelioma, and this has been taken into account in the construction of Fig. 1.

This study showed that chrysotile tends to be more carcinogenic than amosite when equal masses of dust are injected, thus confirming the experience of other workers (SMITH *et al.*, 1965; WAGNER *et al.*, 1973). That the heated chrysotile proved to be the least carcinogenic in our assay is probably due to the radical effect of heating to 850°C, resulting in almost total destruction of the fibrous chrysotile component. It might therefore have been more appropriate to describe this sample as a forsterite preparation. Within the other chrysotile samples, there does appear to be a correlation between fibre

length and carcinogenicity. Thus, the wet-dispersed chrysotile (*probably* the longest) was the most carcinogenic, the parent chrysotile the least, with the other two samples in between.

Previous workers have used estimates of fibre size to account for differences in the carcinogenicity of asbestiform minerals, using either phase contrast or electron optical methods of estimation. The consensus now strongly favours the view that fibre length is the most important descriptor of carcinogenic potential of asbestos sample. Thus studies by Stanton and Wrench (1972) and Stanton *et al.* (1977) indicated that the carcinogenicity of several types of mineral fibre correlated best with the number of fibres of length exceeding 8 μm and a diameter of less than 0.25 μm.

Assessments of fibre size were undertaken in the present study, but an association between increasing fibre size and biological activity is not obvious from Figs. 1, 2 and 3. Figures 2 and 3 summarize the information acquired in fibre length, expressed as the cumulative relative proportions of fibres over certain stated lengths, and based upon different preparations of the test materials. Fibre numbers were not estimated for the samples used in this study. Length distributions previously obtained from the original airborne samples taken as part of the inhalation studies showed the chrysotile clouds to contain a relatively higher proportion of longer fibres than the amosite clouds (Davis *et al.*, 1978). However, when the airborne samples taken during the collection of the 'elutriated' dusts for injection were assessed, the amosite samples were shown to have a greater relative proportion of longer fibres (Fig. 2). When these same elutriated samples were prepared for microscopy using a liquid dispersion procedure somewhat similar to the injection process, there were no statistical differences between the length distributions of the five samples examined (Fig. 3). Similarly, when the elutriated samples were subjected to sufficient ultrasonication in suspension to provide the degree of dispersal necessary for *in vitro* toxicity tests (Wright *et al.*, 1980), the differences in length observed between the five samples were not statistically significant.

It is considered that these variations are mainly associated with the problems of preparing accurate reproducible samples for the electron microscopy, rather than with limitations of resolution associated with the use of the scanning electron microscope for size estimations. It is possible that the degree of dilution and dispersion of fibre samples necessary to provide preparations of sufficient technical quality for size estimation (particularly from the liquid slurries used for injection) may introduce a degree of disaggregation not representative of the sample *in vivo*.

However, Fig. 3 provides the best estimate available of the fibre size distributions of five of the samples injected in the present study and it can be seen that the samples had a similar relative length distribution. Despite this, there is some support for the general concept of Stanton and his co-workers (1972, 1978) that mineral fibre carcinogenicity is related to fibre length. It is known that experimental chrysotile preparations (including the UICC reference samples) tend to contain substantially more fibres per unit mass than amosite, using either phase contrast microscopy (Davis *et al.*, 1978, 1980) or electron optical techniques (unpublished observations). Although we were unable to obtain sufficient information on the number of fibres per unit mass in the present study, it is reasonable to assume a similar trend, with chrysotile samples containing more fibres per milligram than the amosite. Given the similar relative size distributions, it follows that the chrysotile samples contain more fibres of any specific length. It is not, however, possible to discriminate between an effect due to length, or to

some other parameter of particle size such as surface area. Whilst STANTON and LAYARD (1978) did not find a strong correlation between carcinogenicity and their estimate of the total surface area of 17 glass fibre preparations implanted intrapleurally, there was a link with estimated surface area amongst their longer fibre preparations, suggesting a possible role for the fibre surface area exposed as a result of incomplete phagocytosis.

Table 4 shows that both the time for development of the first mesothelioma, and the mean mesothelioma survival time, correlate well with the calculated cumulative survival function, confirming that preliminary information may be obtained on the carcinogenicity of a given particulate material by examination of the time taken for the first tumour to develop. With this in mind, it is possible to predict that several other dusts currently being studied at this Institute will finally be shown to be much less carcinogenic than asbestos. These include a ceramic (aluminium silicate) sample, and several forms of calcium silicate, none of which have yet produced mesothelial tumours, although the animals have survived for 650 days to date. The observed reasonable correlation of the injection experiments with *in vitro* cell viability assays is also noteworthy. Whilst it is accepted that not all cytotoxic dusts are carcinogenic, and some of the results of our viability assays are sufficiently close to be within the limits of experimental variability, there is broad agreement that the cytotoxic fibrous particles were also more carcinogenic. This is despite the fact that prolonged (2 min) ultrasonication of the *in vitro* dust samples was required to achieve adequate dispersion whilst the injected samples received no such treatment.

It is possible to conclude from this study that the relative carcinogenicity of several similar dust samples may be reasonably assessed from the mean tumour latent period rather than the total number of tumour-bearing animals produced. This does, however, presuppose that the mechanism of carcinogenicity is the same for each dust type under investigation, a reasonable assumption under the present circumstances. This study has produced relatively little information on the mechanisms of asbestos carcinogenesis, although there is some evidence that the more active samples contained more longer fibres. However, more information on the interrelationship of particle size, number, and surface area is required before length can be shown to be the principal determinant of carcinogenicity. Examination of these factors, together with investigations into the influence of particle dose, animal age and sex on the incidence of peritoneal mesotheliomas, is in progress.

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A COMPARISON OF THE PATHOLOGICAL EFFECTS IN RATS OF THE UICC REFERENCE SAMPLES OF AMOSITE AND CHRYSOTILE WITH THOSE OF AMOSITE AND CHRYSOTILE COLLECTED FROM THE FACTORY ENVIRONMENT

J.M.G. DAVIS, S.T. BECKETT, R.E. BOLTON & K. DONALDSON

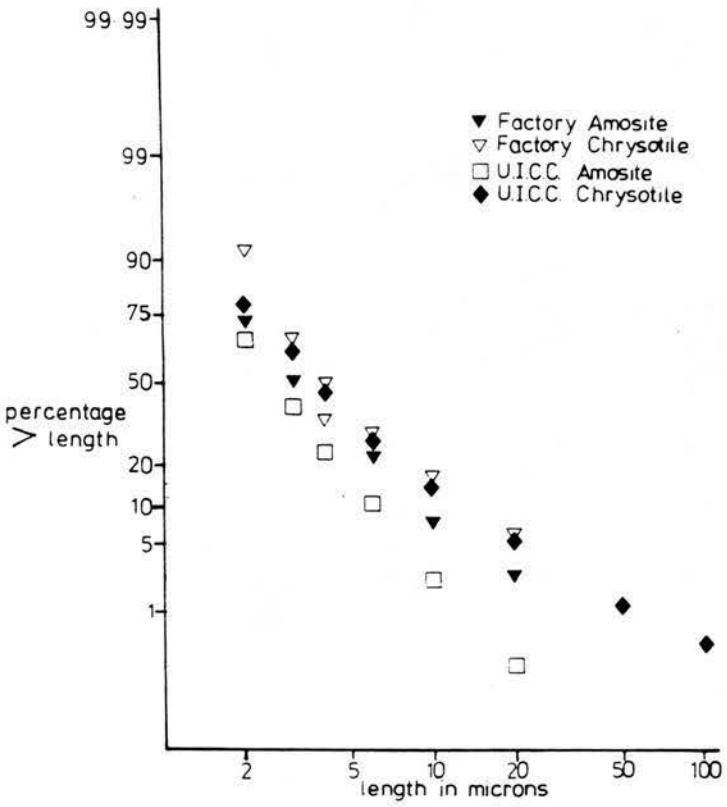
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Following recommendations made at the New York Academy of Sciences' meeting on the Biological Effects of Asbestos in 1964, the UICC Working Group on Cancer and Asbestos organized the preparation of standard reference samples of the most important asbestos types, so that research workers in different countries could undertake experiments using identical material (Timbrell & Rendall, 1971/72). The physical and chemical characteristics of these minerals were described by Timbrell (1970), and these UICC materials have now been utilized in many animal experiments undertaken by a number of workers. However, the characteristics of dust clouds generated from the reference samples are in many ways different from those of factory dusts. For this reason, it was decided to undertake inhalation studies using rats to compare the effects of UICC amosite and chrysotile with those of amosite and chrysotile samples collected from the factory environment. These samples were taken from the bag filters of factories using only amosite or chrysotile so that there was no cross contamination with different asbestos types, although there was considerable contamination with materials other than asbestos.

The chrysotile sample was found to contain only 60% of chrysotile by weight, while the amosite sample was 90% amosite. The nonasbestos component of these dust samples was not characterized. Groups of 48 rats were exposed for a period of 12 months to dust clouds with a concentration of 10 mg/m^3 of air. After the dusting period, small groups of animals were killed for estimations of lung dust content and levels of pulmonary fibrosis, but the majority were allowed to live out their full lifespan in order to study the development of pulmonary neoplasms.

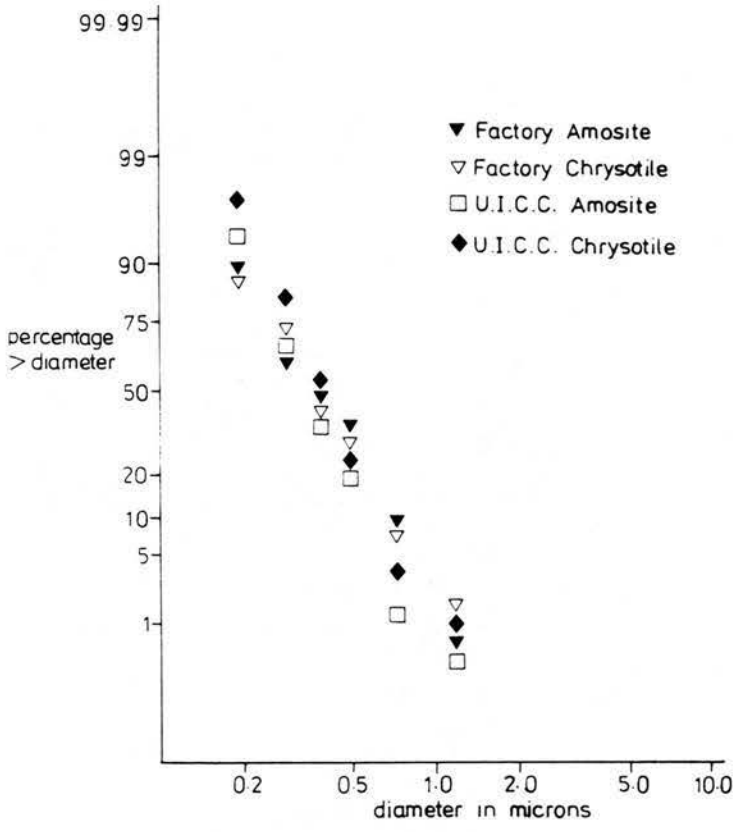
During the dusting period, a number of dust samples were collected from each chamber on membrane filters for measurement of fibre length and diameter distributions, which were undertaken using a Cambridge Instruments S600 scanning electron microscope. The results of these measurements (Figs 1 & 2) indicate that the UICC chrysotile cloud contained a higher proportion of long fibres than did the factory chrysotile, although the factory fibres tended to be thicker on average. The factory amosite cloud contained more long fibres than UICC amosite, but once again the factory fibres were thicker.

FIG. 1. LENGTH DISTRIBUTIONS OF FIBRES LONGER THAN 0.6 μm (SCANNING ELECTRON MICROSCOPE MEASUREMENTS)



At the end of the 12-month dusting period, the mean weights of asbestos in lungs of the different groups were as follows: UICC chrysotile, 1417 μg; factory chrysotile, 2003 μg; UICC amosite, 9169 μg; and factory amosite, 12,710 μg. This means that rats treated with the factory samples, both of which contained impurities, had accumulated more asbestos than animals exposed to the pure UICC samples. The reasons for this may be different for the two asbestos types. In a series of short-term experiments, Middleton et al. (1979) showed that animals dusted in darkness, when they are most active, accumulated three times more UICC chrysotile in their lungs than rats dusted in

FIG. 2. DIAMETER DISTRIBUTIONS OF FIBRES BROADER THAN 0.2 μm (SCANNING ELECTRON MICROSCOPE MEASUREMENTS)



daylight. There was no similar effect with UICC amosite. They concluded that during the day the animals slept with their noses buried in each others' fur, and this acted as a filter for the long curly fibres of chrysotile but did not stop the shorter fibres of amosite. Since the factory chrysotile dust contained fewer long fibres, it may well be that the filtration effect was less and lung dust deposition greater compared with the UICC material. The higher lung burden of factory amosite is more difficult to explain but may be due to the different fibre dimensions of this material, affecting both deposition and retention.

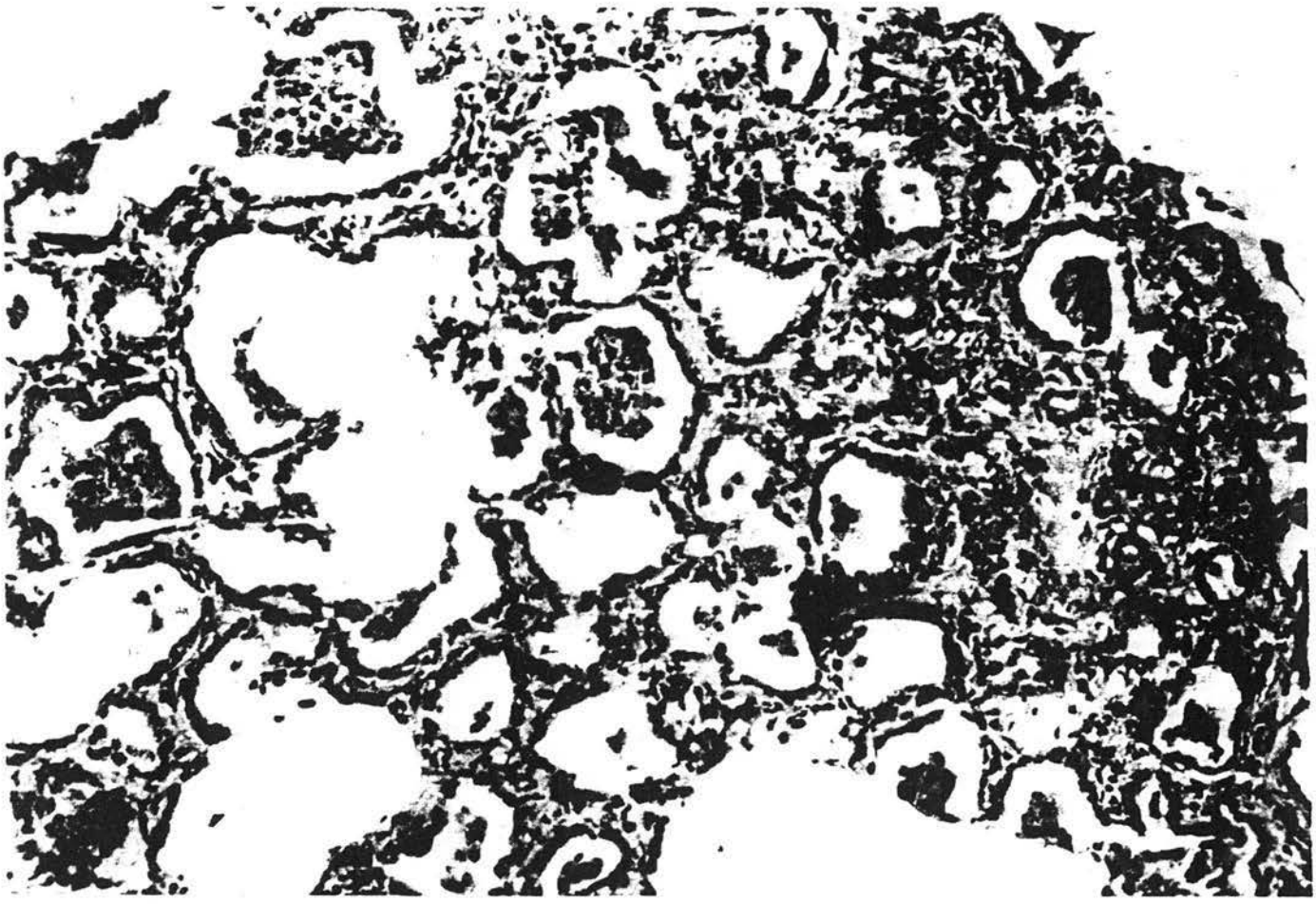
The levels of pulmonary interstitial fibrosis found in the four groups of animals at 12, 18 and 29 months after the start of dusting were recorded as a percentage of total lung tissue area, as reported by Davis et al. (1978). The results, illustrated in Table 1 together with the number of pulmonary tumours found, show that interstitial fibrosis is slow to develop and that only small amounts were present at the 12-month stage. In the older animals, however, it frequently became quite severe (Fig. 3). These results indicate that the sample of factory chrysotile produced very similar levels of lung pathology to UICC chrysotile. The levels of interstitial fibrosis and the

Table 1. Levels of interstitial fibrosis and numbers of pulmonary tumours produced by UICC amosite and chrysotile and by amosite and chrysotile samples collected from the factory environment

Exposure	No. of animals	Level of interstitial fibrosis (% total lung area)	No. of pulmonary tumours	
			Benign	Malignant
UICC chrysotile				
12 months	4	0.48		
18 months	4	0.9		
29 months	6	9.15		
lifespan	43		7	8
Factory chrysotile				
12 months	4	0.0		
18 months	4	0.78		
29 months	6	7.7		
lifespan	42		8	3
UICC amosite				
12 months	4	0.87		
18 months	4	0.12		
29 months	6	2.6		
lifespan	40		2	0
Factory amosite				
12 months	4	0.0		
18 months	4	0.75		
29 months	6	8.5		
lifespan	37		0	0

numbers of benign tumours found in the lung tissue were extremely close, and the only difference was the smaller number of bronchial carcinomas produced with the factory dust. However, with the number of animals involved, this difference was not statistically significant. With the two amosite samples, it was found that the factory dust was more fibrogenic than the UICC sample, and levels of interstitial fibrosis in animals treated with factory amosite were as high as with either of the chrysotile samples. However, both amosite samples showed little carcinogenicity, and the factory amosite produced neither benign nor malignant tumours.

FIG. 3. AN AREA OF INTERSTITIAL FIBROSIS FROM THE LUNG OF A RAT 29 MONTHS AFTER THE START OF EXPOSURE TO A SAMPLE OF AMOSITE FROM THE FACTORY ENVIRONMENT (× 250)



These findings raise some interesting points regarding the importance of fibre length in the pathogenic effects of asbestos. Stanton & Wrench (1972) and Stanton et al. (1977) summarized theories on fibre length and pathogenicity and suggested that the most dangerous fibres were over 8 μm in length and less than 1.5 μm in diameter. Davis et al. (1978) reported experiments in which the different UICC asbestos samples had been administered to rats at different dose levels and confirmed that the chrysotile clouds, which alone contained significant numbers of fibres over 20 μm in length, were the most pathogenic. In this case, the results could be interpreted as indicating that carcinogenicity and fibrogenicity were associated with fibre length to the same degree. In the present study, however, the absence of most of the longest fibres from the factory chrysotile sample as compared with UICC chrysotile reduced the number of malignant tumours, but the number of benign adenomas and the levels of interstitial fibrosis in the two experiments were almost identical. With factory amosite, as compared with the UICC material, an increase in the number of relatively long fibres was associated with an increase in the levels of interstitial fibrosis, but no tumours at all were produced.

Wagner et al. (1974) found that amosite produced fewer pulmonary tumours in rats than other asbestos types after a 12-month inhalation period; it may indeed be that amosite *per se* is a material of relatively low carcinogenicity, although Selikoff et al. (1972) reported significant numbers of tumours in amosite-exposed workers. A more likely explanation of the findings reported in the present paper, however, is that while fibrogenicity and carcinogenicity both depend on the presence of relatively long fibres in dust clouds, different lengths are involved in each process, and tumour production requires the longest fibres. The effect of fibre diameter is still obscure. In the present study, both chrysotile samples had a higher proportion of fibres over 1 μm in diameter than amosite, but chrysotile is much more likely to undergo longitudinal splitting within the tissues to produce very thin fibres. The scanning electron microscope fibre sizing technique used for this work did not permit estimations of the proportions of fibres below 0.2 μm in diameter because of resolution problems. It was not, therefore, possible to study the importance of very thin fibres. Future animal inhalation studies within our Institute will include transmission electron microscope sizing of dusts from both dust clouds and lung tissue, which may resolve this problem.

SUMMARY

Inhalation studies were undertaken in rats to compare the pathogenic effects of samples of UICC amosite and chrysotile with those of amosite and chrysotile samples collected from the factory environment. Fibre length and diameter studies on the four dust samples showed that the UICC chrysotile cloud contained more long fibres than factory chrysotile, although the factory fibres tended to be thicker. The factory amosite cloud contained more long fibres than UICC amosite, but again the factory fibres were thicker. The factory dusts contained considerable amounts of impurities; in spite of this, the lung dust content of asbestos at the end of 12 months' dusting was higher with factory dusts than with UICC material. The UICC chrysotile and factory chrysotile produced similar levels of interstitial fibrosis, but the factory dust produced fewer malignant lung tumours. The factory amosite produced much more interstitial fibrosis than UICC amosite, but neither amosite cloud produced any malignant pulmonary tumours. These results are discussed in relation to current theories on the importance of fibre dimensions in the pathogenesis of lung disease.

RESUME

Les auteurs ont entrepris des études par inhalation chez le rat afin de comparer les effets pathogènes d'échantillons d'amosite et de chrysotile de l'UICC à ceux d'échantillons d'amosite et de chrysotile recueillis en milieu industriel. Les études de la longueur et du diamètre des fibres dans les quatre échantillons ont montré que les fibres longues étaient plus nombreuses dans le nuage de poussières de chrysotile de l'UICC que dans le chrysotile d'usine, alors que les fibres d'usine étaient souvent plus épaisses. L'amosite d'usine contenait plus de fibres longues que l'amosite de l'UICC, mais les fibres d'usine étaient également plus épaisses. Les poussières d'usine contenaient des impuretés en quantité considérable; néanmoins, après 12 mois d'empoussièrement, la teneur des poussières pulmonaires en amiante était plus forte avec les poussières d'usine qu'avec les échantillons de l'UICC. Le chrysotile de l'UICC et le chrysotile d'usine occasionnaient des degrés comparables de fibrose interstitielle, mais les poussières d'usine provoquaient moins de tumeurs pulmonaires malignes. L'amosite d'usine produisait bien plus de fibrose interstitielle que l'amosite de l'UICC, mais aucun des deux nuages de poussières d'amosite ne provoquait de tumeurs pulmonaires malignes. Ces résultats sont examinés à la lumière des théories actuelles sur l'importance des dimensions des fibres dans la pathogénie des maladies pulmonaires.

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THE EFFECTS OF INTERMITTENT HIGH ASBESTOS EXPOSURE (PEAK DOSE LEVELS) ON THE LUNGS OF RATS

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Summary.—Four groups of rats were treated by inhalation with the UICC preparations of amosite or chrysotile in order to explore the effects of intermittent high dust concentrations (peak dosing). For each of the 2 asbestos types one group of rats was treated for 5 days each week, 7 h a day, for 1 year. Two other groups were treated with amosite or chrysotile at 5 times the previous dose for 1 day each week for 1 year. Results showed that the lung dust levels of both chrysotile or amosite in the lungs of rats after the 12-month inhalation period were similar regardless of whether "peak" or "even" dosing had been used. During the following 6 months, asbestos was cleared from the "peak" chrysotile group more slowly than the "even" chrysotile group but clearance from the "peak" amosite group was faster than that found after "even" dosing with amosite. Levels of early peribronchial fibrosis were generally lower for the "peak" dosing groups than for "even" dosing although levels of interstitial fibrosis were slightly higher following "peak" dosing. The incidence of pulmonary neoplasms did not differ between the "peak"-dosing and "even"-dosing experiments. These findings therefore give no indication that short periods of high dust exposure in an asbestos factory would result in a significantly greater hazard than would be indicated by the raised overall dust counts for the day in question.

INHALATION STUDIES published by a number of authors have shown that the laboratory rat is a suitable model to use in the study of asbestos-related lung disease. In humans asbestos exposure is known to result in the development of pulmonary interstitial fibrosis, bronchial carcinoma and pleural or peritoneal mesothelioma, and similar conditions can be produced experimentally in rats. Gross and de Treville (1967), Wagner *et al.* (1974), Reeves, Puro and Smith (1974) and Davis *et al.* (1978) have reported interstitial fibrosis and bronchial carcinomas following experimental inhalation of several asbestos types. Wagner *et al.* (1974) reported the occurrence of occasional pleural mesotheliomas following asbestos inhalation and Davis *et al.* (1978) also found one peritoneal mesothelioma.

However, all these studies used a constant even dose of the various asbestos types and this is not closely comparable to the situation encountered by workers in modern asbestos mills and factories. In these workplaces the overall asbestos exposures are generally very low but occasional breakdowns in the ventilation to any piece of machinery or even periods of maintenance work on machinery can produce high localized dust levels for short periods of time. It has been suggested that these short "peak" doses, which hardly change the overall daily dust counts for the area or factory concerned, may be dangerous because the pulmonary clearance mechanism of exposed workers may become saturated (Holmes, 1972). This could result in a level of asbestos retention far greater than would occur if the same dose was spread over a long period. It was considered important to test this hypothesis under experimental conditions and the present paper reports the findings from experiments using the UICC samples of amosite and chrysotile.

MATERIALS AND METHODS

For previous inhalation studies on the effects of asbestos dust, it has been the practice in our laboratory to expose animals to a constant dust dose for 7 h each day, 5 days each week, for a total period of 12 months, an experimental regime hereafter referred to as "even" dosing. In order to obtain information on the effects of "peak" dosing, however, it was decided to expose 2 groups of animals to 5 times the usual dose for 1 day each week. Previous studies (Davis *et al.*, 1978) had used constant doses of UICC amosite at 10 mg/m^3 and chrysotile at both 10 mg/m^3 and 2 mg/m^3 of respirable dust. It would have been desirable, therefore, to study "peak" exposures using dust levels of amosite and chrysotile at 50 mg/m^3. This was possible with amosite but not with chrysotile, since it was found that at high densities the fibres flocculated within the inhalation chamber and it was not feasible to obtain levels of respirable dust much above 20 mg/m^3. It was decided, therefore, to compare the effects of a cloud of chrysotile at 10 mg/m^3 administered for 1 day each week with previously obtained data on the effects of "even" dosing with this material at 2 mg/m^3. The "peak" amosite studies utilized a 50 mg/m^3 cloud and the results were compared to "even" dosing at 10 mg/m^3.

The dust clouds were generated using a modified Timbrell dust generator (Timbrell *et al.*, 1970) and the inhalation chambers were of similar design to Timbrell's. The dust was size selected by a cyclone system (Beckett, 1975) before being added to the chamber air stream. This ensured a higher proportion of respirable dust in the clouds. Gravimetric monitoring was carried out during dusting and the daily mass concentration measurements were obtained for all the chambers. The NCB MRE sampler (Cassella Type 113A—Dunmore, Hamilton and Smith, 1964) was used to measure the concentrations in the amosite chambers. At 10 mg/m^3 with chrysotile, this instrument had been found to undersample and a vertical elutriation system (Beckett, 1975) was therefore used to monitor the chrysotile clouds.

Additional dust samples were obtained for the estimation of fibre dimensions using the standard sampling method described by the Asbestosis Research Council (1971). Each membrane filter sample was taken using an open Gelman filter holder facing downwards at a flow rate and sampling time calculated to give an optimum density for the microscopical examination (1–3 fibres per graticule area). Fibre length and diameter distributions were obtained partly by phase-contrast microscopy and partly by scanning electron microscopy (Beckett, 1973), using a Cambridge Instruments S600 scanning electron microscope.

Studies on the effects of "peak" dosing of asbestos utilized groups of 48 male random-bred white SPF Wistar rats of the AF/HAN strain. These were exposed for a period of 12 months and groups of 4 animals were killed at 12 and 18 months after the start of dusting for the estimation of pulmonary fibrosis as well as dust deposition and retention. The experiment was terminated at 29 months for comparison with previous studies from this unit (Davis *et al.*, 1978) and lungs from 6 animals in each group were used for estimations of advanced fibrosis. Lungs from the remaining animals were examined only for the presence of pulmonary neoplasms. Tissue used for histological examination was fixed with 10% formol saline solution and embedded in paraffin wax: lungs were fixed by inflation *in situ* until they filled the thoracic cavity. Sections were stained with either haemotoxylin and eosin, van Giesen's method for collagen or Gordon and Sweet's stain for reticulin. Levels of pulmonary fibrosis were calculated using the methods previously described by Davis *et al.* in 1978. Both lungs and heart were embedded together and sections were cut in the coronal plain to include parts of all lobes. Sections were cut at 4 different levels in each block and were at least 1 mm apart, and groups of serial sections were mounted from each of these levels for use with the different staining techniques. For all lesions the H. and E. sections were scanned with the light microscope using an eyepiece graticule consisting of a 1cm square subdivided into 100 units of 1 mm^2. Viewing magnification was × 60. The area of regions of interstitial fibrosis was estimated for each slide by counting the number of grid squares involved and presenting the results as a percentage of total lung area in the section. An average figure for the animal was produced by combining the result from all 4 sections. The early peribronchial fibrotic lesions were usually much smaller than one grid square at the magnification involved and since they were associated with the respiratory bronchioles they were also widely scattered. For this type of small lesion, the calculations were based on the number of squares that contained the small areas of fibrous tissue and the results from all four sections were again presented as a percentage.

Asbestos retained in the lungs of selected animals was recovered by a low-temperature ashing process. This was conducted in a stream of oxygen excited by a radio frequency discharge (Gleit and Holland, 1962). Any residual lung salts were removed by washing the samples in 3 ml of cold (20°) 0·2M HCl before gravimetric estimations of the amounts of asbestos recovered were made using the infra-red spectrophotometric techniques described by Middleton, Beckett and Davis (1977). The amounts of dust retained in the lungs of rats killed 12 and 18

months after the start of dusting was estimated from ashed residues of the left lung in each case, the right lung being retained for histological examination. Studies in this laboratory have shown that the asbestos content ratio between left and right lungs following experimental asbestos inhalation in rats is 0·6:1 and this correction factor was therefore used to calculate the total pulmonary dust burden of each animal.

RESULTS

The results of "peak" dosing studies using amosite and chrysotile have been compared throughout with the results of experiments where the same dust types were administered to rats at the "even" dose levels. The dust parameters for the 4 chambers during the dusting period are given in Table I. The mass concentrations obtained were all very close to the target levels. During the study, a series of dust samples was obtained on Nuclepore filters and these were utilized for measuring size distribution of the fibres using a scanning electron microscope. The fibre length and diameter distributions from the peak dusting chambers appeared identical to those obtained from the same dust types administered at "even" dose levels (Figs 1 and 2). The chrysotile cloud had a higher proportion of fibres over 10μm in length than the amosite and only the chrysotile cloud had any significant proportion of fibres as long as 50 μm.

The survival times for the animals for the 4 inhalation chambers are shown in Table II. These indicate that there were no overall differences in longevity between animals treated with different asbestos clouds. Histological examination of lung tissue from animals in the peak dusting studies showed types of dust lesions identical to those present in "even"-dosed animals. The earliest lesions consisted of deposits of dust and granulation tissue around the respiratory bronchioles and alveolar ducts. In animals treated with chrysotile the granulation tissue contained many giant cells as well as macrophages and fibroblasts but giant cells were rare

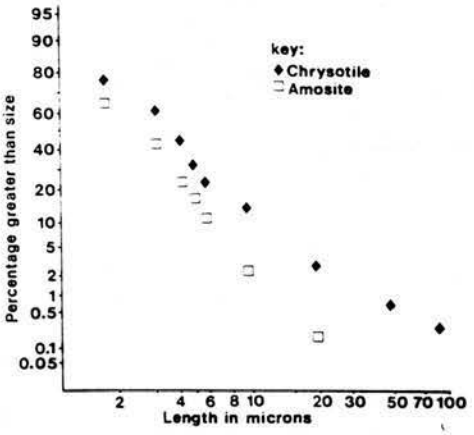


FIG. 1.—Length distributions of fibres longer than 0·6 μm for the chrysotile and amosite clouds used in the study of high intermittent doses. (Scanning electron microscope measurements.)

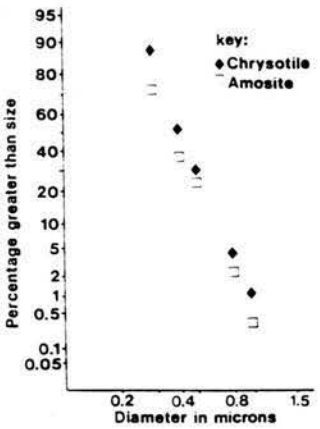


FIG. 2.—Diameter distribution of fibres broader than 0·2 μm for the chrysotile and amosite clouds used in the present study of high intermittent doses. (Scanning electron microscope measurements.)

TABLE I.—*The mean mass of the 4 UICC asbestos clouds over the exposure period*

	Chrysotile (even dosing)	Chrysotile (peak dosing)	Amosite (even dosing)	Amosite (peak dosing)
Target concentration (mg/m^3)	2·0	10·0	10·0	50·0
Mean concentration achieved (mg/m^3)	2·0	9·0	10·0	49·2
Mean ratio of total to respirable dust	1·3 : 1	1·1 : 1	1·15 : 1	1·13 : 1

TABLE II.—*Number of animals surviving in the different inhalation groups throughout the study. The experiment was terminated at 29 months. Groups of 4 animals from each chamber were killed at both 12 and 18 months*

Dust cloud	Months after start of exposure			
	12	18	24	29
Chrysotile 10 mg/m³ (peak dose)	48	43	29	7
Chrysotile 2 mg/m³ (even dose)	48	40	26	7
Amosite 50 mg/m³ (peak dose)	48	44	31	8
Amosite 10 mg/m³ (even dose)	47	40	22	11

in animals treated with amosite. These early lesions contained considerable amounts of reticulin but only small amounts of collagen were found at this stage. In some cases the normal epithelial lining of the respiratory bronchioles, alveolar ducts and associated alveoli became replaced by rounded epithelial cells of bronchiolar type. It was not, however, possible to determine whether or not this was due to hyperplasia of the bronchiolar lining or metaplasia of the alveolar epithelial cells. This change was usually found associated with areas of peribronchiolar granulation tissue and fibrosis but it could occur on its own. More advanced changes consisted of the thickening of alveolar septa over quite large areas of lung tissue (Fig. 3). In these areas of interstitial change the alveoli were lined with rounded cells, probably Type 2 pneumocytes, and there was an increase in the thickness of the reticulin network in the septal walls. No collagen was present in the early stages but in the oldest animals some areas of interstitial fibrosis showed very marked thickening of the alveolar septa which stained strongly positive for collagen.

In some areas, however, an alternative to advanced fibrosis was the continued growth of the rounded alveolar epithelial cells with subsequent compression of the alveoli to produce some adenomatous appearance. In some cases definite adenomas had formed in these areas. In a few animals small areas of squamous metaplasia of the alveolar epithelium were also found.

Quantitative estimations of these lesions in both animals subjected to "peak" doses and those which had received the same amount of asbestos at an "even" rate are given in Table III. From these data it can be seen that for chrysotile the levels of early peribronchiolar fibrosis were significantly lower following peak dosing than even dosing ($P < 0{\cdot}05$). With amosite, however, there were no significant differences between the 2 dusting regimes. Similarly there appeared to be no progression of the small lesions of peribronchial fibrosis after the end of the dusting period with either method of dosing. In fact the oldest animals of each group showed less peribronchiolar fibrosis than those examined at either 12 or 18 months after the start of dusting. However, this might be due to an increase in areas of interstitial fibrosis which enclosed and masked earlier areas of fibrosis close to the bronchioles. The extension of bronchial epithelium to alveolar ducts and alveoli did not differ significantly between peak and even dosing for either amosite or chrysotile. Levels of pulmonary interstitial fibrosis were similar for the peak and even dose groups of both asbestos types at both 12 and 18 months from the start of dusting. At 29 months, however, animals from both the peak dosing experiments appeared to have more pulmonary interstitial fibrosis than those treated with the same type of asbestos at "even" dose levels although these differences were not statistically significant.

The incidence of neoplasms of the lung and mesotheliomas found in the 4 experimental groups is shown in Table IV. The figures for the peak and even-dose studies show no significant differences. The peak amosite study, however, did produce 2 bronchial carcinomas where none had occurred with even dosing of the same material. The tumour incidence from sites



FIG. 3.—An area of pulmonary interstitial fibrosis in the lung of a 32-month-old rat after the inhalation of chrysotile asbestos. × 200.

other than lung and excluding mesotheliomas is shown in Table V and it can be seen that there were no significant differences between the 4 groups of animals under study.

The weights of asbestos extracted from the lungs of animals in the different inhalation groups are summarized in Table VI. This shows that the amount of chrysotile present at the end of the 12-month dusting period is extremely close regardless of whether the inhalation dose was administered evenly during the week or concentrated into a single day. In animals treated with amosite, however, the retention of inhaled dust at the end of the dusting period was higher for "peak" dosing than for "even". Long-term clearance of chrysotile from the rat lungs appeared to be slower following "peak" dosing, while clearance of amosite appeared faster in the "peak" dosing experiment. In all the cases, however, statistical examination of the figures showed no significant differences.

DISCUSSION

The figures for the deposition and retention of asbestos dust reported in the present study appear to refute the idea that short periods of very high exposure to asbestos result in a much higher lung burden of retained dust than might be expected from the greater density of the "peak" dust clouds. The amounts of dust found in the lungs of chrysotile-treated animals at the end of the dusting period for both "peak" and "even" dosing were extremely close. For amosite, animals in the "peak" dosing group did appear to retain more dust than those subjected to "even" dosing, but with only 4 animals in each group the figures were not significantly different. There were no significant differences between the long-term clearance of chrysotile and amosite regardless of whether dust had been administered by either peak or even dosing. These figures suggest that short periods of high dust exposure in asbestos factories are unlikely to result in a much greater lung dust bur-

TABLE III.—*Mean levels and ranges of lung fibrosis produced in rats by clouds of UICC chrysotile and amosite administered at different dose rates (parameters as described in Methods section)*

	Chrysotile 2 mg. Even dose			Chrysotile 10 mg. Peak dose			Amosite 10 mg. Even dose			Amosite 50 mg. Peak dose		
Time after start of exposure (months)	12	18	29	12	18	29	12	18	29	12	18	29
Peribronchiolar fibrosis	10·7 (7·8–12·7)	9·9 (7·5–11·77)	7·53 (5·2–9·0)	6·8 (4·5–8·9)	6·1 (3·7–8·8)	3·9 (2·5–6·5)	4·12 (3·0–5·5)	5·1 (3·8–5·9)	4·2 (2·5–5·5)	6·2 (4·6–7·4)	5·4 (3·2–7·5)	2·9 (1·6–3·8)
Extension of bronchial epithelium to alveolar ducts and alveoli	1·7 (1·1–2·5)	4·03 (2·8–6·6)	1·05 (0·5–1·5)	2·3 (1·2–3·4)	2·6 (1·3–4·0)	1·5 (0·6–2·3)	2·27 (1·6–3·2)	3·9 (1·0–6·0)	3·05 (1·8–5·5)	1·8 (1·3–2·5)	2·1 (1·6–3·3)	1·6 (1·3–2·0)
Interstitial fibrosis	0·35 (0–1·2)	0·83 (0–2·9)	3·86 (0–7·2)	1·3 (0–5·2)	1·05 (0–4·21)	6·8 (0·89–19·5)	0·87 (0–3·24)	0·12 (0–0·4)	2·58 (1·1–5·1)	0·0	1·18 (0–4·6)	5·8 (1·0–11·4)
No. of rats in sample	4	4	6	4	4	6	4	4	6	4	4	6

TABLE IV.—*Pulmonary tumours found in the lungs of rats following either "even" dosing or "peak" dosing at equivalent levels of UICC chrysotile and amosite*

Type of tumour	Chrysotile 2 mg/m³ even dosing 40 animals	Chrysotile 10 mg/m³ peak dosing 43 animals	Amosite 10 mg/m³ even dosing 40 animals	Amosite 50 mg/m³ peak dosing 44 animals
Adenoma	6	4	2	4
Adenocarcinoma	1	1	0	2
Squamous carcinoma	1	1	0	0
Mesotheliomas	1	0	0	0

den than would be indicated by the overall dust counts for the day in question and are in agreement with previously reported experimental work. Thus a series of short-term inhalation experiments (Middleton *et al.*, 1977, 1979) and a long-term inhalation study (Davis *et al.*, 1978) both indicated that, in proportion to the density of the dust cloud, there was no large increase in asbestos dust deposition for exposure levels between 1 mg/m³ and 10 mg/m³. In both long- and short-term studies, the lung dust burden of amphibole dusts was found to be proportionally slightly higher for high doses than for low but for chrysotile the amount of dust in lungs at the end of the dusting period was actually considerably reduced with increasing dust cloud density. In the long-term studies, clearance during the 6 months following the cessation of dusting was slightly slower for high chrysotile doses than for low, but clearance following high doses of amphibole was actually faster. In the short-term studies it was found that the rate of clearance was independent of the type of asbestos or the dust dose.

TABLE VI.—*Calculated weights of asbestos recovered from lung tissue at 7 and 182 days after the end of the dusting period*

Asbestos cloud	No. of animals	Days after exposure	Mean lung dust content (μg)	Percentage clearance between 7 & 182 days
Chrysotile 2 mg/m³	4	7	526	66%
	4	182	180	
Chrysotile 10 mg/m³ (peak dose)	4	7	501	47%
	4	182	270	
Amosite 10 mg/m³	4	7	9169	24%
	4	182	7020	
Amosite 50 mg/m³ (peak dose)	4	7	11226	35%
	4	182	7331	

For methods of calculation see text.

TABLE V.—*Sites of tumours other than lung*

Site of tumour	Chrysotile 2 mg/m³ (even dosing) 40 animals		Chrysotile 10 mg/m³ (peak dosing) 43 animals		Amosite 10 mg/m³ (even dosing) 40 animals		Amosite 50 mg/m³ (peak dosing) 44 animals	
	B	M	B	M	B	M	B	M
Subcutaneous connective-tissue tumours		1			2	3	2	2
Peritoneal connective-tissue tumours		1		1	1	1		
Osteosarcomas		1						
Testicular tumours	1				4		2	
Squamous tumours of the epidermis				2	1			
Parotid tumours								2
Adrenal tumours	1					1		1
Thyroid tumours		1			1			
Lymphoma/leukaemia		1		2				
Pancreatic tumours							2	
Total	2	5	0	5	9	5	6	5

There was thus no evidence of greatly increased dust retention with increasing exposure at what by human standards are extremely high dose levels. From this it seems particularly unlikely that there would be an excessive build-up of lung dust because of "peak" doses considerably lower than those examined experimentally.

In this connection, however, it is important to consider just how different the factory situation is from the experimental design used in this study. In factories the overall dust levels are extremely low, usually well below 2 fibres per ml, so that a peak dose caused by a temporary machine defect probably lasting less than 1 h could easily be 100 times higher than this base-line and still result in a dust cloud of less than 1 mg/m^3 of air. A cloud of UICC chrysotile with a mass of 2 mg/m^3 has approximately 400 fibres per ml over 5 μm in length (Davis *et al.*, 1978). It was unfortunately not possible to compare the pathological effects of this extreme differential between "even" and "peak" doses in the laboratory. The dust load retained in a pair of rat lungs after a 12-month dusting period at 2 fibres/ml would have been too low to estimate reliably. It is also most unlikely that dusting at this level would have produced any detectable pathological changes in the lungs of rats over a 2½–3-year life span. The equivalent "peak" dust cloud of 0·5 mg/m^3 of air for 1 h each week would result in an overall dose after 12 months of 140 times less than the 2mg dose used as a base-line for the peak experiments in the present study. This also would probably have been too low a dose to produce any detectable pathological change over the life span of a rat.

Regardless of the amount of dust retained in lung tissue following "peak" dosing, however, there did appear to be some differences in the levels of tissue damage resulting from this experimental procedure as compared to "even" dosing. Although there were no significant differences in tumour production in these experiments, the levels of early peribronchiolar fibrosis were significantly lower following "peak" inhalation of chrysotile and in the later stages of the experiment the levels of interstitial fibrosis found after "peak" dosing of both amosite and chrysotile were higher compared to the "evenly" dosed animals. In this case the differences were not statistically significant. It is possible that these tissue changes may indicate differences in the dust deposition patterns with "peak" or "even" dosing that do not show up in levels of total retained dust. It may be that short periods of high dose levels result in more dust remaining in the alveoli rather than being cleared towards the terminal bronchioles. There would thus be less tissue reaction near to the bronchioles and more in the lung parenchyma. This problem may be further elucidated by additional studies on long-term deposition and clearance of asbestos dust.

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THE EFFECT OF DOSE OF ASBESTOS ON MESOTHELIOMA PRODUCTION IN THE LABORATORY RAT

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INTRODUCTION

The association between asbestos exposure and primary mesothelioma of the pleura and peritoneum has been repeatedly demonstrated in epidemiological studies (NEWHOUSE 1973, BECKLAKE 1976, SELIKOFF and LEE 1978). Ever since WAGNER (1962) showed that mesothelial tumours could be produced in rats by direct intrapleural inoculation of asbestos preparations, animal experiments have been used to assess the tumorigenic potential of fibrous particulates (SMITH et al 1965, WAGNER and BERRY 1969, POTT and FRIEDRICHS 1972). SMITH et al (1973), POTT et al (1976), STANTON and WRENCH (1972), and WAGNER et al (1973) all showed that there was a dose response effect when different asbestos or glass fibre samples were injected at different mass doses. However, the range of doses used in these studies was small and the lowest dose used was 0·5 mg of dust. The present study was undertaken to explore the shape of the dose response in more detail and to examine the effects of very small amounts of asbestos.

In our laboratory we have routinely used the intraperitoneal injection route to compare dust types, and the first of these comparisons was published recently (BOLTON et al 1982) where seven asbestiform preparations were compared at a single dose of 25 mg per rat. During the course of this work it became clear that the peritoneal cavity was a more sensitive site for mesothelioma induction than the pleural cavity since a greater proportion of animals developed tumours, and in a shorter time, than with comparable doses inoculated into the pleural cavity by other workers.

Accordingly, the intraperitoneal injection route was adopted in the present study to examine the relationship between mesothelioma production and dust dose for the three main varieties of asbestos. In order that the results could be integrated with inhalation studies using the same materials, both 'elutriated' (BOLTON et al 1982) and standard forms of the UICC samples were injected. The results obtained from 'elutriated' samples of UICC chrysotile (A) and UICC crocidolite form the basis of this presentation. Assays of effects of amosite treatment, comparison of standard and elutriated forms of all three dusts, and detailed fibre size assessments, are currently in progress and will be reported at a later date.

MATERIALS AND METHODS

A total of 489 male AF/HAN random bred SPF Wistar laboratory rats was used, 8-10 weeks old at the time of injection, housed 4 per cage, with ad libitum access to standard pelleted laboratory diet and tap water. They were stratified by age and randomly allocated into their 17 treatment groups as shown in Table 1. Each animal received a specified dose as a single intraperitoneal injection of dust suspended (without ultrasonication) in 2 ml of Dulbeccos phosphate buffered saline. Doses ranged from ·01 mg per rat up to 25 mg per rat. The chrysotile and crocidolite samples examined in the present report were derived from the UICC standard reference samples and are described as 'elutriated' to signify that they were collected from airborne asbestos clouds generated as part of related inhalation studies (BECKETT 1975) by using an absolute filter assembly in the ducting between the dust generator and the animal exposure chamber.

All animals were examined daily, killed when distressed or moribund and a necropsy performed. Presence or absence of mesothelioma at necropsy was recorded for each animal. Patterns of times to death associated with mesothelioma, in the face of losses due to other competing causes of death, were anlysed by calculating survival functions in each treatment group. These were calculated by the product-limit method (KAPLAN and MEIER 1958) using the package BMDP (DIXON 1981). One useful summary statistic for comparison of survival curves is the time point at which survival falls below 0·5, i.e. the estimated median survival time. This incorporates more information about the response than does the mean survival time (used in BOLTON et al 1982) in situations where only some of the animals produced mesotheliomas, as in the lowest doses of the present study. The median provides valid comparison across the doses, although it cannot be directly estimated when only a small proportion of animals respond positively, so that the survival curve associated with mesothelioma does not fall below 0·5.

TABLE 1 : Summary of the experiment plan, the percentage of animals with mesothelioma, and the estimated median survival time for both chrysotile and crocidolite treatment groups.

Dose per rat (mg)	Number of rats(1)		Percentage of animals with mesothelioma		Estimated(2) Median Survival Time (days)	
	Chrysotile	Crocidolite	Chrysotile	Crocidolite	Chrysotile	Crocidolite
25.0	27	0	96	-	454	-
15.0	21	22	90	77	467	652
10.0	22	19	91	53	476	743
7.5	24	22	83	68	516	841
5.0	24	22	79	45	506	818
2.5	32	32	69	59	613	910
0.5	32	31	81	29	693	1002
0.05	32	31	41	26	903	?(3)
0.01	48	48	8	0	?(3)	No tumours

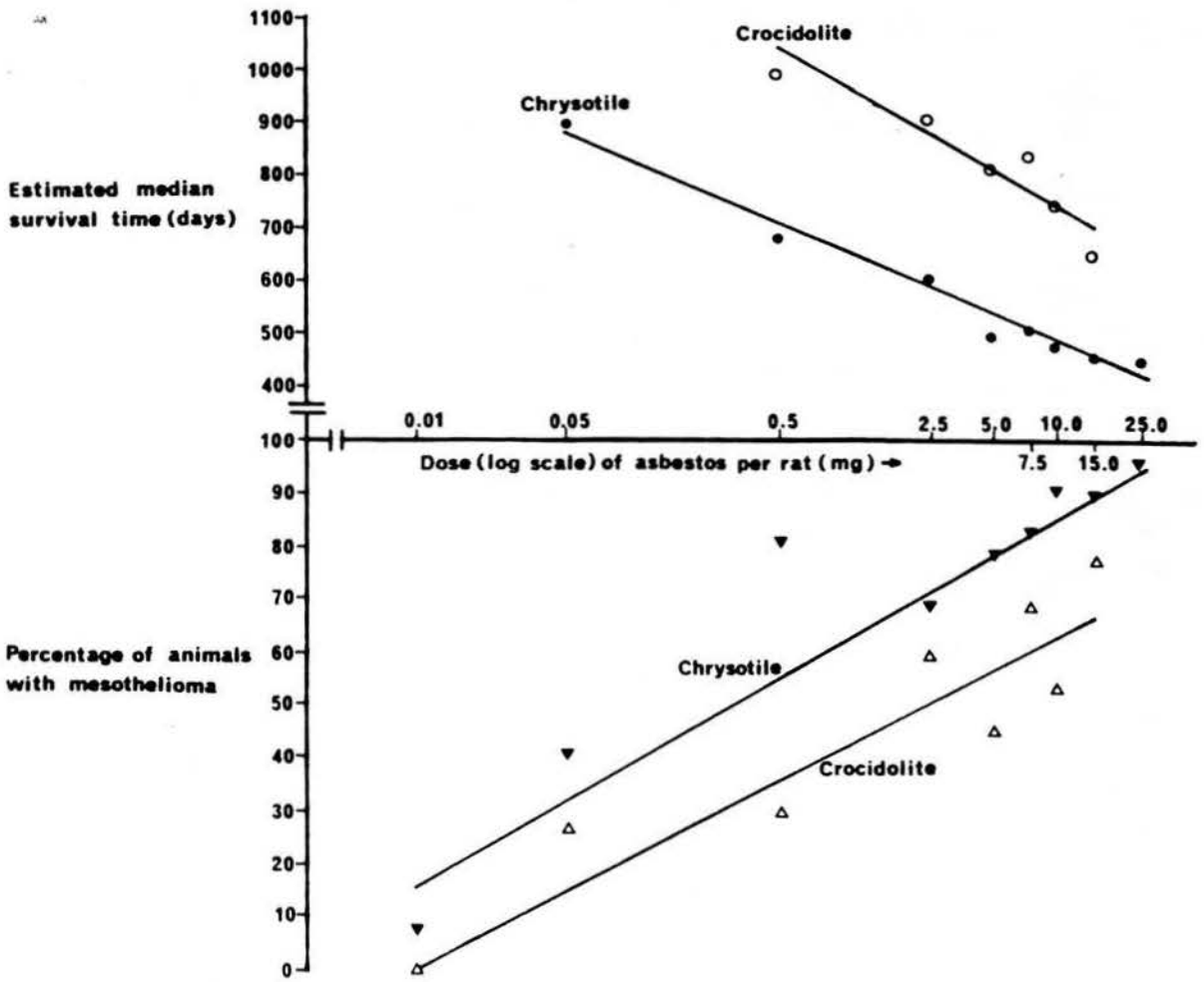
(1) = Number of rats with reliable information

(2) = Estimated using procedure described in text

(3) = Not calculable - refer to text

The results summarised in Table 1 show that both chrysotile and crocidolite produce a graded dose response for mesothelioma development when injected into the rat peritoneal cavity. The assay can be shown to be both internally consistent and experimentally repeatable (data to be reported separately). There appears to be a consistent difference between the two dusts, with chrysotile producing tumours in a greater proportion of animals at any dose level. (The crocidolite sample failed to produce a mesothelioma in any of the 48 animals each injected with 0·01 mg of dust.) In addition, the results for both dusts show that a reduction in the mass of dust injected is associated with a progressive increase in the time taken for tumours to appear (the 'latent' period). This relationship is particularly clear when the response is assessed by estimating the median survival time.

Figure 1 : Estimated median survival time and percentage of animals with mesothelioma, plotted against dose of asbestos (on a log scale).



In Fig. 1 median survival time and the percentage of animals with mesotheliomas are plotted against dose (on a log scale) for each asbestos type. For both dusts the data appear to fit the straight lines that have been added by eye, and there is no evidence of a threshold dose below which the hazard associated with dust injection is zero. The absence of tumours at the-0·01 mg dose of crocidolite is not evidence of such a threshold, since differentiation of small probabilities from a zero level requires much larger treatment groups than are used here. The progressively increased tumour induction period seen with reduced doses of both chrysotile and crocidolite would indicate that there may be dose levels that would not produce tumours within the life span of the rat. However, these doses appear to be extremely small and perhaps several orders of magnitude below the 0·01 mg per rat which was the lowest dose used in the present study.

In addition to providing data on the dose response effects of chrysotile and crocidolite, this study demonstrates the merit of dose response assays for the investigation of the tumorigenicity of fibrous dusts particularly where the aim is to rank the activity of dusts against known standard samples. However, analysis of survival can also provide useful information where comparisons are made between several dusts injected at a single, relatively high, dose. In this situation, the more active dusts are likely to elicit a tumour response in the majority of animals, and comparisons using the proportions of animals affected can therefore be an insensitive index of activity. (BOLTON et al 1982)

Throughout this account the 'dose' of an injected dust has been expressed in terms of mass, but in full recognition that the current consensus of scientific opinion favours fibre length as the most important single descriptor of asbestos pathogenicity. Detailed particle size assessments of each dust are currently in progress and will be presented at a later date.

It can be concluded that the asbestos dusts used in the present study have produced clear evidence of a dose-response with nc effective minimum threshold and that chrysotile was considerably and consistently more active than an equivalent mass of crocidolite.

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The pathogenic effects of fibrous ceramic aluminium silicate glass administered to rats by inhalation or peritoneal injection

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For many years it has been realized that the industrial use of asbestos can involve considerable health risks. Workers exposed to asbestos dust may develop pulmonary interstitial fibrosis (asbestosis), bronchial carcinomas or mesotheliomas (1).

Because of the hazards of asbestos use, industry has attempted to substitute other types of fibres, many of them man-made. However, knowledge accumulated over the past few years indicates that, for glasses at least, this cannot be done with impunity. As early as 1968 Gross et al. (2) reported that many types of mineral fibre could stimulate the production of pulmonary ferruginous bodies that were similar to asbestos bodies. Potentially more serious, however, was the report by Stanton and Wrench (3) that some preparations of glass fibres implanted into the pleural cavities of rats produced mesotheliomas in similar numbers to those produced by asbestos fibres. This work was later expanded by Stanton et al. (4), and it was suggested that the most carcinogenic size for mineral fibres was > 8 um in length and < 1.5 um in diameter, and that the chemical composition of fibres was not important. Davis (5), Pott et al. (6) and Wagner et al. (7) confirmed the carcinogenicity of glass fibres following intrapleural or intraperitoneal injection into rats, and Wright and Kuschner (8) reported pulmonary fibrosis following the intratracheal injection of long glass fibres into guinea pigs. No fibrosis occurred following the injection of short glass fibres.

So far, inhalation studies with glass fibres have shown little pathological tissue reaction at all. In 1976 Gross (9) reported studies in which rats and hamsters had been exposed to high airborne concentrations of three types of glass fibre with an average diameter of about 1 um. Almost no fibrosis was found and no tumours developed. A similar study was reported by Lee and his co-workers (10). Here the dust concentration used was also high and some alveolar proteinosis occurred but no fibrosis and no tumours were found. In a follow-up paper (11) the same group reported studies in which rats, hamsters and guinea pigs were exposed for three months to a dense cloud of glass fibre dust. No

fibrosis occurred in any of these species but two benign pulmonary adenomas were found in both the rats and the guinea pigs. Hardy (12) exposed rats to glass fibre for eight weeks with a follow-up period of four weeks. No effects on pulmonary function were noted during this period and no pulmonary fibrosis developed.

At the same time that experimental studies have been in progress, human populations of workers exposed to glass fibre have also been examined. Here again, the results have so far been negative. In 1968 Wright (13) examined chest radiograms from 1400 men in the American glass fibre industry who had been exposed for at least 10 years. He found no evidence of radiological abnormality. In 1971 Gross et al. (14) examined the lungs obtained at autopsy of 20 workers exposed during the manufacture of glass fibre. Very little dust was present in these lungs and no dust-associated pathology. In 1973 Hill et al. (15) examined a group from the British glass fibre industry. Only 70 men were involved, but their mean exposure was 20 years and both chest radiograms and lung function data were available. Once again no evidence of lung damage could be detected. Enterline and Henderson in 1975 (16) and Bayliss et al. in 1976 (17) reported studies of the incidence of pulmonary tumours in two groups of American glass fibre workers consisting of 416 and 1448 men. They found that the incidence of pulmonary tumours was slightly less than in the general population.

So far, no information is available on the pathogenic effects of the ceramic aluminium silicate glass forms of man-made mineral fibre. The present paper reports the findings from a long-term inhalation and injection study in which this material was administered to rats.

Materials and Methods

A group of 48 SPF Wistar rats of the AF/HAN strain was exposed to fibrous ceramic aluminium silicate glass (ceramic fibre) dust for 7 hours a day, 5 days a week, for a total of 224 days during a period of 12 months. The animals were approximately three months old at the start of dusting. A batch of 40 undusted animals was maintained within the same unit as controls during the same time period. The planned dust concentration was 10 mg/m^3 of respirable dust. Initially the bulk ceramic insulation material was passed through two steel rollers to break up the longest fibres which were often several millimetres long. Finally the dust clouds were generated with a Timbrell dust generator and inhalation chambers, as described by Timbrell et al. (18) but modified as described by Beckett (19). Size-selecting the airborne dust by a cyclone system prior to injecting it into the chamber airstream ensured a high proportion of respirable dust in the clouds. Gravimetric monitoring was carried out during all dust exposure time. Daily mass concentrations were measured with an open filter-holder to collect total dust, and an NCB MRE sampler (Casella Type 113A, Dunmore et al. (20)) to collect respirable dust. The

dust generating system and chamber ventilation were adjusted in response to these measurements so as to produce the target mean concentration.

On 100 separate days additional dust samples were obtained for the estimation of fibre numbers and dimensions by means of the standard sampling method described by the Asbestosis Research Council (21). Each membrane filter sample was taken with an open Gelman filter-holder facing downwards at a flow rate and sampling time calculated to give an optimum density for counting by phase-contrast microscope. All fibres longer than 5 um were counted provided that they had an aspect ratio of at least 3 : 1 and their diameters were less than 3 um. Some samples were obtained with Nuclepore filters for subsequent examination by a Cambridge Instruments S600 scanning electron microscope at a (10 000x) magnification higher than available by optical microscopy (500x). Some of the samples originally collected on Nuclepore filters were also examined by transmission electron microscopy with an AEI CORA microscope. For both light and electron microscope counts, fibres were considered to be all particles with an aspect ratio greater than 3 : 1.

At the end of the 12-month dusting period four dusted animals were killed and six months later four more were killed. The experiment was terminated at 32 months for comparison with previous long-term inhalation studies from this unit, by which time seven dusted animals remained alive. Four control animals were killed 12 months after the start of the experiment but the remainder were allowed to live out their full life span or until the experiment was terminated. Lung tissue from all dusted animals from the fixed killing dates at 12 and 18 months, and six animals from the final killing date of 32 months, was used for estimations of pulmonary fibrosis and examined for the presence of pulmonary neoplasms. Lungs from the remaining animals were examined only for the presence of neoplasms. Tissue used for histological examination was fixed with 10% formal saline solution and embedded in paraffin wax. Lungs were fixed by inflation in situ until they filled the thoracic cavity. Sections were stained by either haematoxylin and eosin, Van Giesen's method for collagen, or Gordon and Sweet's stain for reticulin. Lungs and heart were embedded together and sections were cut in the coronal plane. Sections were cut at several different levels in each block, at least 1 mm apart, and the groups of serial sections were mounted from each of these levels for use with the different staining techniques. For quantitative estimations of pulmonary interstitital fibrosis four slides from each animal were examined with an electronic image analyser (Graphic Information Systems Ltd., GDS1). The areas of interstitial fibrosis were calculated and expressed as a percentage of total lung tissue. Finally the results from all animals in each group were averaged.

For the four dusted animals killed 18 months after the start of dusting, the left lung was used for dust extraction and only the right was available for histological study. Dust retained in the lungs was

recovered by a low-temperature plasma ashing process (22) with a Nanotech P100 apparatus. The residues were washed in 0.2 M HCl at room temperature before the amounts of ceramic fibre recovered were estimated by means of the infra-red spectrophotometric techniques described by Beckett et al. (23). Studies in this laboratory have shown that in rats the dust content ratio between left and right lungs after experimental inhalation of fibrous dust such as asbestos is 0.6 : 1; this correction factor was therefore used to calculate the total pulmonary dust burden of each animal.

In addition to the inhalation studies, the ability of the ceramic fibre to produce mesotheliomas was examined by means of the intraperitoneal injection assay. A dose of 25 mg of dust suspended in 2 ml of Dulbecco's phosphate buffered saline was injected under ether anaesthetic into the peritoneal cavities of a group of 32 rats of the AF/HAN strain. The dust was collected from the inhalation exposure system and was as similar as possible to the dust entering the inhalation chamber (24).

Results

The target mean mass concentration of 10.0 mg/m^3 of respirable dust was achieved with some variation from day to day, as indicated by a standard deviation of 4.8 mg/m^3. The total dust mass concentration was 8.4 mg/m^3 (standard deviation 9.6 mg/m^3). Phase-contrast microscope counts of fibres (longer than 5 um, narrower than 3 um, and aspect ratio greater than 3 : 1) gave a concentration of 95 fibres/cm^3. This mean number concentration is less than those previously found for a series of UICC standard reference samples of asbestos (25), which exceeded 500 fibres/cm^3. This difference in number for the same respirable mass concentration is due not only to differences in size distribution but also to the presence of considerable numbers of non-fibrous particles in the ceramic dust cloud (Fig. 1). By light microscope estimation, the ratio of particles (more than 1 um diameter) to fibres (longer than 5 um) was approximately 4 : 1.

Fibre length distributions for the fibres > 5 um in length were obtained by both light microscopy and scanning electron microscopy; the numbers of fibres sized were 2000 and 900 fibres respectively. The results were almost identical (Fig. 2). This was because the diameters of almost all fibres longer than 5 um were found to be > 0.3 um; thus, theoretically, they should all have been visible on light microscope examination. While long and thin fibres were extremely rare, however, complete length and diameter distributions obtained by scanning electron microscope of all fibres longer than 0.04 um showed the presence of large numbers of short thin fibres (Fig. 3 and 4). Approximately 90% of fibres were shorter than 3 um and less than 0.3 um in diameter. Most of these short thin fibres did not have the neat cylindrical shape of the larger ceramic fibres and in outline were often irregular (Fig. 5). Their method of formation has not yet been determined. While they made up the bulk of

the dust cloud by numbers, they could have represented only an extremely small proportion by mass. They could have been present in the original bulk sample of ceramic fibre or been formed by the fragmentation of larger particles during dust generation. The same problem arises with the many non-fibrous particles present in the dust cloud.

Throughout the study the animals tolerated the exposure regime well and none died during the dusting period. The numbers of animals surviving at varying time points throughout the remainder of the study are given in Table 1; these figures are better than any from groups previously exposed to asbestos (25). The figures for the control animals were very similar. Histological examination of lung sections from the groups of dusted animals killed at the end of the dusting period and six months later revealed a different pattern of pulmonary damage from that previously found in rats exposed to asbestos (25-27). There were very few areas of peribronchiolar fibrosis of the type found with some forms of asbestos, especially chrysotile; detailed quantitative estimations were not undertaken therefore. Often those areas of fibrosis that were present could be recognized with certainty only at magnifications higher than were usually used for these estimates. Instead of peribronchial fibrosis the lungs of animals treated with ceramic aluminium silicate fibre showed large areas of alveolar proteinosis (Fig. 6). Within the proteinaceous material many dust particles were visible with the light microscope. At the first killing date much of the dust was extracellular, but at the second the alveoli affected with proteinosis more frequently contained foamy macrophages as well as protein, and these cells usually contained many of the dust particles. Most of the visible dust appeared to be particulate rather than fibrous and those fibres that were visible were relatively thick, with diameters of 2-3 um. They ranged from 5 um to 50 um in length. However, the reaction of the rat lung tissue to these fibres appeared to have been different from that due to asbestos, in that many fibres had become coated with Perls positive material to become ferruginous bodies (Fig. 7). Asbestos fibres are rarely coated in rat lungs but other species produce ferruginous bodies readily.

Lungs of animals from the final killing date showed only small amounts of alveolar proteinosis, but where this did occur the proteinaceous material still contained some extracellular dust particles as well as dust-containing macrophages. However, in general much less dust was visible with the light microscope than at earlier dates. Most of this remaining visible dust was contained in pulmonary macrophages, aggregations of which occurred in the alveoli close to the respiratory bronchioles. By the end of the study many of the dust-containing cells also contained masses of Perls positive material, but with the light microscope it was not possible to determine whether this had originated from fragmented ferruginous bodies.

One animal from each of the first killing dates at 12 and 18 months had small amounts of interstitial fibrosis in its lungs although this

occupied less than 0.01% of total lung area in each case. However, this type of lesion occurred in most of the older animals and some were severely affected (Fig. 8). On average, 5.0% of the lung area of the six animals examined from the final killing data had interstitial fibrosis; this figure ranged from 0.2% to 14.5% for individual animals. Within these areas of interstitial fibrosis only small amounts of dust were visible and this was mainly particulate. Two of the control animals from the final killing date at 32 months had very small areas of interstitial fibrosis but in each case these did not exceed 0.01% of total lung tissue area.

In the present study eight animals in all were found with pulmonary neoplasms. One tumour was a benign adenoma and three were carcinomas. Two of the carcinomas showed only a squamous histological pattern but the other had areas of both squamous and adenocarcinomatous appearance. In addition to these tumours, however, which were of similar type to those found in rats after asbestos treatment, some animals in the present study had pulmonary deposits of tumours which appeared to be malignant histiocytomas. These consisted of irregularly shaped cells of epithelioi type interspersed with multinucleate giant cells (Fig. 9). In four animals the tumours were obviously malignant with very large masses occupying most of one or more lung lobes. In one of these cases multipl nodules were also present in the pleural cavity, and the mediastinal lym nodes were involved, but in the others the tumour appeared restricted to the lung tissue. A further six animals showed small areas of histologic pattern similar to the larger tumours, in the centres of areas of interstitial fibrosis. While in these cases there was no definite indication of malignancy it appeared likely that these were early stages of tumour development. No pulmonary tumours of any type were found in t control animals.

In addition to tumours primarily associated with the lung, eight benign and eight malignant tumours, including one peritoneal mesothelion were also found in other tissues in the group of animals exposed by inhalation to ceramic aluminium silicate. Similar numbers of non-pulmonary tumours were found in the control animals (Table 2). Thre animals from the dust-exposed group and three from the controls had two neoplasms at autopsy. In two of the cases from the dust-exposed group c of the neoplasms was present in the pulmonary tissue.

The lung-dust burden of four rats killed six months after the end c dusting was extracted and its mass estimated with infra-red spectro-photometry. The mass of ceramic aluminium silicate in a complete lung ranged from 2800 ug to 6800 ug, with a mean of 4130 ug. This means tha the mass of ceramic fibre recovered from the rat lungs at this point wa approximately half of the figure found after inhalation of the same mas of UICC crocidolite or amosite, although it was more than six times hig than the figure found after inhalation of UICC chrysotile (25).

After the intraperitoneal injection of 25 mg of elutriated ceramic aluminium silicate dust, three animals developed peritoneal tumours. This is in contrast to findings with most asbestos preparations, which show that at this dose level more than 90% of animals usually develop mesotheliomas (24). Only one of the three tumours was a typical multinodular mesothelioma, with blood-stained ascites. The other two consisted of large single masses with few, if any, minor nodules. The histological picture of the large masses was similar to that of a fibrosarcoma. In addition, the first tumour produced by aluminium silicate fibre did not occur until approximately 850 days after injection, in contrast to some types of chrysotile asbestos where the first tumours occurred in as little as 200 days.

Discussion

With the same types of generator as those previously used for different kinds of asbestos, it has proved possible to generate respirable clouds of ceramic aluminium silicate glass fibres. However, the number of fibres > 5 um in length and < 3 um in diameter, as seen with the light microscope, was only 95 fibres/cm^3 in a cloud with a respirable mass of 10 mg/m^3 of air. This compared with figures of approximately 500 fibres/cm^3 and 2000 fibres/cm^3 for previously examined clouds with the same respirable mass of UICC amosite and chrysotile (25). The reasons for the low fibre number in the case of ceramic fibre were that almost all the relatively long fibres were thick and that the dust cloud contained much particulate dust. There were also large numbers of short thin fibres, visible only by electron microscopy, but these could have constituted only an extremely small proportion of the total mass.

Regardless of the number of long fibres in the dust cloud, particles of ceramic fibre predominated at all times in histological sections and relatively few fibres could be found, although the mass of retained dust was relatively high. It is possible that most of the long but relatively thick fibres were deposited in the larger bronchial tubes and were rapidly cleared, and only particles and short thin fibres reached the alveoli in any numbers; or that the ceramic fibres are apt to break up in lung tissue fluids, with the result that few long fibres remain for any length of time. However, the ceramic fibre used in these studies is known to be particularly resistant to chemical attack by both acids and alkalis in laboratory conditions, and it is difficult to envisage chemical conditions within lung tissue that could cause it to break up.

Whether the process of ferruginous body formation could be associated with the subsequent break-up of ceramic fibre is uncertain, although Botham and Holt (28) have suggested that this process contributes to the break-up and removal of asbestos fibres in the lungs of some animal species. These workers believed the process was mechanical but Jaurand et al. (29) have reported that the chemical leaching of chrysotile fibres

in lung tissue was more marked in the case of fibres in the centre of ferruginous bodies than in the case of uncoated fibres.

Regardless of these considerations, the inhalation of ceramic aluminium silicate dust in rats produced considerable pathological change including malignancy. However, the pattern of lesions was significantly different from that found with asbestos (25). Peribronchiolar fibrosis, which is particularly marked in rats treated with chrysotile and which reached its maximum by the end of the dusting period, was almost non-existent in animals treated with ceramic fibre. However, interstitia fibrosis, also common with chrysotile, did occur in animals treated with ceramic fibre, to a lower but not significantly different degree from tha occurring in chrysotile treated animals.

From this it would appear that large numbers of long thin fibres are probably needed to produce peribronchial fibrosis but that such fibres ma not be necessary for the development of interstitial fibrosis. Since in the studies mentioned, crocidolite and amosite clouds, which contained many short thin fibres, caused little interstitial fibrosis, the short fibres are unlikely in themselves to be the main cause of interstitial fibrosis in the experiments with ceramic fibre. Factors other than fibre length must therefore be considered. The occurrence of large areas of alveolar proteinosis in rat lungs at the end of the dusting period indicates that the ceramic dust (fibres and particles) may have a toxic effect on the lung tissue. Perhaps damage is done to the alveolar walls at this stage which, although not visible with the light microscope, nonetheless results in interstitial fibrosis as the animals age.

While relatively large numbers of neoplasms occurred in the lung tissue of animals treated with ceramic aluminium silicate, the pattern o tumour development was different from that previously seen with asbestos (25). Only one benign pulmonary adenoma was found, a finding comparable to that obtained previously with amphibole asbestos types and the same number of rats, but significantly less than the six or seven adenomas found in previous experiments with UICC chrysotile ($P < 0.05$). However, although the amphibole asbestos types at a dose of 10 mg/m^3 produced no malignant pulmonary tumours, three bronchial carcinomas occurred in animals treated with ceramic fibre; since eight bronchial carcinomas were found in animals treated with UICC chrysotile, the ceram fibre takes an intermediate place, in that the number of tumours it produced was not significantly different from the numbers produced by either of the other two substances. However, the finding of malignant histiocytomas in the group of animals treated with ceramic aluminium silicate is completely different from any results so far published from experimental studies related to asbestos. In previous inhalation studie with asbestos in this laboratory an occasional animal was found with pulmonary deposits similar to the malignant histiocytomas. However, deposits were also present in other organs, and the lung did not appear be the primary site. They were therefore classified under the general

heading of lymphomas. In the present study all these tumours appeared to involve mainly lung tissue and there was evidence of systemic involvement in only one animal. This suggests that this type of tumour occurred in direct response to the inhalation of ceramic fibre, but at present there is no information as to why this specific response occurs with ceramic fibre but not with asbestos.

The occurrence of tumours caused by a nor asbestos dust in rats was also reported by Wagner and Wagner (30). In this case the tumours resulted from the intrapleural injection of quartz and grew mainly in the pleural cavity, being diagnosed as thymomas. However, some of the histological patterns reported were very similar to the malignant histiocytomas found in the present study.

The ability of ceramic fibre to cause pulmonary neoplasms contrasts with the small numbers of mesotheliomas that developed following intraperitoneal injection. Only three tumours were produced by this dust when close to thirty would have been produced by similar doses of a number of asbestos types (24). Work by Stanton et al. (3,4) has suggested that the mineral fibres most likely to cause mesotheliomas after intrapleural implantation are those > 8 um long and < 1.5 um in diameter. Since the ceramic fibre dust used in the present study had relatively few fibres of this size per cm^3, as well as much particulate material and many short thin fibres, the low production of mesotheliomas is in keeping with Stanton's suggestions. However, it had appeared likely that the fibres most able to produce mesotheliomas were also likely to be the most carcinogenic to lung tissue. The findings from the present study suggest that this may not be the case.

Since the inhalation of dust from ceramic aluminium silicate insulation material has produced both pulmonary fibrosis and neoplasia in rats, the use of these man-made mineral fibres in industry should be undertaken with caution.

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MMMF

Table 1. The survival pattern of rats treated with ceramic aluminium silicate glass fibre dust by inhalation[a]

	Months after the start of dust exposure			
	12	18	24	3
Number of surviving dusted rats Group size, 48	48	44	33	
Number of surviving controls Group size, 40	39	32	29	

[a] The survival pattern of a group of control animals is included fo comparison. The experiment was terminated at 33 months after the start dusting. Groups of four treated animals were killed at both 12 and 18 months. A group of four control animals was killed at 12 months.

Table 2. Sites of tumours, other than lung

Organ system	Ceramic fibre		Control	
	Benign	Malignant	Benign	Malignant
Digestive/peritoneal	3	3[a]	3	2
Urinogenital	3	-	1	2
Endocrine	1	-	2	1
Musculo, skeletal and integumentary	1	2	3	2
RE/vascular	-	3	2	2
Total	8	8	11	9

[a] Includes one peritoneal mesothelioma.

Fig. 1. Light microscope photograph of a sample of the ceramic aluminium silicate glass fibre used in the present study; magnification, 4



Note: this dust was collected from the air in the inhalation chamber.

Fig. 2. Length distribution of ceramic aluminium silicate fibres longer than 5 um

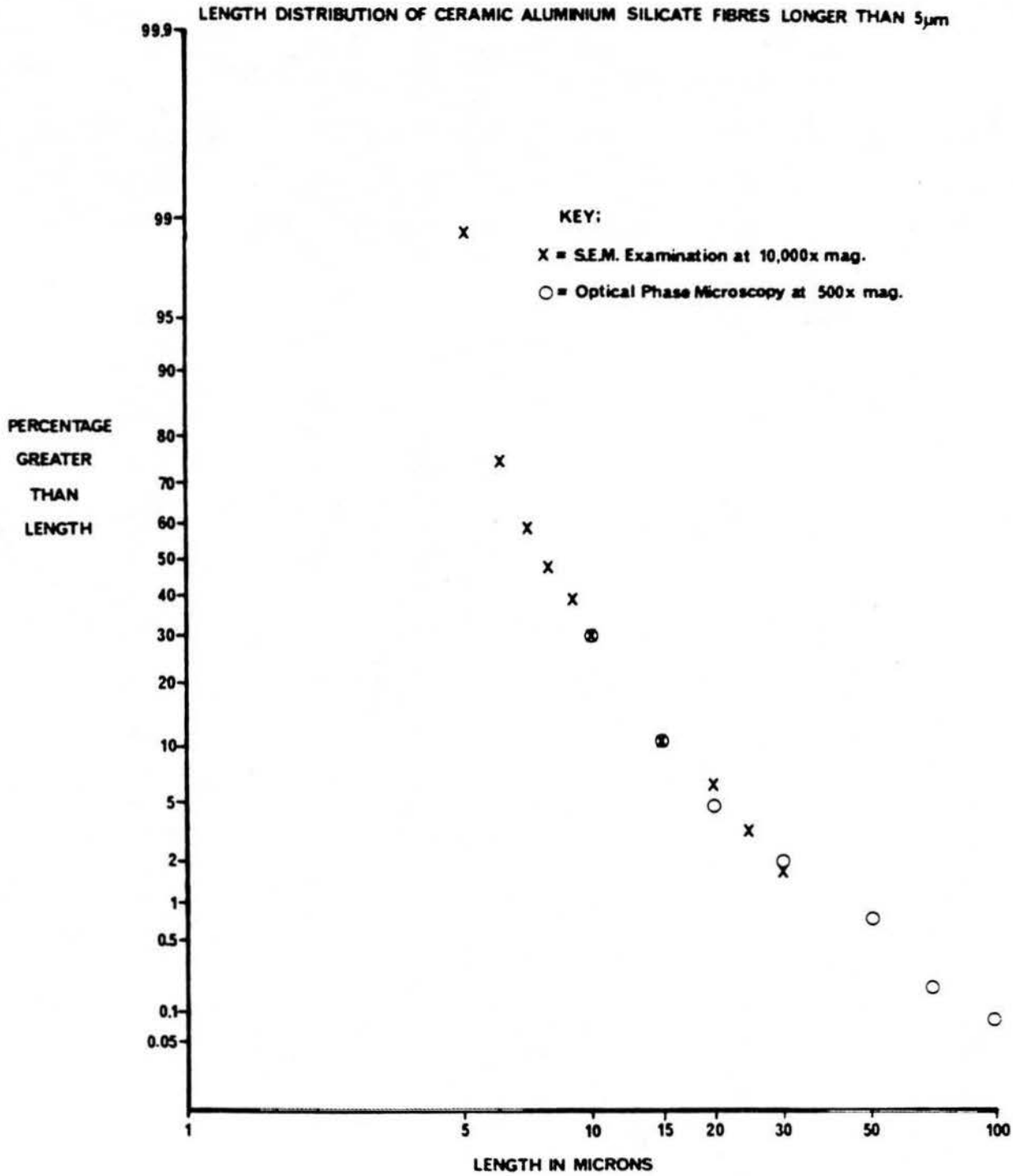
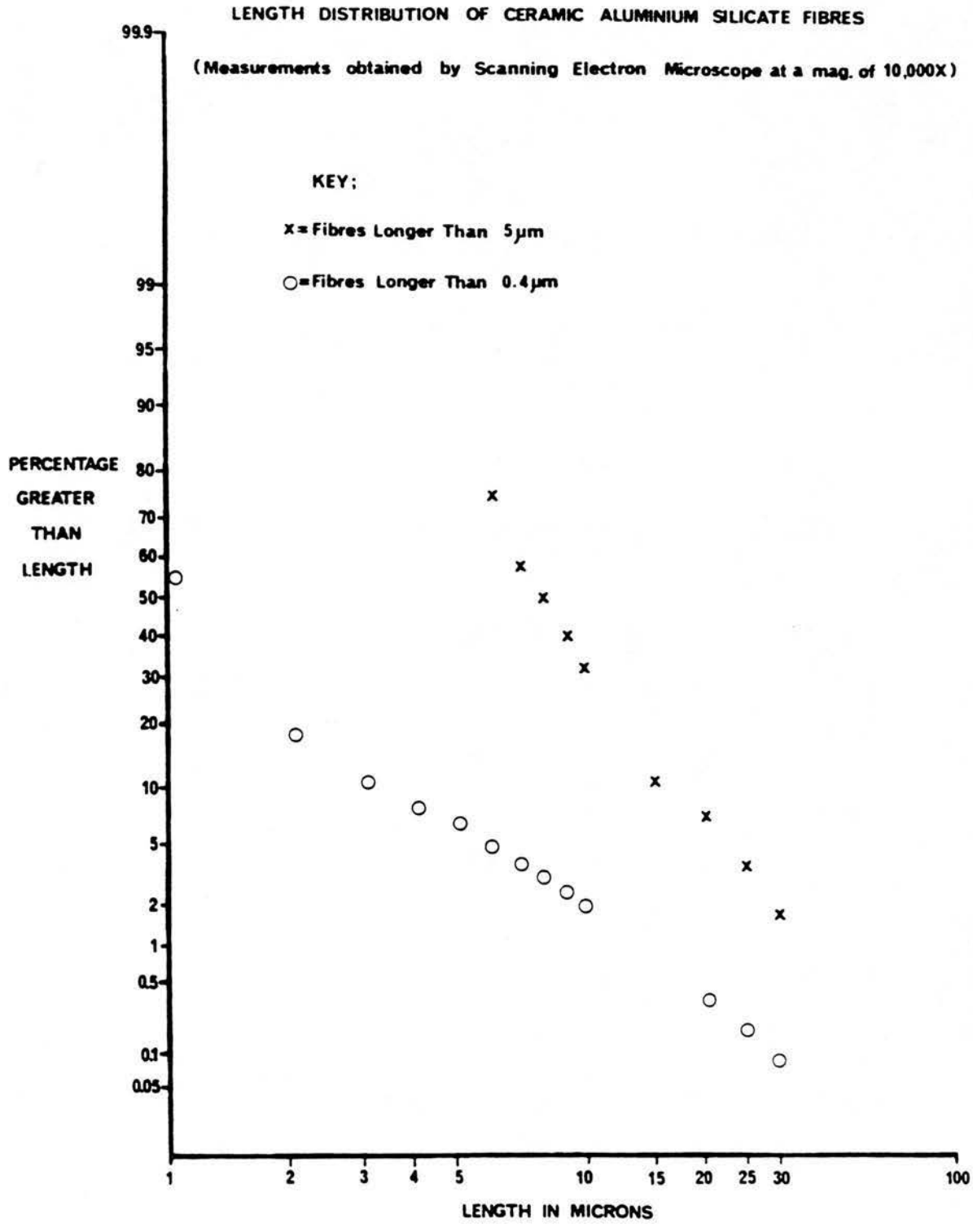
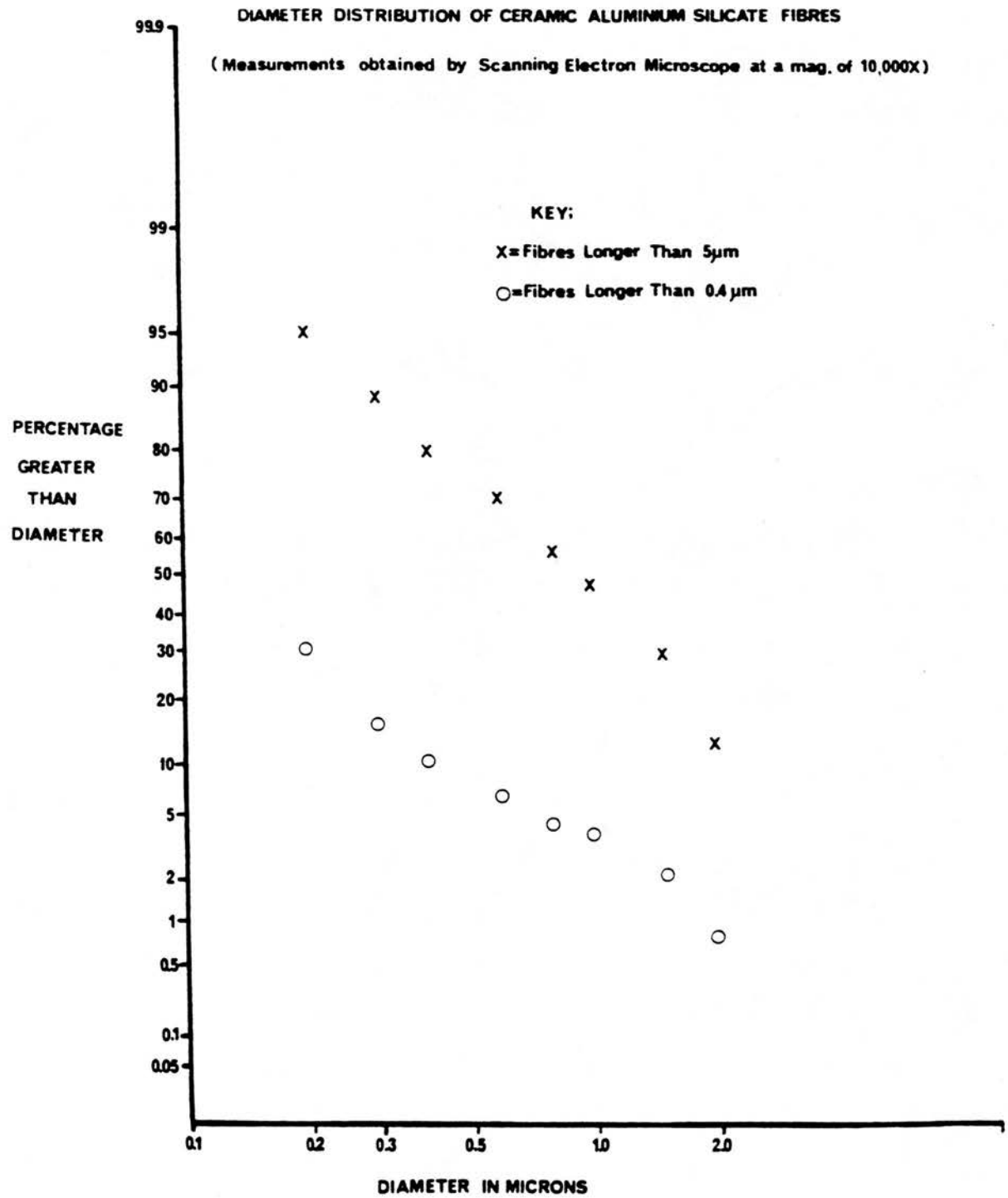


Fig. 3. Length distribution of ceramic aluminium silicate fibres



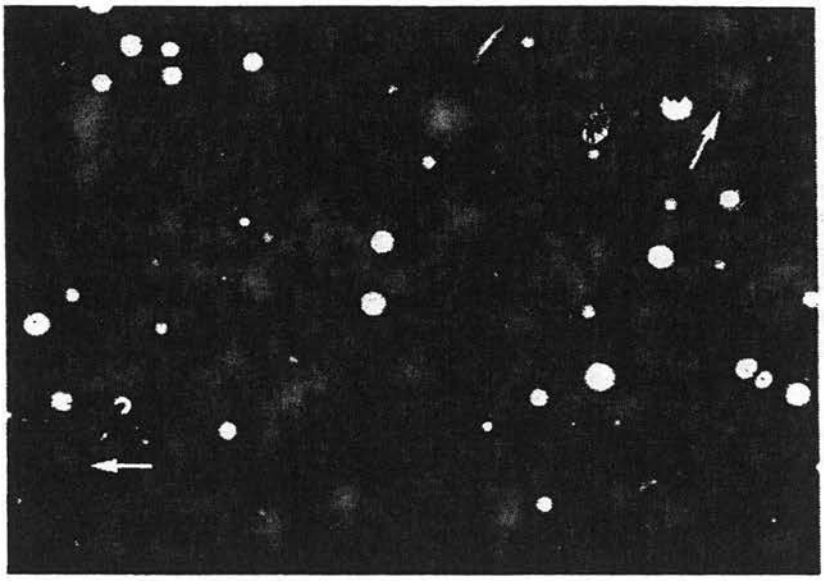
Note: measurements obtained by scanning electron microscope at a magnification of 10 000x.

Fig. 4. Diameter distribution of ceramic aluminium silicate fibres



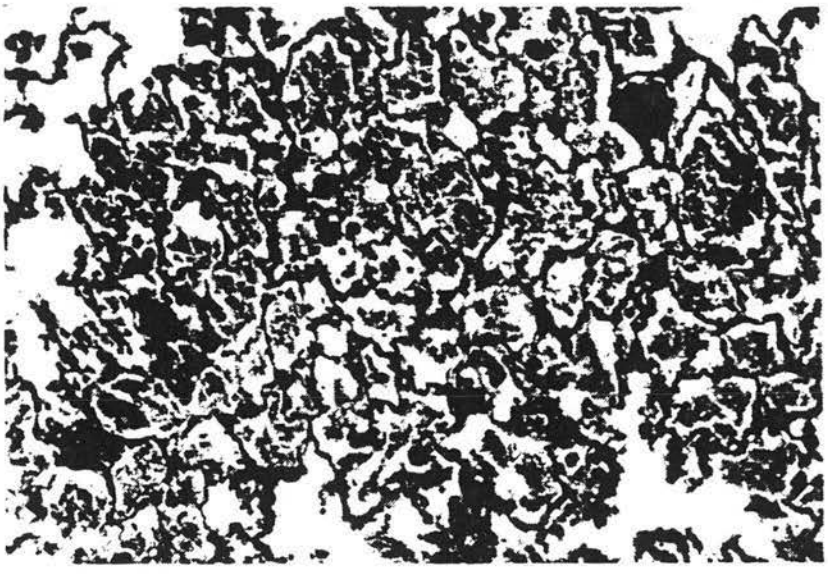
Note: measurements obtained by scanning electron microscope at a magnification of 10 000x.

Fig. 5. A transmission electron microscope photograph of small dust particles from the ceramic fibre cloud; magnification, 6000x



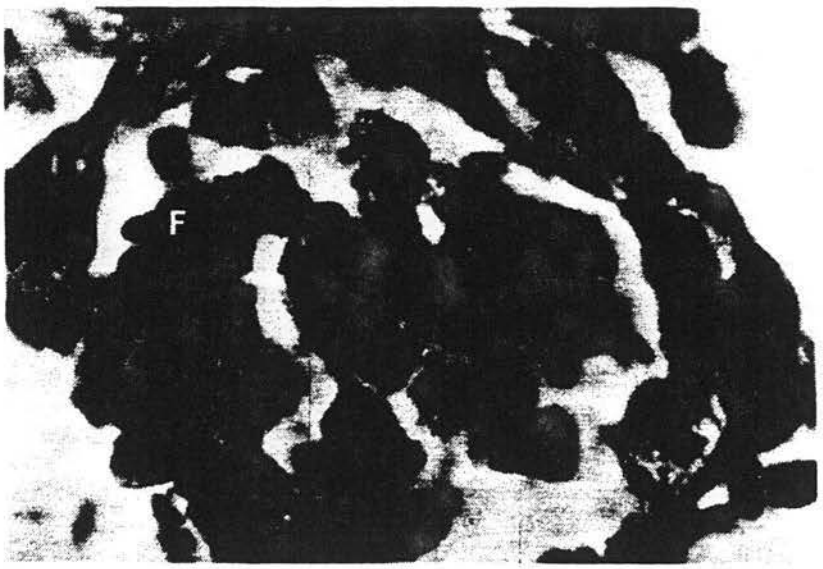
Note: elongated particles counted as fibres because their aspect ratio was greater than 3 : 1 are arrowed.

Fig. 6. An area of alveolar proteinosis from the lungs of a rat after 12 months of dusting with ceramic fibre; magnification, 250x



Note: the alveolar septa are not thickened but the alveolar spaces are filled with proteinaceous material.

Fig. 7. An alveolar space from the lungs of a rat 33 months after the start of dusting with ceramic fibre; magnification, 1500x



Note: the alveolus is largely filled with macrophages containing particulate dust, but one ferruginous body (F), 25 um in length, is present.

Fig. 8. An area of interstitial fibrosis from the lungs of a rat 33 months after the start of dusting with ceramic fibre; magnification, 250x

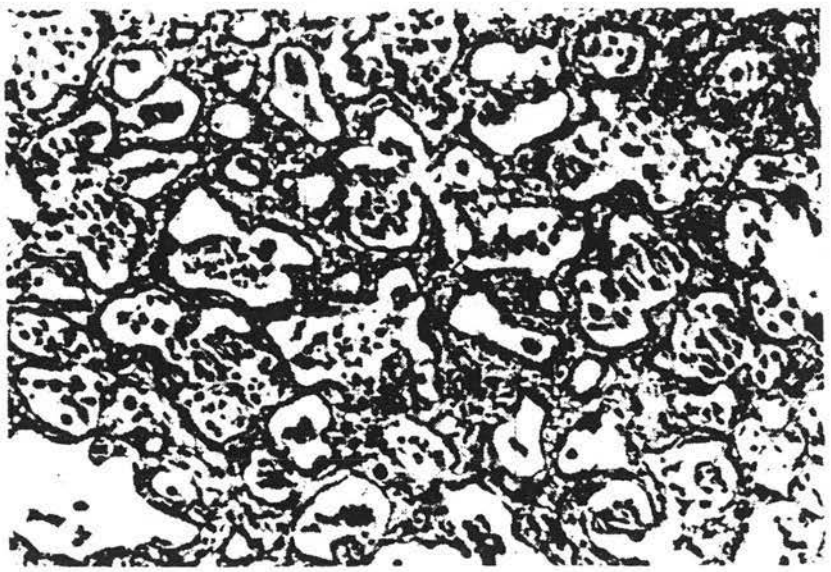
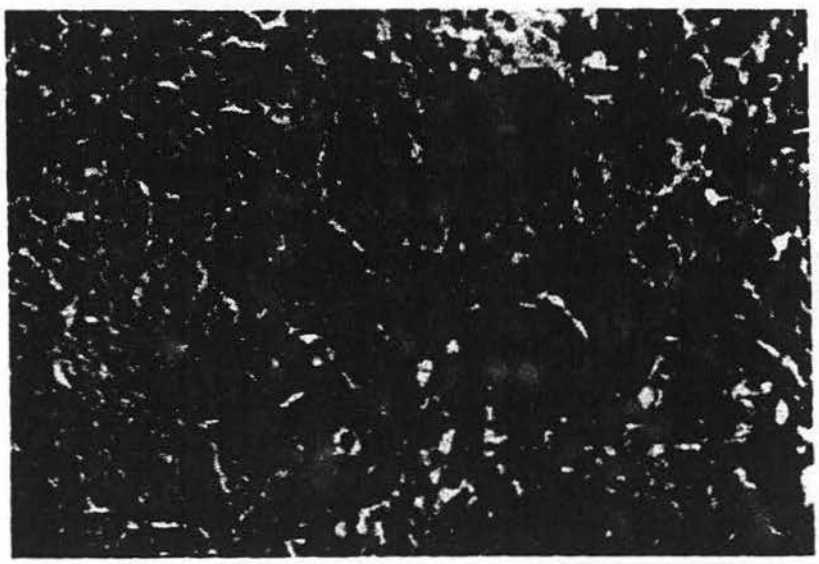


Fig. 9. The histological pattern shown by a malignant histiocytoma from the lungs of a rat treated with ceramic fibre dust; magnification, 450x



Note: most of the cells are of epithelioid type, but several multinucleate giant cells are also present.

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The pathogenicity of long versus short fibre samples of amosite asbestos administered to rats by inhalation and intraperitoneal injection

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Summary. For many years it has been accepted that fibre dimensions are the most important factor in the development of asbestos related disease with long fibres being more dangerous than short for all types of asbestos. This information has been derived from *in vitro* experiments and injection or implantation experiments since the kilogramme quantities of specially prepared dusts that are necessary for long term inhalation have not been available. The present study has taken advantage of the availability of a sample of amosite produced so that almost all fibres were less than 5 μm in length. The effects of this dust were compared to dust prepared from raw amosite that contained a very high proportion of long fibres. Previous data from studies with UICC amosite, which was intermediate in length, were also available for comparison. At the end of 12 months of dust inhalation, significantly more short fibre amosite was present in the lung tissue compared to the long but while the long fibre dust caused the development of widespread pulmonary fibrosis, no fibrosis at all was found in animals treated with short fibre. One third of animals treated with long fibre dust developed pulmonary tumours or mesotheliomas but no pulmonary neoplasms were found in animals treated with short fibre dust. Following intraperitoneal injection, the long fibre amosite produced mesotheliomas in 95% of animals with a mean induction period of approximately 500 days. With short fibre dust, only a single mesothelioma developed after 837 days. In previous inhalation studies with UICC amosite, relatively little pulmonary fibrosis had developed and only two benign pulmonary tumours. This would suggest that to produce a significant carcinogenic response in rat lung tissue amosite fibres must be longer than those in the UICC preparation. Following the injection of UICC amosite, however, mesotheliomas developed in the same proportion of animals and with the same mean induction period as with long fibre dust. From this it would appear that while very short fibres exhibit little carcinogenicity to either lung or mesothelial tissues, mesotheliomas can be produced by dust preparations consisting of shorter fibres than are needed to produce tumours.

Keywords: amosite asbestos, fibre length, pulmonary fibrosis, inhalation, injection

The inhalation of asbestos dust may produce both pulmonary fibrosis and neoplasia in man and experimental animals and many experimental studies have been undertaken

in an attempt to determine the mechanisms by which asbestos fibres produce these pathogenic effects. A number of early studies suggested that the most important factor in fibrogenesis was the silica content of asbestos which stimulated collagen production by chemical action (Beger 1934, Kuhn 1941). As early as 1946, however, King *et al.* (1946) administered chrysotile fibres cut on a special microtome at lengths of 15 μm and 2.5 μm to rabbits by intratracheal injection. They reported a greater tissue reaction from those animals that had recieved the long fibre sample. Later, Vorwald *et al.* (1951) reported that animals which had inhaled chrysotiie fibres in the 20–50 μm range had more pulmonary fibrosis than those breathing only dust with fibres below 3 μm in length. Scymczykiewicz & Wiecek (1960) obtained similar results when they administered fibrous and amorphous asbestos dust to guinea-pigs by intratracheal injection. They did not, however, give details of the asbestos type employed.

Hilscher *et al.* (1976) extended these studies using both the intratracheal and intraperitoneal injection of finely ground chrysotile and crocidolite. They found these fibres produced little or no fibrosis in either site. In contrast, longer fibres of the same asbestos types resulted in considerable fibrosis in both regions. Davis (1972) conducted a series of experiments using the intrapleural injection of a number of different mineral types including long and short fibre chrysotile. The short fibre samples were either synthetic chrysotile with a maximum crystal length of 1 μm or chrysotile fragmented by ultrasonic treatment until all fibres were below 1 μm in length. While the long fibre samples produced extensive fibrosis, the short fibre specimens produced almost no tissue reaction. Wright & Kuschner (1977) reported that following the intratracheal injection of long and short samples of both asbestos and glass fibre all the long fibre samples produced considerable fibrosis while the short samples did not.

The fact that fibre dimensions were important in carcinogenesis as well as fibrogenesis was demonstrated by the work of Stanton *et al.* (1972, 1977, 1981). Stanton's group implanted numerous samples of carefully sized fibrous dust into pleural cavities of rats and reported that fibres >8 μm in length and <1.5 μm in diameter appeared most effective in producing mesotheliomas. These findings have been confirmed by Pott & Friedrichs (1972) and Pott *et al.* (1976) using intraperitoneal rather than intrapleural implantation of fibrous dusts.

Until recently it was difficult to examine the importance of fibre length on pulmonary pathology in long term inhalation studies because large amounts of specially prepared dusts are required for such work. In 1981, however, the Manville Corporation in the USA prepared several kilograms of short fibre amosite and supplied the Institute of Occupational Medicine in Edinburgh with sufficient of this material to undertake the present study.

Materials and methods

Amosite samples used for dust cloud generation. Both the long and short amosite dusts used in these studies were prepared from the same batch of South African amosite. The short fibre amosite sample was prepared and characterized by the Manville Corporation in the USA by grinding in a ceramic ball-milled system followed by sedimentation in water. The resulting materials was so fine that only 37% of particles had an aspect ratio $>3:1$ and were therefore regarded as fibres. The mean length of these fibres was 2.68 μm while the mean length of all particles was 1.42 μm. The crystallinity of both the fibrous and non-fibrous particles was checked by transmission electron microscopy (TEM). All particles examined showed a high degree of crystallinity as indicated by the presence of characteristic spot patterns seen in electron diffraction and well ordered periodic lattice fringes seen in high resolution images. No evidence of damage to the crystal lattice or loss of crystallinity induced during the prep-

aration process could be found. In addition analysis of individual particles was undertaken in the TEM by energy dispersive X-ray spectroscopy. The elemental composition of each particle was consistent with that of reference amosite samples from South Africa. The long fibre amosite sample was prepared at this Institute from the same batch of amosite that was used to prepare the short fibre material.

Dust cloud generation and monitoring. The dust inhalation phase of this study was undertaken in inhalation chambers of the type described previously by Beckett (1975). The animals were housed, fed and watered in the chamber throughout the exposure phase.

For the generation of the long fibre amosite cloud, the modified Timbrell dust generator (Beckett 1975) proved satisifactory but dust clouds generated from the short fibre material with this apparatus were found to contain aggregates. For this dust, therefore, a fluidized bed generator (Marple *et al.* 1978) (TSI model 3400) was used. In order to reduce the risk of dust aggregation even further both dust clouds were exposed to a thallium 204 source of β-particles to reduce the electrostatic charge on the airborne fibres before entry into the exposure chambers (Liu & Pui 1974). These techniques produced dust clouds consisting almost entirely of individual fibres or particles.

The mass concentration of the dust in the chambers was monitored daily by sampling throughout the 7 h of exposure using both an open filter holder facing vertically downwards and a Casella MRE113A dust sampling instrument (Dunmore *et al.* 1964). The former sampler monitored the 'total' dust concentration and the latter monitored the respirable dust concentration. The target mean respirable dust airborne concentration of 10 mg/m^3 was achieved by adjusting the generators in responses to each previous days measurement.

Fibre number concentrations and fibre size distributions for the experimental dust clouds were assessed from membrane filter snatch samples collected on 90 separate days. Fibres were counted from all these samples using phase contrast optical microscopy (PCOM) at a magnification of $\times 600$ and fibre sizing was undertaken by scanning electron microscopy (SEM) at a magnification of $\times 10\,000$. With PCOM only fibres with a length > 5 μm, a diameter < 3 μm and an aspect ratio of greater than 3:1 were considered (ARC 1971, AIA 1979). With SEM examination all fibres longer than 0.4 μm were measured if their aspect ratio was greater than 3:1.

Animal inhalation studies. For the inhalation studies groups of 48 SPF male Wistar rats of the AF/Han strain were exposed to dust clouds of either long or short amosite for 7 h each day, five days a week for a total of 224 days during a period of 12 months. The animals were 10 weeks old at the start of dusting. Two batches of 36 and 25 undusted animals were maintained within the same unit as controls during the same overall time period.

Four animals from each experimental group were killed at the end of the 12 months dusting period and four more were killed 6 months later. The remaining animals were left for their full life span except that the study was terminated when the number of survivors in one group (the long fibre treatment group) had dropped to six. Estimations of early fibrotic lesions were limited to the small groups of animals from the first two killing dates. However, for the more advanced alveolar interstitial fibrosis occurring in the oldest animals, all those dying within 2 months of the final killing date were included. In practice this produced groups of 18 animals treated with long fibre amosite and 23 animals treated with short fibre amosite. Groups of 9 and 13 control animals of similar age were included in this estimation of intersitial fibrosis. Lungs from all animals in this study were examined histologically for the presence of neoplasms. Samples were also taken for histology from all

other organs showing macroscopic abnormalities. Tables 3 and 4 include only animals surviving 18 months or more after the end of dusting since we have found that few neoplasms, and particularly pulmonary neoplasms, develop in the AF/HAN rats before this time.

Tissue used for histological examination was fixed with Karnovsky's fixative and embedded in paraffin wax. Lungs were fixed by inflation at a standard pressure of 30 cm of fixative. Subsequently, the tracheas were ligated and the lungs excised and immersed in fixative. Sections were cut in the coronal plain at 1 mm intervals and were stained by either haematoxilin and eosin, Van Giesen's method for collagen or Gordon and Sweet's stain for reticulin.

Measurement of pulmonary fibrosis was undertaken by similar methods to those previously published by Davis *et al.* (1978) except that an electronic image analyser (Graphic Information Systems Limited, GDS1) was available for use in conjunction with the light microscope (Davis *et al.* 1985). Single lung sections were examined and the sections selected to contain the maximum area of lung parenchyma. As previously described, interstitial fibrosis was estimated using a $\times 2$ microscope objective lens and is expressed as a percentage of total lung tissue area. Peribronchiolar lesions are more numerous and smaller and so the lung tissue was scanned with an eyepiece graticule covering a tissue area of 2.92 mm^2 and divided into 100 squares. A $\times 4$ objective lens was used. Peribronchiolar lesions were recorded as a percentage of squares containing lesions of this type.

Lung dust estimations were performed on animals from the first two killing dates. Only the left lung was used so that the right lung was available for histological studies. (Studies in this laboratory have shown that the ratio of dust content between left and right lungs following experimental inhalation of fibrous dust such as asbestos in rats is approximately 0.6:1 and this correction factor was therefore used to estimate the total pulmonary dust burden for each animal). Dust retained in the lungs was recovered by a low temperature plasma ashing process using a Nanotech P100 apparatus. Following tissue ashing the amosite residues were washed in 0.2 M HCl at room temperature before estimations of the amounts of retained fibre were made using the infra-red spectroscopy technique described by Bolton *et al.* (1983).

Animal injection studies. In addition to the inhalation studies the ability of the long and short amosite preparations to produce mesotheliomas was examined using the intraperitoneal injection assay. A dose of either 25 mg or 10 mg of dust suspended in 2 ml of Dulbecco's phosphate buffered saline was injected under ether anaesthetic into the peritoneal cavities of groups of 24 rats of the AF/Han strain. The dust was collected from the animals inhalation chambers by an elutriation process chosen to select the respirable fraction of the dust cloud (Bolton *et al.* 1982). The short fibre dust was dispersed in the saline by ultrasonic treatment. All animals were allowed to live out almost all of their dull life span and were killed when moribund.

Statistical techniques. Survival functions for each experimental group were estimated using the product-limit method (Kaplan & Meier 1958). The survival curves for the different series were tested for homogeneity using the Generalized Wilcoxon (Breslow) statistic in the statistical package BMDP (Dixon *et al.* 1983). In order to compare the overall mortality of the four groups of animals, the death of an animal as part of a planned kill was treated as control, all other deaths being treated as responses. Survival curves were also estimated for the response of developing a non-pulmonary tumour. All deaths of animals without non-pulmonary tumours were recorded as censoring events, whilst non-pulmonary tumours were treated as responses irrespective of whether the death was planned or unplanned.

Comparisons of levels of pulmonary fibrosis and lung dust burdens were made using the generalized linear models facilities in the statistical package GENSTAT (Alvey *et al.* 1977). Differences between the induction period for pulmonary and non-pulmonary tumours were analysed using the Mann Witney *U*-test (Siegal 1956).

Results

Dust characterization

The respirable and total dust masses achieved for both dusts in this study are shown in Table 1 as are the fibre numbers >5 μm and >10 μm seen by PCOM.

The short fibre amosite preparation contained very few fibres >5 μm in length (Fig. 1) but the long fibre amosite dust cloud contained a far higher proportion of long fibres than any other amosite preparation previously examined in this laboratory (Davis *et al.* 1978; 1982) with over 11% of all fibres >10 μm in length and 3% >25 μm in length as seen by SEM. The diameter distributions of the two dusts were close as would be expected since the same batch of amosite was used in their preparation but they were not identical. The long fibre preparation in general consisted of slightly thicker fibres. Figs. 2 and 3 illustrate the fibre size distributions obtained by SEM. The percentages of fibres exceeding a given length (or diameter) are plotted against that length.

Table 1. Figures for dust mass and fibre number for the long and short fibre amosite dust clouds

	Long fibre amosite	Short fibre amosite
Total dust mass (mg/m^3 of air)	11.9	11.6
Respirable dust mass (mg/m^3 of air)	10.0	10.0
Fibres >5 μm in length (per ml of air)	2060	70
Fibres >10 μm in length (per ml of air)	1110	12

When plotted on log-probability axes, the data are close to straight lines, indicating that the size distributions are approximately log-normal. Whilst there is a large difference in the length distributions of the two fibre samples (here indicated by the large separation between the two lines), the diameters of the short and of the long fibres are very similar. The slopes of the lines for short amosite are slightly steeper than those for the long-fibre amosite, indicating that the variances in the (logarithmically transformed) lengths and diameters for short amosite are slightly less than the corresponding variances for long amosite.

Pathological findings

There were no significant differences between the overall survival of animals treated with either long or short fibre amosite or the two groups of controls. Animals treated with the long fibre amosite preparation developed the same types of pathological change previously reported in similar inhalation studies from this Institute (Davis *et al.* 1978; 1982; 1985). At the end of the 12 month dusting period the main lesions present were deposits of granulation tissue around the terminal and respiratory bronchioles (Fig. 4). This granulation tissue consisted mainly of macrophages and fibroblasts but some foreign body giant cells were also present. At the end of the dusting period there was marked reticulin staining in the perobroncial deposits although relatively little collagen could be demonstrated by Van Gieson's stain. In lesions from older animals, however, collagen staining increased in intensity. In animals killed after the end of dusting, the overall area of peribronchiolar fibrosis per section was similar but the proportion of lung tissue occupied by these lesions was significantly less (Table 2) ($P<0.01$ variance ratio test). However, the number of lesions per lung section remained the same. The differences was partly due to the increase in size of the rat lungs during this period but also to the fact that there

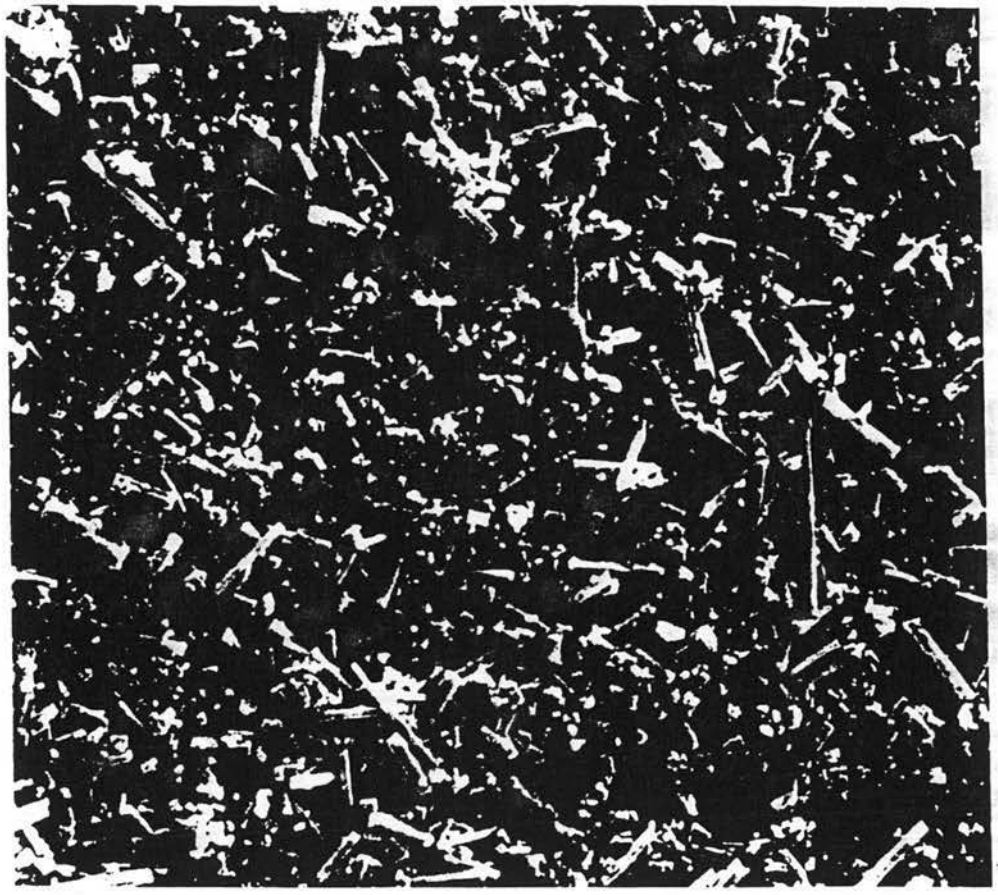


Fig. 1. Scanning electron microscope photograph of the bulk sample of short fibre amosite used in the inhalation and injection studies. × 6000.

appeared to be some contraction of the loose granulation tissue originally formed. As these fibrotic lesions aged, the epithelial cells lining adjacent alveoli frequently became cuboidal in shape (Fig. 5). Animals treated with the short fibre amosite preparation showed no evidence of peribronchiolar fibrosis at any stage. At the end of the dusting period the lungs contained very large numbers of pulmonary macrophages packed with dust particles but these cells remained almost entirely free in the alveolar spaces. Sometimes large groups of these cells were aggregated in alveoli close to respiratory bronchioles (Fig. 6). There was no formation of granulation tissue or thickening of alveolar septa at these points.

In general from about 18 months onwards, areas of lung tissue in animals exposed to long fibre amosite showed a progressive thickening of alveolar septa. In its earliest form this thickening was caused almost entirely by hyperplasia of type II pneumocytes but later there was considerable deposition of reticulin and eventually collagen in the septal walls (Fig. 7). Accumulations of dust were frequently visible among the fibrous tissue in the thickened septa. As shown in Table 2 these areas of alveolar interstitial fibrosis became more extensive as

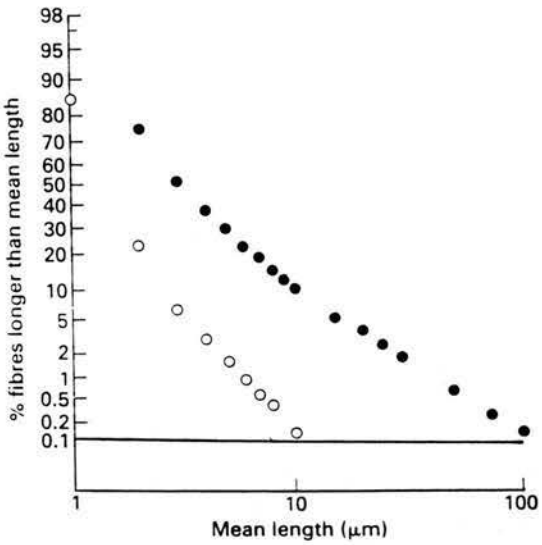


Fig. 2. Fibre length distributions of the long and short fibre amosite dust clouds. SEM sizing at 10 000 magnification 0.4 μm: diameter < 3 μm: aspect ratio > 3 to 1. ○, short fibre amosite (700 fibres sized); ●, long fibre amosite (700 fibres sized).

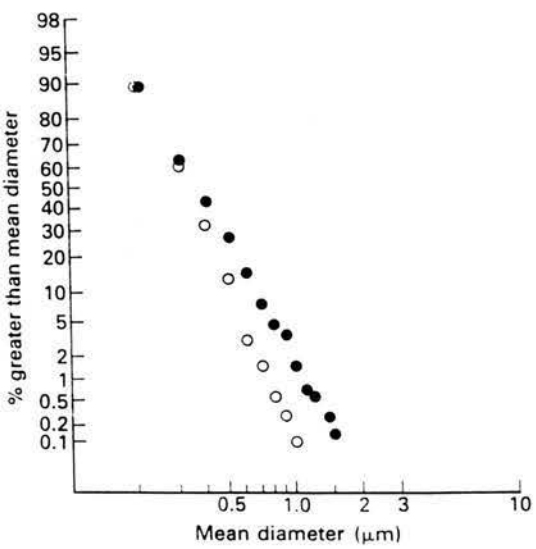


Fig. 3. Fibre diameter distributions of the long and short fibre amosite dust clouds. SEM sizing at 10 000 magnification. >0.4 μm diameter < 3 μm: aspect ratio > 3 to 1. ○, short fibre amosite: (700 fibres sized); ●, long fibre amosite (700 fibres sized).

the animals aged. In the oldest animals a mean of 11.0% of lung tissue was involved with interstitial fibrosis in the long fibre treatment group. The corresponding figure for animals treated with short fibre amosite was 0.15% which is comparable to figures seen in old control animals. In some areas the interstitial fibrotic element became progressively more marked with time but in some lesions hyperplasia of the epithelial cells lining the tissue spaces became more pronounced to produce a pattern of adenomatosis.

Three pulmonary adenomas, eight pulmonary carcinomas and two pleural mesotheliomas were found in the group of animals treated with long fibre amosite but no pulmonary tumours developed in the short fibre treatment group (Table 3). One adenoma and one carcinoma were found in control animals. The carcinoma was the first spontaneous malignant primary pulmonary tumour that we have ever found in our strain of rats was a small lesion (2 mm in diameter) which was not responsible for the death of the animal. One peritoneal mesothelioma was found in each of the group treated with amosite dust. However, both of these tumours were found in association with a testis and appear to have developed from the epithelial covering of the tunica vaginalis as reported from untreated populations of rats (Gould 1977). It is probable therefore that neither of these mesotheliomas was associated with amosite treatment.

The numbers of tumours occurring in other tissue sites for the two groups of rats treated with amosite and the two control groups is illustrated in Table 4. Differences in the proportions of animals developing non-pulmonary tumours were not statistically significant when the time at risk was taken into account. The relatively high number of tumours in control group one appears to be related to the lack of planned kills in control groups which allows a higher proportion of animals to reach advanced age and increases their chance of developing a tumour.

One difference between the development of



Fig. 4. Foci of loose granulation tissue in the walls of respiratory bronchioles and alveolar ducts from the lungs of a rat killed at the end of 12 months inhalation of long fibre amosite. ×450.

pulmonary and non-pulmonary tumours was noticeable in this study. Non-pulmonary tumours began to be recorded after only 1 year of the study and in the animals treated with long fibre amosite the mean period of these tumours was 694 days from the start of dusting. In contrast, the mean induction period of pulmonary tumours was 891 days. This difference is statistically significant at the $P<0.002$ level.

Lung dust burden

The retained dust burden of amosite in animals treated with either the long or short preparations is illustrated in Table 5. Less dust was present in the lung parenchyma of animals treated with long fibre amosite than in those treated with short fibre at both the first and second killing dates. Analysis of variance shows that this difference is significant ($P<0.01$). The figures suggest proportionately greater clearance of short fibres than long during the 6 months between the first and second killing dates. However, with the small group sizes, this difference was not significant.

Injection studies

The results from experiments in which doses of either 25 mg or 10 mg of the long and

Table 2. Mean levels of pulmonary fibrosis produced by long and short fibre amosite dusts

	Long amosite			Short amosite			Control group 1	Control group 2
Time after start of exposure (months)	12	18	27–29	12	18	27–29	27–29	27–29
No of rats examined	4	4	18	4	4	23	9	13
Peribronchiolar fibrosis	15.6 (14.5–17.5)	11.2 (11.0–12.2)	—	0	0	—	0	0
Interstitial fibrosis	0	0.45 (0–1.8)	11.0 (0.4–34.6)	0	0	0.15 (0–2.8)	0.5 (0–3.0)	0

See text for units.
Figures in brackets are ranges.



Fig. 5. Section of terminal and respiratory bronchioles from the lungs of a rat killed 6 months after the end of 12 months inhalation of long fibre amosite. Fibrotic foci are less marked than in Fig. 4. but now some alveoli are lined with cuboidal epithelium. × 350.

short amosite dusts were injected into the peritoneal cavity of rats are illustrated in Table 6. Data from previously published work with UICC amosite (Bolton *et al.* 1982) is included for comparison. The long fibre amosite produced mesotheliomas in almost all animals with a mean tumour induction period of 520–530 days. These figures are very similar to results obtained with UICC amosite. The short fibre amosite preparation produced only a single mesothelioma with an induction period of 837 days.

Discussion

The results from the long term inhalation studies reported in this paper are in agreement with findings in many previous publications which showed the relatively low pathogenicity of short asbestos fibres. The short fibre amosite dust cloud used in the present study contained only 1% of fibres > 5 μm in length and 0.1% > 10 μm and was thus the shortest amosite preparation that has been available for long term inhalation experiments. After a 1 year period of dust inhalation at 10 mg/m^3 this short fibre amosite failed to produce either pulmonary fibrosis or pulmonary tumours within the lifetime of the laboratory rat. The long fibre amosite sample with 30% of fibres > 5 μm and 11% of fibres > 10 μm was longer than

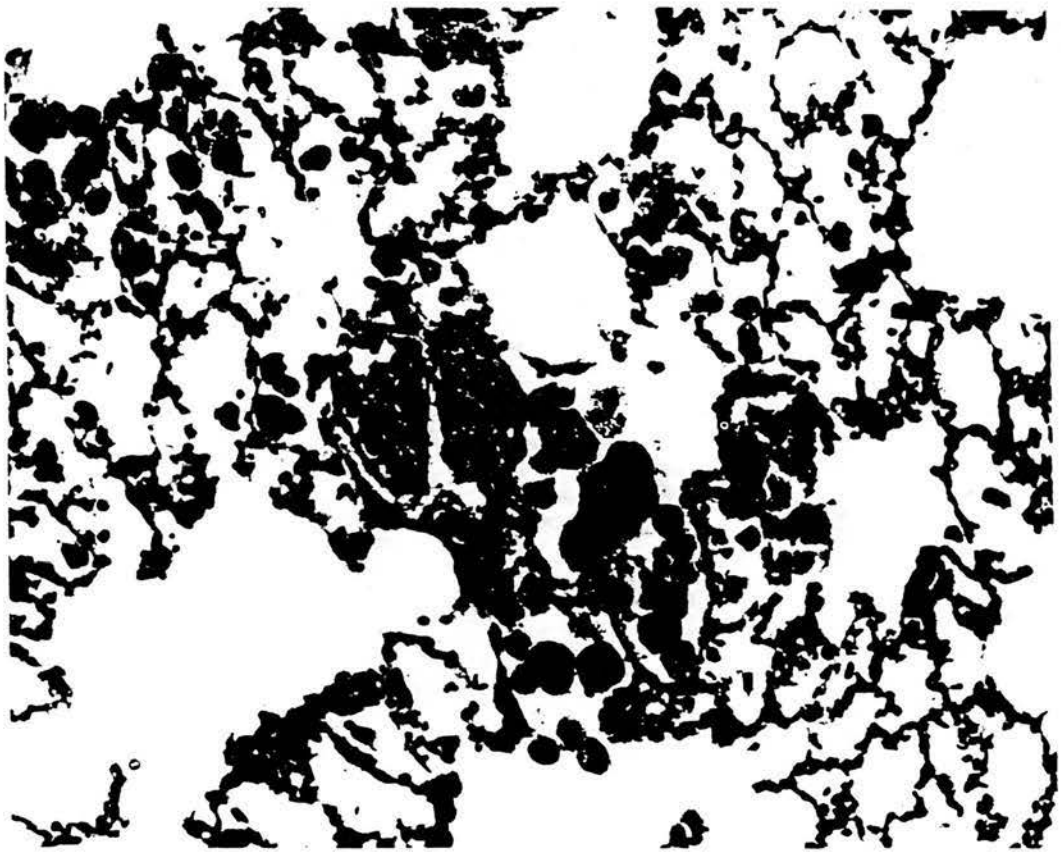


Fig. 6. Section of lung tissue from a rat killed at the end of 12 months inhalation of short fibre amosite. Many pulmonary macrophages packed with dust are present and these are particularly aggregated around the respiratory bronchioles. Apart from the presence of these cells, however, the lung structure appears normal. ×400.

any other amosite preparation we have examined and produced pulmonary tumours in over 30% of animals and large amounts of peribronchiolar fibrosis and alveolar interstitial fibrosis. The UICC standard reference sample of amosite which was tested previously in our laboratory (Davis *et al.* 1978) had a fibre length distribution roughly midway between the present long and short fibre preparations. It was found to be only moderately fibrogenic compared to other asbestos dusts we have examined at the same airborne concentration (10 mg/m^3) and produced only two benign pulmonary adenomas in a group of 40 rats. Thus it would appear that to produce a significant carcinogenic response in rat lung tissue in studies of this size, amosite fibres must be longer than those in the UICC preparation. These findings, however, contrast with those from experimental injection studies. In these the long fibre amosite preparation produced more than 90% of mesotheliomas in a group of rats while the short dust produced only a single tumour. Results from injection studies with UICC amosite (Bolton *et al.* 1982) were in this case almost identical to those from the long fibre amosite dust. From this it would appear that while very short fibres exhibit little carcinogenicity to either lung or

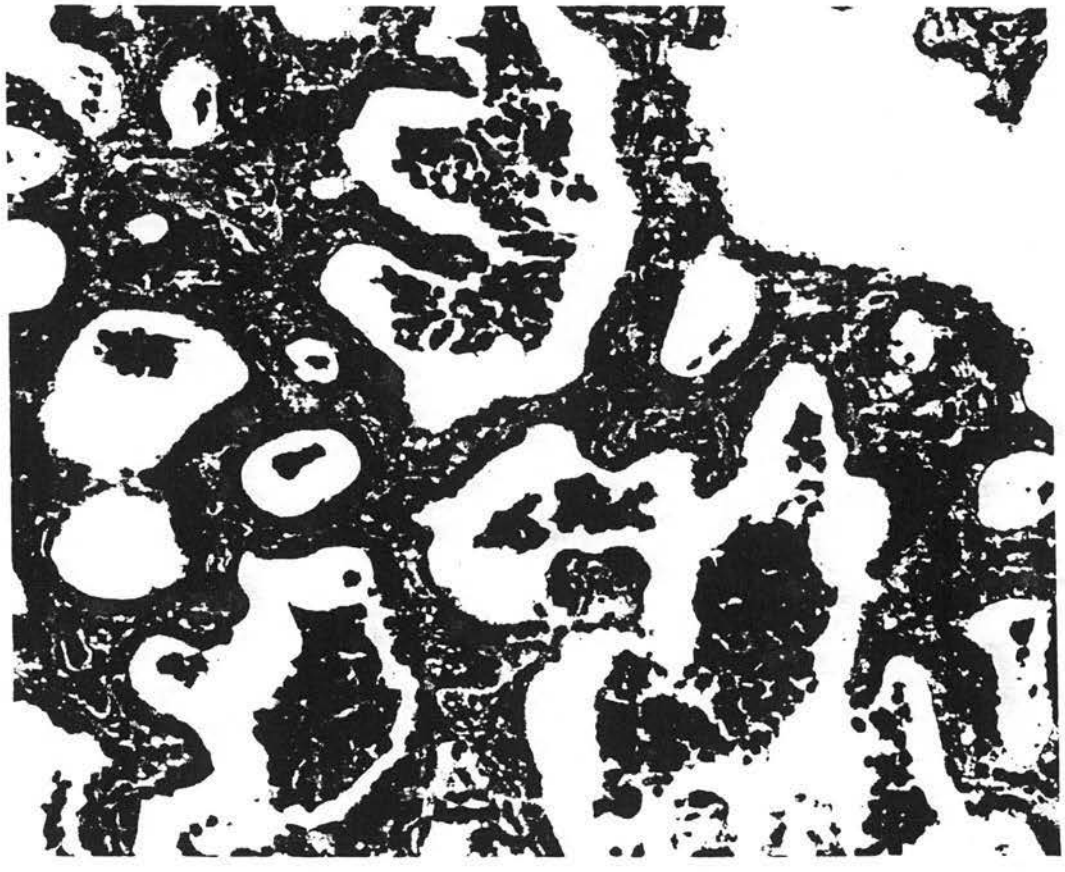


Fig. 7. Section of lung tissue from a rat killed 20 months after the end of twelve months inhalation of long fibre amosite. The normal lung architecture has been replaced by a lesion similar to human 'honeycombing' where the lung spaces no longer corresponde to the original alveoli. The honeycomb space are lined by cuboidal epithelium and the thickened septa between them are fibrosed but also contain many cells. × 400.

Table 3. Numbers of pulmonary tumours and mesotheliomas found in rats treated with three different preparations of amosite asbestos and in two control groups of these animals

Tumour type	Long amosite	Short amosite	UICC amosite*	Control group 1	Control group 2
No of rats	40	42	43	36	25
Adenoma	3	0	2	1	0
Adeno-carcinoma	3	0	0	0	0
Squamous-carcinoma	4	0	0	1	0
Undifferentiated-carcinoma	1	0	0	0	0
Pleural mesothelioma	2	0	0	0	0
Peritoneal mesothelioma	1	1	0	0	0

*Davis *et al.* 1978.

Table 4. Numbers of tumours occurring at sites other than lung

	Long amosite		Short amosite		Control group 1		Control group 2	
	40		42		36		25	
Number of rats examined	B	M	B	M	B	M	B	M
Organ System								
Digestive/peritoneal	—	—	—	2	1	3	1	—
Urinogenital	—	1	1	1	—	2	2	—
Endocrine	3	—	—	—	6	2	3	—
Musculo. skeletal and integumentary	1	4	1	7	—	5	2	3
Reticulo-endothelial/ vascular	—	5	—	3	—	3	—	1
Totals	4	10	2	13	7	15	8	4

B. Benign: M. Malignant.

Table 5. Estimates of the lung dust content of rats treated with long and short fibre samples of amosite asbestos.

Time after end of dusting	Long amosite	Short amosite
0	3570 μg (1590)	5640 μg (370)
6 months	3080 μg (370)	4470 μg (580)

The figures quoted are the means of groups of four rats in each case.

Figures in brackets are standard deviations.

mesothelial tissues at the doses examined. mesotheliomas can be produced by dust preparations consisting of shorter fibres than are needed to produce pulmonary tumours. Taken together these findings may explain both the comparative rarity of mesotheliomas in both rats and humans following dust inhalation and also their characteristically long latent period between first exposure and tumour development. Fibres >25 μm in length are unlikely to be transported through lung tissues to reach the pleural or peritoneal mesothelium. If this length were necessary to transform mesothelial cells then mesotheliomas might not occur at all even in heavily exposed individuals. Medium length fibres (8–15 μm) probably represent a size that is difficult but not impossible to move. After a long period of time enough of these may reach mesothelial tissues to produce

Table 6. Mesothelioma production in rats following the intraperitoneal injection of three samples of amosite dust

	Long amosite		Short amosite		UICC amosite
	25 mg	10 mg	25 mg	10 mg	25 mg
Number of animals developing mesotheliomas	20 (95%)	21 (88%)	1 (4%)	0	30 (94%)
Mean tumour induction period in days	520	535	837	N/A	505

mesotheliomas in a few individuals. Short fibres (shorter than 5 μm) are relatively easily moved (Morgan *et al.* 1978) but do not appear to be very carcinogenic.

While almost all workers, who have reported experimental studies on fibrous dusts, agree that 'short fibre' preparations are at least relatively harmless, a series of papers by Kolev (1976; 1982; 1984) has reported that crocidolite or anthophyllite ground until all dust particles were isometric could produce mesotheliomas in rats and the isometric crocidolite sample apparently produced more mesotheliomas than a fibrous control sample of crocidolite. These results are difficult to evaluate since detailed size distributions of the injected dust samples were not given but one possible explanation comes from the photographs and histological descriptions of some of the tumours produced. These are described as a giant cell type and a sarcoid type. This type of histological pattern was found by Wagner and Wagner (1972) and Wagner (1976) following the intrapleural injection of isometric quartz particles and the tumours were classified as thymomas. Tumours of similar giant cell and sarcoid type (classified as malignant histiocytomas) were also found in the pleural cavities and lungs of rats treated ceramic fibre dust (Davis *et al.* 1984). Although this dust did contain some long fibres the majority of dust particles were isometric. From these results it seems possible that rats respond to a number of isometric dusts with a formation of tumours that may look superficially like mesotheliomas but are in fact of reticuloendothelial origin.

From a very large amount of published experimental work there is now a general concensus that fibre dimensions are the major factor in disease production and recently Pott & Ziem (1983) suggested that because of this attempts to produce less dangerous asbestos materials by modifying the fibre surfaces would not prove successful. Bignon & Jaurand (1983), however, reported that acid treatment of chrysotile caused a reduction in fibrogenicity and carcinogenicity and suggested that while fibre dimensions were important the surface chemistry of fibres does play a part in disease production. As the authors point out, however, the findings of Bignon's group need not be taken as proof that the chemical composition of fibres is a factor in carcinogenicity. It is likely that changes in fibre chemistry could also effect fibre length and durability and that these factors may influence the experimental results.

Acknowledgments

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Effects of the Inhalation of Dusts from Calcium Silicate Insulation Materials in Laboratory Rats

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The effects of respirable dust from three commercially produced calcium silicate insulation materials were examined in laboratory rats by long-term inhalation and injection techniques. These calcium silicate products have been used as replacements for asbestos in the insulation of the engine rooms of ships, and the particle size distribution of the dust clouds generated for the experimental study closely matched those found in ships during the installation of this type of material. One year of exposure to a dust cloud of 10 mg/m^3 of respirable dust had no discernible effect on the length of survival of treated animals compared to controls. No pulmonary lesions were found that appeared associated with the inhalation of calcium silicate per se, but one sample did contain significant amounts of quartz and this did produce a few small pulmonary nodules. While two small pulmonary neoplasms, one malignant and one benign, were found in dusted animals, neither was the cause of death, and the incidence was not significantly different from the control group where no tumors were found. One peritoneal mesothelioma was found in an animal from one of the inhalation groups, but this was considered to be a spontaneous tumor as none of over 100 animals injected intraperitoneally with 25 mg of calcium silicate developed these tumors. While the white blood cell count of dusted animals, compared to controls, was significantly raised in all treated groups at the end of the dusting period, these figures were within the published normal ranges for laboratory rats. It was concluded that the three tested calcium silicate products were harmless to the rats of this species at the doses tested. © 1986 Academic Press, Inc.

INTRODUCTION

Asbestos has been used as an insulation material in ships for many years. The high resistance to heat and chemical corrosion of some asbestos types has made them particularly useful in engine rooms and for service ducting. However, as a result of the identification of serious hazards associated with human exposure to airborn asbestos dusts, the asbestiform minerals are being replaced by other materials where possible. In modern ships, the main substitute insulation materials have been calcium silicate composites.

The calcium silicates are a complex group of natural and man made minerals many of which have been used in cements and autoclaved building materials. These exhibit a wide range in the proportions of calcium and silicon and in the degree of hydration, depending on the chemistry of the initial components and on the temperature and water pressure at the time of formation.

The main component of the calcium silicate insulation materials used in these experiments was the mineral tobermorite $Ca_5(OH)_2Si_6O_{16} \cdot 4H_2O$; more particu

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larly it was the type known as the 11 Å form on the basis of the elementary layering of its crystal lattice (Taylor, 1974). It is a mineral of moderate strength which is not stable in acid conditions, although it is not substantially attacked by carbon dioxide unless left in very moist atmospheres. Calcium carbonate and quartz are both used in the commercial manufacture of tobermorite, and residues of both components are normally found in the final products. Various other additive components such as man made mineral fiber, cellulose fibers, amorphous silica, and haematite were also present in the insulation materials.

Calcium silicate insulation products are dusty when handled on installation or removal, and while no harmful effects have yet been detected in dockyard workers, concern has been expressed that the long-term inhalation of any dusty material could prove dangerous. So far there have been no published epidemiological studies on workers using calcium silicate insulation products and little experimental work has been undertaken with these materials.

Schepers *et al.* (1955) exposed guinea pigs, rats, and hamsters to two varieties of commercial hydrous calcium silicate dust ($Ca_2SiO_4H_2O$ and Ca_3SiO_5) by inhalation. Some animals were exposed for as long as 36 months. The dust induced some peribronchiolar fibrosis, and epithelialization of atelectatic alveoli was seen. Also, in the period 1965–1970, P. F. Holt at Reading University (personal communication) undertook an inhalation study using guinea pigs which were exposed to finely divided calcium silicate powder prepared from insulation material. However, no pathological effects of dust inhalation were found and the results were not published.

Some *in vitro* testing of calcium silicate insulation products similar to those used in the present study has been undertaken. Wright *et al.* (1980) found that three separate samples of calcium silicate produced marked hemolysis with sheep erythrocytes and they were also cytotoxic toward $P388D_1$ cells, a permanent line of transformed mouse cells with many macrophage characteristics. Hunt *et al.* (1981) also found that three samples of calcium silicate composites from the same source produced hemolysis of rabbit erythrocytes but less than chrysotile asbestos at a similar dose. This paper reported that the calcium silicate samples were also toxic to rabbit alveolar macrophages *in vitro*, and when fibroblast cultures were treated with two of the dust samples, hydroxyproline production was depressed in one case but maintained at control levels in the other.

Various studies have been undertaken on the health hazards of some types of calcium silicate not used primarily for insulation purposes. In 1978, Boehlecke *et al.* reported a study of 104 men who had worked with wollastonite ($CaSiO_3$), a naturally occurring calcium silicate which is fibrous in some instances. Four cases showed abnormalities on chest radiographs consistent with early pneumoconiosis. Subsequently, Hahon *et al.* (1980) found that this type of wollastonite enhanced the induction of interferon by influenza virus in mammalian cell cultures, but the mineral per se did not induce interferon.

The purpose of the present project was to examine the effects in rats of dust from three calcium silicate insulation materials currently used for insulation purposes in ships. The full details of the study are available as a Technical Memo-

randum (TM/83/1), copies of which can be obtained from the librarian at the Institute of Occupational Medicine in Edinburgh.

MATERIALS AND METHODS

Outline of project. The study utilized both inhalation and injection routes of exposure of animals to calcium silicate dusts. The main emphasis was placed upon the consequences of prolonged inhalation, but an intraperitoneal injection assay was undertaken to assess the possible potential for mesothelioma production. Outbred white male Wistar SPF rats of the AF/HAN strain were used throughout, maintained on standard pelleted laboratory diet and tap water. Forty-eight animals were placed in each of the four inhalation chambers. One chamber was reserved for a control treatment group receiving only filtered air, and the other chambers were used to test three commercially available calcium silicate composites. Dust exposure was for 7 hr/day, 5 days/week, for a total of 224 days over an elapsed period of 12 calendar months. A mass concentration of 10 mg/m^3 of respirable dust was selected to provide a direct comparison with the known effects of prolonged exposure to similar concentrations of asbestos dusts.

Animals were identified individually and examined daily throughout their natural lifespan for evidence of abnormalities. Twelve animals were killed from each of the four experimental groups when they were removed from the exposure chambers at the end of the dusting period, and the remainder were kept for their full lifespan. The experimental phase of the study was terminated when the final surviving animals from all groups were killed to provide good quality histological material 19 months after the end of the dusting phase.

The survival of animals within the four experimental groups was examined using Cox's proportional hazard model (Cox, 1972). Planned kills were treated as censoring events and the control group was compared with the combined dust-treated groups and with each dusted group individually. The weights of all animals in the study were recorded monthly, but a statistical examination of these figures for the four groups of animals was complicated by the fact that the body weight data was obtained from surviving animals. Since different numbers of animals were available at each time of weighing, and since there was no information on the effect of death of an animal upon the mean body weight of survivors, only a graphical comparison of the four groups was undertaken.

Hematological data were obtained from animals both before the start of inhalation and at the end of the dusting period when the 12 animals from each group were killed. These animals were also used for an analysis of lung dust content and histological examination for early evidence of harmful effects of dust inhalation.

Dust cloud generation and analysis. A preliminary hygiene survey was undertaken in the occupational environment over a period of 1 week sometime prior to the animal experimental phase. The respirable dust concentration ranged between 0.8 and 1.8 mg/m^3, the total particle number concentrations (<2 μm) between 60 and 200 particles/ml, and the ratio of total to respirable dust varied considerably from 1.1 to 1.8, depending upon the sampling location. The dust

concentrations were, as expected, below the TLV for nuisance dust, and therefore below the target concentrations used in the animal experimental studies. Man-made mineral fibers present in small amounts in the original bulk insulation products were rarely seen in the dust clouds. Information from this preliminary survey was used to ensure that the dust generation system that was developed was capable of producing a dust cloud with similar physical characteristics to that found in the dockyards.

All three types of calcium silicate insulation material were supplied as standard product slabs 50 mm thick, and they were sawn into smaller pieces as required. Following trials with various methods of generating dust clouds from the bulk insulation product, it was found that a rotating wire brush was the most suitable practical method of generating a cloud of airborn dust containing a wide range of particle sizes. A jet of compressed air was used to carry the dust into the chamber ventilation air flow. The animal inhalation chambers and methods of dust cloud control have been described fully elsewhere (Beckett, 1975). Airborn dust samples were taken regularly from each chamber throughout the exposure phase, and these were used to provide estimates of the total and respirable mass concentrations, the size distributions, and the mineral composition of the dusts. The apparatus and techniques used in the dust sampling are summarized in Table 1. Full details of the sampling methods may be found in the references cited in Table 1, and in Davis *et al.* (1983).

Histopathology. At autopsy, tissue was taken from all major organs for histological examination. Specimens were fixed in 10% formal saline and embedded in paraffin wax. Lungs were fixed by instillation prior to excision from the thoracic cavity. Sections from all tissues were routinely stained with hematoxylin and eosin (H & E). Van Gieson's stain for collagen or Gordon and Sweet's reticulin stain were used for the study of pulmonary fibrosis, and other stains were used selectively to assist in specific histopathological diagnoses.

Serial sections were cut from the lungs of all animals autopsied, with groups of sections being mounted at approximately 1-mm intervals through the block. This procedure provided material for detailed examination from six to eight levels of each lung. Three levels of all tissues taken from those animals ($n = 36$) involved in the final kill were also taken for detailed examination. Examination of multiple levels of tissues from other animals was only undertaken when required for the indentification of specific lesions.

Interstitial and peribronchiolar fibrosis were estimated by the methods published previously (Davis *et al.*, 1978). A square eyepiece graticule, divided into 100 subunits, was used in the light microscope. This was used to estimate the area of tissue damage in the case of interstitial fibrosis. The lesions of peribronchiolar fibrosis, however, were scattered and much smaller than one of the graticule subunits at the objective magnification used ($\times 4$). These lesions, therefore, were quantified by counting the number of graticule squares containing lesions and expressing this as a percentage.

Lung dust analysis. Lungs were available for dust recovery and analysis from 6 of the 12 animals killed from each of the four experimental groups at the end of the dust exposure phase. Only one lung from each animal was assessed, the

TABLE 1

METHODS OF DUST SAMPLING AND ANALYSIS USED FOR DUSTS FROM THE INHALATION CHAMBERS AND BULK MATERIALS

Sampling equipment[a]	Filter	Analysis	Frequency
Total dust			
50-mm diam head	50-mm sartorius membrane	Mass	Daily
25-mm diam Gelman head	25-mm silver membrane	XRD qualitative[b]	Monthly
25-mm diam Gelman head	25-mm sartorius membrane	Infrared qualitative[c]	Monthly
25-mm diam Gelman head	25-mm Millipore	Fiber and particle counts[d]	100 filters over inhalation period
Respirable dust			
Casella MRE 113A[e]	50-mm sartorius membrane	Mass	Daily
Casella MRE 113A	50-mm sartorius membrane	Infrared qualitative	Monthly
IOM Vertical Elutriator[f]	50-mm sartorius membrane	Mass	Daily
"Higgins Cyclone"[g]	25-mm silver membrane	XRD qualitative	Monthly
Bulk materials			
—	—	Infrared qualitative	Monthly
—	—	XRD qualitative	Monthly

[a] These were open sampling heads used facing downward.
[b] Klug and Alexander (1954).
[c] Dodgson and Whittaker (1973).
[d] Walton and Beckett (1977).
[e] Dunmore *et al.* (1964).
[f] Beckett (1975).
[g] Higgins and Dewell (1967).

contralateral lung being preserved in each case by instillation of formal saline to provide material for histological examination.

The lungs were dried at 110°C for 48 hr, and then incinerated in a muffle furnace at 600°C for approximately 72 hr. The resulting ash was washed with 0.01 M hydrochloric acid, and then filtered onto 25-mm-diam 0.2-μm pore-size polycarbonate membrane filters (Nuclepore) using distilled water. The insoluble residues were washed off the filters into platinum crucibles, dried, and mixed with 250 mg of potassium bromide for preparation of disks for infrared analysis.

This procedure was adopted after a number of techniques for the extraction and recovery of the calcium silicates had been examined. It was confirmed during these investigations that most components of the dusts were soluble in mild reagents such as slightly acidic solutions and phosphate buffered saline when stored for up to 6 weeks. This was taken as evidence for the likely solubility of calcium silicate components *in vivo*, although no specific tests of solubility under more controlled prolonged biological conditions were undertaken. It was further confirmed that the unstable nature of the likely principal component (tobermorite) made direct recovery of the calcium silicate dusts from pulmonary tissues impractical. Under these circumstances it was considered reasonable to convert any labile components of the calcium silicate dusts to more stable silicates by heating the samples, and to assess the residues in terms of a general silicate content.

Hematology. Blood samples were taken from the tail veins of all 192 experimental animals 5 days prior to the start of the 1-year inhalation phase, and 3 days after the cessation of exposure. These same samples (20 μl) were used for estimations of hemoglobin by standard colorimetric assays and for estimations of packed cell volume after hematocrit centrifugation.

A more detailed hematological examination (requiring 2 ml blood per animal) was undertaken on 12 animals of the same stock specifically reserved for preexposure screening. These animals provided the baseline hematology. Information on the effect of prolonged inhalation exposure on hematological parameters was obtained from blood samples taken from the 48 animals (12 from each experimental group) killed after the end of the 1-year dusting phase. Duplicate measurements of all blood samples were obtained using a Coulter Counter (Model S). Parameters recorded were total red cell count, hemoglobin content, packed cell volume (Hematocrit), mean corpuscular volume, mean corpuscular hemoglobin content, and total white cell count. Differential white cell counts were performed manually according to the method of Dacie and Lewis (1975).

Intraperitoneal injection studies. Apart from the inhalation studies, dust preparations from the three calcium silicate materials were injected into the peritoneal cavities of three groups of 36 rats to assess their potential for mesothelioma production. An elutriated form of each dust was used for injection that was collected from airborn clouds (generated for the inhalation studies) by using an absolute filter assembly in the ducting between the dust generator and the animal exposure chamber. Each animal was given a single injection of 25 mg of dust suspended in 2.0 ml of saline. All animals were allowed to live out their full lifespan.

RESULTS

Dust Cloud Generation and Analysis

The measurements for the airborn dust clouds used in the rat inhalation study are summarized in Table 2 and Fig. 1. All three dust clouds were very similar in particle size and number, and the fiber concentrations were below the detection limits for light microscopy (<0.01 fibers/ml) in each case. The infrared (ir) spectra for the "respirable" and "total" dust samples from the three types of insulation material were all very similar, and characterized by the absorbance spectrum of tobermorite. They also had small absorbance maxima indicating the presence of calcium carbonate (880 cm^{-1}) and amorphous silica (800 cm^{-1}), but quartz could not be recognized in any of the spectra. The X-ray diffraction (XRD) analyses confirmed the presence of calcium carbonate in all of the dust samples, and also indicated the presence of quartz in samples A and C. There was very little variation from month to month in the quartz or amorphous silica contents of the respirable dusts. The quartz content (ca. 1%) of sample A was consistently higher than in the others. Quartz was never positively identified in respirable dust from sample B. The amorphous silica component of all three types of respirable dust was either manmade mineral fiber or silica derived from the chemical disintegration of the tobermorite.

Animal Survival and Weights

In an examination of the survival times of the animals, the combined dust-treated groups were compared with the control group and then each treated group was compared individually with each other. No significant differences were found: in no case was the $P = 0.3$ level exceeded. It may be concluded, therefore, that there was no sign of any treatment-related effects upon the survival of animals used in this study. A substantial proportion of animals survived in excess of 1000 days. The 80% mortality point—sometimes taken as a guideline for termination of long-term chronic toxicity studies—occurred at approximately 1030 days.

Similarly, there were no statistically significant differences among the mean autopsy body weights of the experimental groups. All four groups behaved similarly and the mean values of the control animals fell between those of the dusted groups. All groups showed a decline in body weight at the time they were removed from the dusting chambers, but this was followed by full recovery and was probably related to the stress of a new caging environment.

Histopathology

Tissues were available for examination from 184 animals (139 dusted and 45 controls). The incidence of pulmonary disease among both control and dusted animals was very low. Bronchial accumulations of lymphoid tissue (Lamb, 1975) were minimal throughout.

All animals treated with the calcium silicate materials showed dust-containing macrophages scattered throughout the alveolar regions of the lung at the end of the exposure period (Fig. 2) but particularly close to the respiratory bronchioles.

TABLE 2
SUMMARY OF DUST MEASUREMENTS FOR THE INHALATION STUDY

Parameter	Sample A			Sample B			Sample C		
	Mean	SD	Coeff var. (%)	Mean	SD	Coeff var. (%)	Mean	SD	Coeff var. (%)
Mass concentration (mg/m^3)									
Total	24.9	16.1	65	21.4	16.0	76	19.2	9.9	52
Respirable	10.05	4.5	45	10.0	6.7	67	10.0	5.7	57
Ratio of total:respirable		2.5			2.1			1.9	
Particle concentration (No./ml)[a]	536	236	44	537	225	42	514	219	43
Fiber concentration (No./ml)[a]		Below detection limit (<0.01)			Below detection limit (<0.01)			Below detection limit (<0.01)	

[a] Based upon optical microscopy.

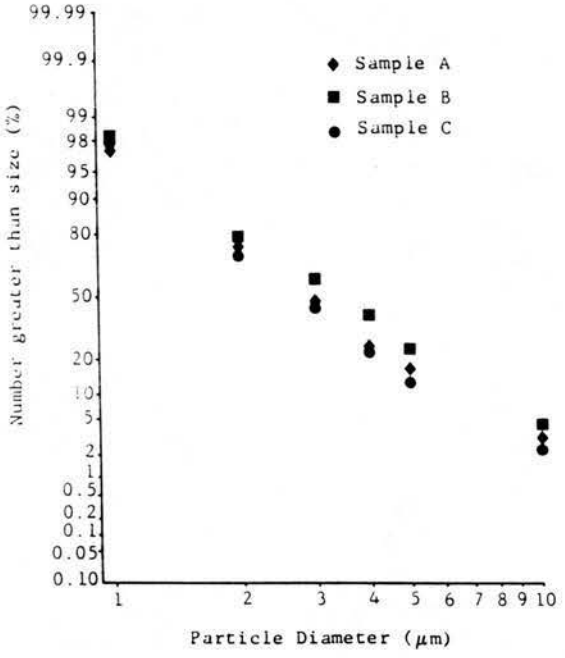


FIG. 1. Size distribution of calcium silicate particles >0.8 μm in diameter. Scanning electron microscope measurements. ×10,000.



FIG. 2. Section of rat lung tissue taken at the end of the dusting period showing accumulation of dust containing macrophages in the alveoli near to a respiratory bronchiole. ×450.

Most of the dust tended to be isometric but occasional fibers were seen in animals exposed to sample C. The frequency of dust-containing macrophages in the histological sections declined substantially after the end of the dust exposure, and those remaining were usually found in clusters often filling an isolated alveolus (Fig. 3). There appeared to be relatively fewer dust-containing cells in animals exposed to sample C than to A or B, and the numbers of cells in animals exposed to sample A appeared to decline more slowly than with the other treatments.

Small amounts of interstitial fibrosis were found in 5 control and 13 dusted animals that had survived more than 10 months after the cessation of dust exposure. This type of change was similar to that reported previously (Davis *et al.*, 1978) from animals treated with asbestos, although it had not been seen in the relatively small groups of control animals used in those studies. It shows a somewhat variable histological pattern (Fig. 4). In all cases the alveolar septum is thickened with abnormal deposits of reticulin and in old animals with collagen as well. However, while the epithelial lining in some areas remains relatively flat, at the other end of the scale the cells have become cuboidal and the appearance is one of adenomatosis. The extent of interstitial fibrosis was quantified by estimating the area of lung tissue involved as a percentage of total lung section, but the amounts present were extremely low (<0.2%) and similar in extent in both dusted animals and controls.

The animals exposed to sample A tended to have peribronchiolar areas of fibrosis not found with any of the other experimental groups, including the con-

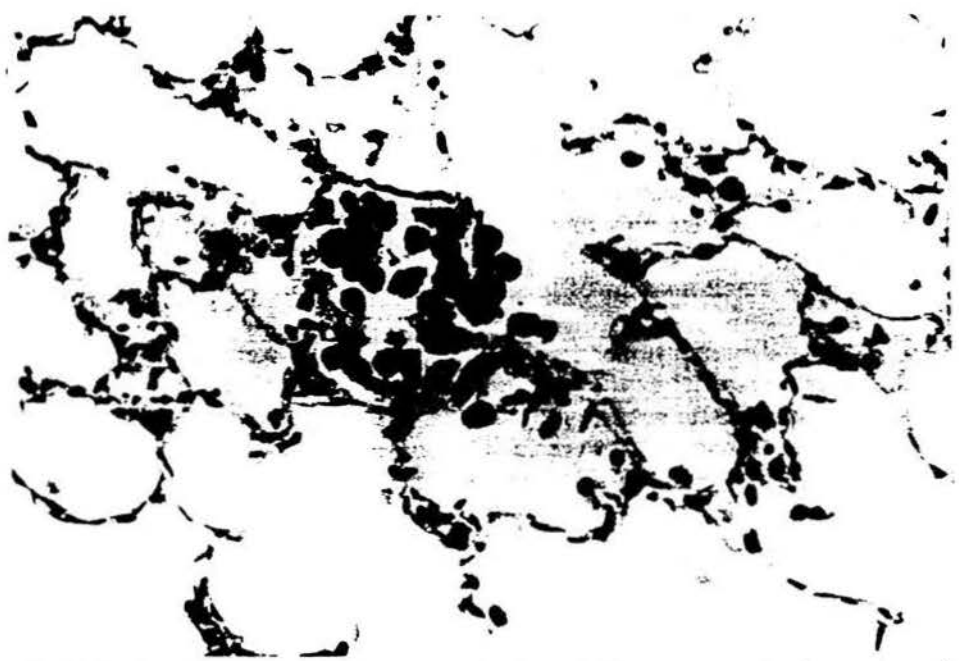


FIG. 3. Section of rat lung tissue taken 1 year after the end of dust exposure showing an aggregation of dust containing macrophages filling a single alveolus. ×450.

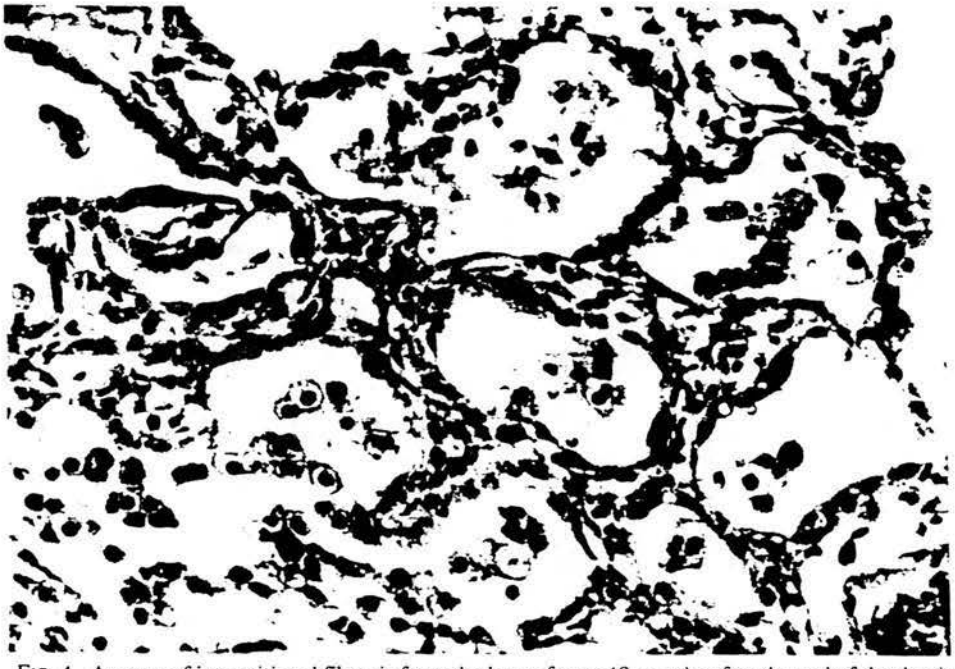


FIG. 4. An area of interstitional fibrosis from the lung of a rat 18 months after the end of the dusting period. ×450.

trols. The lesions were close to the respiratory bronchioles, and consisted of small granulomatous nodules containing a mixture of macrophages and fibroblasts (Fig. 5). A network of reticulin was always present among the cells, and in some of the older animals collagen was also found. These peribronchiolar fibrotic areas were similar to those previously described in animals treated with asbestos (Davis *et al.*, 1978), but were considerably smaller and less frequent than asbestos-induced lesions, and were only found in the older animals. Sixteen animals treated with sample A were found with the nodules of peribronchiolar fibrosis, but only a relatively small amount of lung tissue lesions were present in each case (Table 3).

The mediastinal lymph nodes from all dusted animals were found to contain some particulate material at the end of exposure, and this amount appeared to increase with time postexposure, although it never reached very large amounts. Animals exposed to sample A were found to have more extensive changes than the other groups; the lymph nodes contained numerous areas of closely packed cells of epitheloid type which contained only small amounts of visible dust. These areas were sharply demarcated from the surrounding lymphatic tissue (Fig. 6). Areas of very similar structure have previously been reported from animals treated with quartz dust and have been described by Bruch *et al.* (1977). Lesions of this type were found in 34 out of the 45 animals examined that had been treated with dust A and in all 28 of those animals of more than 800 days of age.

Small primary neoplastic lesions were found in the lungs of two of the dusted

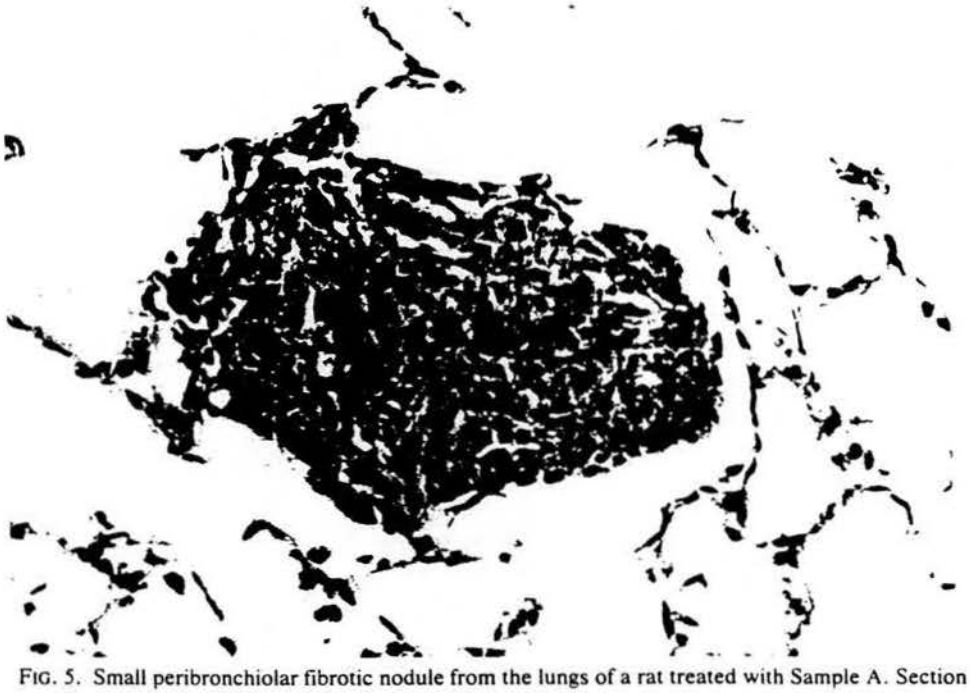


FIG. 5. Small peribronchiolar fibrotic nodule from the lungs of a rat treated with Sample A. Section taken 18 months after the end of the dusting period. ×450.

animals exposed to sample B. The first was a small focal squamous carcinoma (classified as of low grade malignancy) found in the lung parenchyma of an animal killed at 851 days because of a simultaneously occurring malignant basal cell adnexal tumor near the right ear. The other pulmonary tumor was a benign adenomatous lesion 2 mm diam found incidentally in an animal aged 1024 days.

In organs other than the lung no pathological changes were observed that appeared to be related to the inhalation of calcium silicate dust, although old animals

TABLE 3
INCIDENCE OF PULMONARY FIBROSIS IN RATS TREATED WITH CALCIUM SILICATE INSULATION MATERIALS

	Treatment			
	Sample A	Sample B	Sample C	Control
Interstitial fibrosis				
No. of animals	3	5	5	5
Lung area involved (mean %)	<0.2	<0.2	<0.1	<0.2
Peribronchial fibrosis				
No. of animals	16	0	0	0
Total grid squares (mean %)	0.07	0	0	0
No. of animals over 800 days old	28	27	28	29

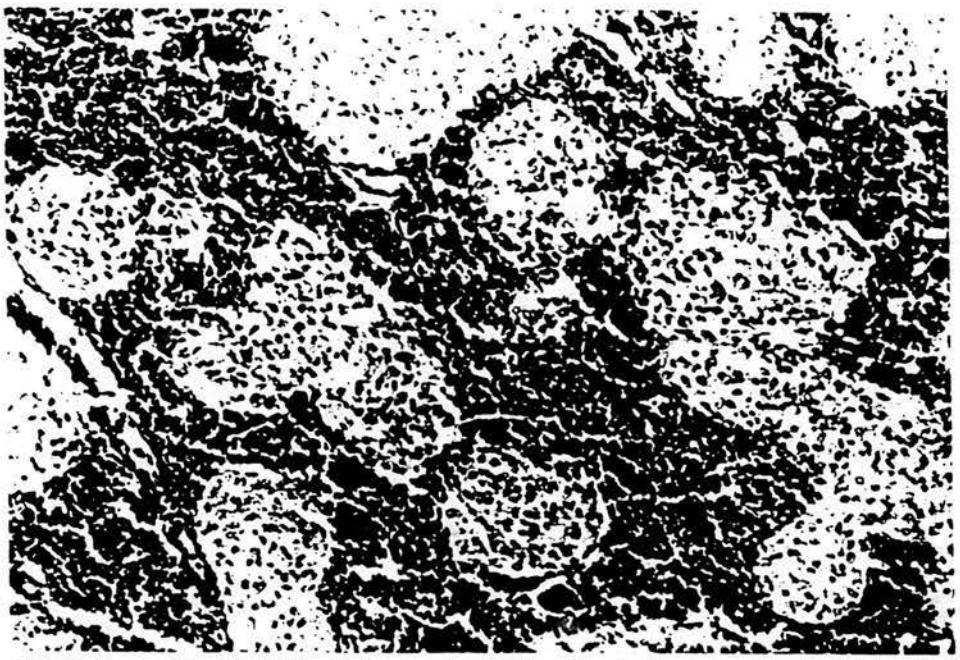


FIG. 6. Areas of "epitheloid" cells in a mediastinal lymph node from a rat treated with Sample A. Section taken 18 months after the end of dusting. ×300.

from all groups including controls showed a number of degenerative changes. The most frequent of these were chronic progressive nephrosis (Gray, 1977) and polyarteritis. Microscopic areas of hyperlasia of the tunica vaginalis were found in some of the oldest animals but the number of dusted animals with these lesions was not significantly different from the controls.

Neoplastic lesions were found in many tissues but the numbers in the three

TABLE 4
THE NUMBER OF BENIGN (B) AND MALIGNANT (M) TUMORS AMONG THE FOUR EXPERIMENTAL GROUPS

	Sample A		Sample B		Sample C		Control	
Organ system	B	M	B	M	B	M	B	M
Respiratory	—	—	1	1	—	—	—	—
Digestive	4	3	2	—	1	3	4	2
Urinogenital	4	—	8	1	3	—	2	2
Endocrine	9	—	7	2	6	—	11	2
Reticuloendothelial/vascular	2	5	5	6	4	4	6	2
Nervous	—	1	1	—	2	—	5	1
Muscular, skeletal, and integumentary	1	1	—	3	—	2	3	2
Totals	20	10	24	13	16	9	31	11

groups of dusted animals and the controls were not significantly different (Table 4). One peritoneal mesothelioma was found in a dusted animal but this was considered to be a spontaneous tumor since the results of the intraperitoneal injection studies were completely negative.

Lung Dust Analysis

As described in the materials and methods section, it was not possible to quantify exactly the amount of minerals in the dusts recovered from the animal lungs, although an attempt was made to assess lung dust residues in terms of general silicate content.

The lung residues from group C were very similar to those of the control residues with indications of only minor amounts of amorphous silica (Table 5). The group B lung residues contained relatively much more amophous silica, with strong absorbance maxima at 800 and 470 cm^{-1}, but no other recognizable minerals. The group A lung residues were found to contain a mixture of amorphous silica and quartz, as indicated by the 800–780 cm^{-1} absorbance doublet which is superimposed upon the amorphous silica absorbance peak at 800 cm^{-1}. It is interesting to note that although the total mass of silicate recovered from the lungs was low and subject to large variation, the proportion of quartz in the group A lung residues was considerably greater than that found in the respirable fraction of the original airborn material. Thus the respirable dust cloud contained approximately 1% quartz by mass, while the lung residues were found to contain in excess of 10% quartz.

Hematology

The results of the hematological assays undertaken during the present study showed that all the examined blood parameters fell within normal ranges both for animals studied before the start of the inhalation period and for the dusted animals and controls at the end of 1 year's dusting. The only difference between the groups was found in the absolute number of white cells. This figure fell in the

TABLE 5

APPROXIMATE MASSES OF DUST AND MINERALS EXTRACTED FROM ANIMAL LUNGS EXPOSED TO THE THREE TYPES OF INSULATION MATERIAL AND FROM CONTROL ANIMALS[a]

Dust type	Mass of silicate in lung (mg)		Mass of quartz in lung (mg)
	A800 cm^{-1}	A470 cm^{-1}	
Sample A	0.8[b] (0.4)[c]	0.7 (0.3)	0.1
Sample B	0.4 (0.4)	0.5 (0.4)	—
Sample C	0.1 (0.1)	0.1 (0.1)	—
Control	0.03 (0.02)	0.06 (0.03)	—

[a] The figures are estimates of whole lung content based upon the analysis of single lungs and recalculated from a left:right lung content ratio of 1:2.2 as evaluated in previous experiments.

[b] Data from the treated animals are summarized to one decimal place only to indicate the imprecision of the silicate estimate of the total lung dust burden.

[c] SD.

control group that had spent a year in a dusting chamber as compared to the young control animals (from 4.3 to 2.9 $\times$ 10^6 cells/ml), but the dusted animals showed a marked increase to an average for the three groups of 5.8 $\times$ 10^6 cells/ml. Although all these figures are within the published normal ranges for laboratory rats (Ringler and Dabich, 1979) the differences between the nondusted controls and the other groups was found to be statistically significant ($P = <0.01$).

Intraperitoneal Injection Studies

In the studies involving the intraperitoneal injection of calcium silicate, no mesotheliomas developed in any of the animals from the three treatment groups. At autopsy, little dust or dust-related fibrosis was visible in the peritoneal cavity.

DISCUSSION

The present study has shown that the inhalation of dust from three varieties of calcium silicate insulation material produced no major pulmonary damage in rats. These animals were exposed for 7 hr/day at a concentration of 10 mg/m^3 *respirable* dust, whereas the present threshold limit value (TLV) for those dusts classified as of nuisance effect only is 10 mg/m^3 of *total* dust. The dusts were generated from calcium silicate products as supplied by the manufacturers prior to use in ships. An exposure level of 10 mg/m^3 was chosen to provide a direct mass comparison with the results of other studies at the Institute involving the effects of asbestos. At these concentrations, relatively large quantities of asbestos are found in the lungs of exposed animals, and considerable pulmonary damage develops in those animals maintained for their full lifespan.

Although, as expected, there were short-term fluctuations in dust concentrations during the dusting phase, the dynamic procedure of monitoring the dust clouds throughout each day of exposure made it possible to achieve mean mass concentrations very close to the target levels. Similarly, the semiquantitative analyses of minerals in the dust samples showed that a consistent cloud was produced throughout the exposure phase, and the composition of the respirable dust remained consistent. Detailed analyses of complex mixtures such as the calcium silicate insulation materials are difficult, but the semiquantitative approach adopted in the present study provides a useful description of both the bulk and the respirable dust samples. It is noteworthy that although the bulk materials were reported to contain modest amounts of manmade mineral and other fibers, relatively few fibers of respirable sizes were generated in the experimental system. This is consistent with the information on the composition of airborn dusts collected in the dockyards, and probably reflects the relative nonrespirability of the fibrous components. It is also noteworthy that although all three bulk materials contained small residual amounts of quartz, only respirable dust from sample A was found to contain any significant quartz component, and this represented only 1% by mass of the respirable dust.

Only small amounts of dust were recovered from the lungs of animals exposed to calcium silicate dusts for 1 year (i.e., approximately one-tenth of the lung burden to be expected following exposure to an equivalent respirable mass of amosite asbestos). These calcium silicate residues were difficult to analyze be-

cause the principal mineralogical component, tobermorite, is unstable in the biological environment and tends to decompose to form carbonate and amorphous silica. Lung dust estimation was based, therefore, on the general silica content as assessed by infrared spectophotometry and may only be taken as an approximate guide to the total lung burden. Despite these difficulties, it appears likely that much of the inhaled dust was removed from the lung tissue while the animals were alive by a process of decomposition and dissolution. Support for this idea may be inferred from the labile nature of individual components of the calcium silicate composites, from the proven solubility of some components in mild reagents *in vitro*, and from the observed substantial decline in histologically visible dust in the lungs of those animals killed during the follow-up period after the end of dusting. One result of the dissolution of the main calcium silicate components was the increased quartz content (from 1 to 10%) in extracted lung dust from animals exposed to sample A. It appears that the dissolution of calcium silicates in the lung has had the effect of increasing the relative proportions of quartz within the remaining dust. This concentration of the quartz component in the lungs of animals exposed to A is in accord with the observed mild quartz-related damage in the lungs and lymph nodes of those animals.

In most animals exposed to the calcium silicate composites, the only signs of dust inhalation were the accumulation of dust-laden macrophages in the alveoli and surrounding respiratory bronchioles. The frequency of these cells declined substantially after the end of the dusting phases so that few signs of dust remained in the oldest animals. This is in marked contrast to animals treated with asbestos at similar dose levels where large amounts of lung damage developed and considerable quantities of histologically identifiable dust remained in the rat lungs throughout the experimental period. A few dusted animals from all three calcium silicate-treated groups showed small areas of pulmonary interstitial fibrosis, but similar amounts were also found in control animals, and it is assumed that at this level the lesion is a spontaneous one in aged rats. This type of pulmonary fibrosis occurs extensively in animals treated with asbestos and with some asbestos preparations; the area of tissue damage in dusted animals is more than two orders of magnitude greater than found in the present study (Davis *et al.*, 1978). The only pulmonary fibrotic lesions that appeared to be related to calcium silicate exposure were the peribronchial nodules found in animals exposed to sample A, and these were related to the quartz content of the lung dust.

There were no differences in the overall tumor levels between the control and any of the dusted groups of animals. The only types of tumors that might have been associated with inhaled dusts were one small pulmonary adenoma and a very early bronchial carcinoma in animals exposed to sample B, neither of which resulted in the death of the animal. In addition, one malignant peritoneal mesothelioma was found in an animal treated with sample C, and this tumor was the cause of death. Both pulmonary tumors and mesotheliomas are rare in rats but do occur spontaneously in a few cases (Maltoni *et al.*, 1982). This is likely to have been the case with the peritoneal mesothelioma found in the present study since the same three calcium silicate materials when injected directly into the peritoneal cavities of rats produced no mesotheliomas in a total of 107 animals.

Since calcium silicate insulation materials were introduced as substitutes for asbestos, a comparison of the pathological effects of these materials is important. The Institute of Occupational Medicine in Edinburgh has published the results of a number of inhalation and injection studies with different varieties of asbestos and other mineral fibers (Davis *et al.*, 1978, 1980a, b; Bolton et al., 1982). These were undertaken using the same strain of rats and the same inhalation chambers as the present study involving calcium silicates. All the asbestos samples tested produced more pulmonary neoplasms and pulmonary fibrosis than calcium silicate. Of particular importance is the contrast seen in the injection studies where the majority of asbestos samples produced mesotheliomas in over 90% of animals. No tumors followed the injection of calcium silicate.

An examination of hematological information showed that there were no differences in most blood parameters between control and dusted animals, which indicate there had been no marked systemic toxicity as a result of dust inhalation. However, white cell counts were significantly higher in the dusted groups than in controls. Pulmonary dust accumulation is known to result in increased number of both neutrophils and macrophages in lung tissue but there is no evidence that this alone produces changed levels in the numbers of circulating white cells. There are, for example, no overall differences between the white cell counts of coal miners and the rest of the population (Boyd *et al.*, 1981). It may be that because calcium silicate is relatively soluble, the dissolution products reach the blood stream and may cause a rise in white cell levels. Since, however, this increase leaves the total still within the normal published range (Ringler and Dabich, 1979) for rats, it does not indicate a serious reaction.

In conclusion, the present study has produced no evidence that inhaled dust (at a respirable concentration of 10 mg/m^3) from three types of calcium silicate insulation material affects the survival or health of laboratory rats.

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A dosimetric approach for relating the biological response of the lung to the accumulation of inhaled mineral dust

J H Vincent, K Donaldson

Abstract

Results from studies of the retention of contrasting mineral dusts inhaled by rats (for periods of up to three months) and the resultant changes in the phagocyte defence system of the deep lung were examined. The dusts used were titanium dioxide (relatively innocuous) and quartz (relatively toxic). The parameters assessed included the accumulation of material in the lung and lymph nodes during chronic exposure and the associated leukocyte response as assessed by bronchoalveolar lavage. The principal findings were that: (*a*) low level exposure to titanium dioxide produced no measurable inflammation (as indicated by neutrophil recruitment) but higher concentrations (30, 50, and 90 mg/m^3) caused the transfer of dust to lymph nodes and first evidence of inflammation; and (*b*) for quartz, there was a more prominent response and earlier transfer of material to the lymph nodes. The suggested relation between changes in the neutrophil population and dust accumulation is discussed in terms of a quantitative dosimetric model, from which implications for assessing and managing human exposures emerge.

The relation between exposure of the respiratory system to harmful dusts and the subsequent development of associated lung disease are both complicated and variable, involving: (*a*) inhalation of particles and their deposition, clearance, and redistribution within the lung; (*b*) integrated dose of "harmfulness" to lung tissue (where harmfulness represents some property of the particulate material relevant to the potential hazard); (*c*) biological responses, including inflammation; and (*d*) tissue damage and repair or destruction. A full description of the disease process requires consideration of all these factors. In this paper we focus attention on some central issues, specifically: (*a*) the kinetic behaviour of particle transfer within the lung, (*b*) its relation to a meaningful definition of integrated dose of harmfulness, and (*c*) the resultant cellular response.

Dust accumulation and alveolar leukocyte response have been investigated recently at the Institute of Occupational Medicine in two separate but complementary studies aimed primarily at elucidating these processes for inhaled coalmine dusts.[1,2] To gain an appreciation of what happens for non-pathogenic and pathogenic dusts, the experiments were carried out using two contrasting insoluble mineral dusts—relatively innocuous titanium dioxide and relatively toxic quartz. The present paper is based on findings derived from bringing together some of the results from these two studies. Full descriptions of the experimental methods used are given in the reports cited and only the essential details will be repeated here.

Deposition, clearance, and redistribution of particles within the lung and associated lymph nodes

Vincent *et al* have described experiments in which titanium dioxide (rutile, mass median aerodynamic diameter 1·5 μm) and quartz (Sikron F600, mass median aerodynamic diameter 2·5 μm) were inhaled by SPF rats of the AF/HAN strain for up to 16 weeks (for five days a week, seven hours a day).[3] Rats were killed in groups of up to four after prescribed periods of exposure and postexposure. Exposure levels for the experiments comprised nominal "respirable" dust concentration ranging widely from 1 to 90 mg/m^3 for the titanium dioxide and from 0·1 to 30 mg/m^3 for the quartz. The results of the analyses of lung and lymph node burdens were used to construct a pharmacokinetic model (fig 1), the main features of which include:

Deposition of particles into "compartments" from which some may be cleared via the mucociliary escalator.

Transfer of some particles to another "compartment" from which they are not cleared (the "sequestration" compartment).

Lung burden scaling in proportion to the exposure level.

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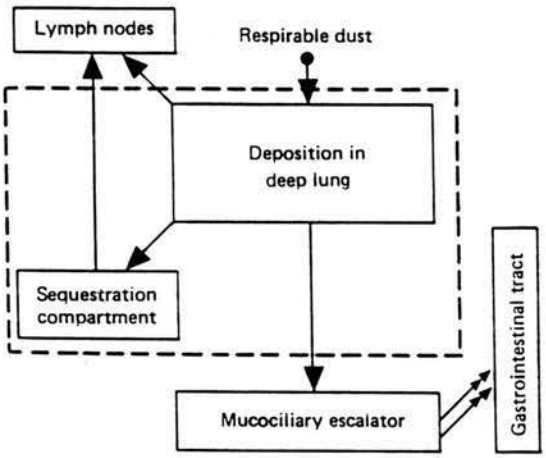


Figure 1 Schematic diagram for pharmacokinetic model describing deposition, clearance, redistribution, and storage of dust within lung.

Transfer of some particles to lung associated lymph nodes, driven by the mass burden in the lung itself but at a rate that does not become significant until the lung burden has exceeded a certain "threshold" value.

Here the term compartment is used to refer to the mathematical features of the lung's response to inhaled particles and not explicitly to its anatomical characteristics, although plausible biological suggestions have been made linking the two in much of the research in this area. It is also worth noting in passing that broadly similar trends had earlier been found in similar experiments with rats inhaling amosite asbestos.[4 5]

Quantitatively, when this model was first applied to the experimental data and its various numerical coefficients estimated, it was suggested that effective overall clearance of dust out of the lung was broadly comparable for the two dusts. A more recent examination of the data, however, indicates that, in fact, the quartz was cleared substantially more slowly than the titanium dioxide (A M Johnston and K Donaldson, unpublished data). So far as transport to lymph nodes is concerned, although the actual rates were of the same order for the two materials, the threshold of lung burden at which transfer "took off" was much lower for quartz. This would account for the apparently more rapid transfer to lymph nodes reported by some other workers.[6]

The main features of this model are broadly consistent with those proposed in recent years by other workers based on results for a range of insoluble materials, including not only quartz and titanium dioxide but also diesel particulate[7] (and S C Soderholm at the EPA diesel emissions symposium, Raleigh, NC, 1981). The same main features also hold for insoluble fibrous dusts such as amosite asbestos.[5] One aspect that remains uncertain, however, concerns the apparent impairment of clearance ("overload") at high lung burdens, an effect first identified in an earlier paper from this laboratory.[4] Morrow has drawn attention to the role of the pulmonary macrophage in the overload phenomenon and to how it might vary for different inhaled materials.[8] Whereas there is indeed strong experimental evidence, from animal inhalation studies, for reduced clearance at high lung burdens during postexposure,[4 6] the evidence is less clear for continuous exposures. For example, from the Vincent *et al* chronic inhalation studies referred to above,[3] the fact that lung burdens scale in direct proportion to exposure level over such wide ranges reflects the absence of any clear cut consequences that might be expected to be associated with overload. On the other hand, from the results of longer term inhalation studies with rats reported by Wolff *et al* for rats exposed to diesel particulate,[9] an overload effect begins to become apparent at high concentrations and for exposure times beyond one year. Elsewhere, in inhalation studies involving toner particulate (as used commercially in the photoreproduction process) the effect is even more pronounced.[10] Clearly, for overload, there remain several unanswered questions, including the suggestion that different kinetics might be required to account for what happens during continuous exposure and postexposure respectively.

Cellular response to particles in the lung

From a parallel programme of experiments carried out in our laboratory, Donaldson *et al* have reported results for the corresponding cellular responses associated with the inhalation of dust.[2] These inhalation studies used the same test dusts and basic exposure regimens as those described above, at nominal respirable dust concentrations of 10 and 50 mg/m^3. One difference worthy of note is that, here, the animals used were syngeneic PVG rats bred under specific pathogen free conditions, but this is not expected to influence substantially the particular conclusions we shall be attempting to draw by combining the two sets of results.

Samples recovered from the rat lungs by bronchoalveolar lavage were assayed to provide a wide range of biological parameters. In this paper, however, attention will be focused on just one indicator of the lung's initial biological response to the dust insult—the accumulation of neutrophils in the bronchoalveolar space. It is a reasonable first assumption that this, being a quantitative measure of the level of inflammation, is an appropriate index relevant to subsequent biological processes leading ultimately to diseases such as pneumoconiosis and emphysema.

For given periods of exposure and postexposure, the numbers of neutrophils recovered by bronchoalveolar lavage were determined. The results are presented in figs 1 and 2, where the neutrophil numbers are plotted as a function of time elapsed since exposure started. The circular symbols refer to animals killed immediately at the termination of exposure, the diamonds to animals killed at the stated time postexposure. They show that, for the titanium dioxide, cell counts for exposure at 10 mg/m^3 did not significantly differ from zero. At 50 mg/m^3, however, a sudden rise in neutrophil numbers occurred between 72 and 105 days since the start of exposure. In the 62 day postexposure phase following 105 days of exposure the count subsequently fell to about half of its end of exposure value. For the quartz, the magnitude of the response was much greater. Even at 10 mg/m^3, there were significant numbers of neutrophils for exposure times beyond about 40 days. At 50 mg/m^3, the onset of recruitment occurred even earlier, at about 10 days. After onset, the neutrophil counts increased steadily during continuing exposure. Most interestingly, however, and in pronounced contrast to the results for titanium dioxide, the numbers went on increasing after exposure had ceased.

Cellular response in relation to particle dose

To begin with, it is relevant to consider the relation between the onset of the cellular response and the onset of lymphatic clearance. From the experiments referred to earlier, Vincent *et al* estimated the lung burden at which clearance to lymph nodes "took off" for titanium dioxide and quartz (1800 and 900 μg respectively).[3] From the pharmacokinetic model derived from the same body of work, the lung burden at which, in the cell studies, the neutrophil count began to rise significantly may also be estimated. For each of the two dust types, it turns out that the latter threshold value is of the same order to magnitude as that for the onset of lymphatic clearance. From such estimates, therefore, it would appear that the two types of response are closely linked.

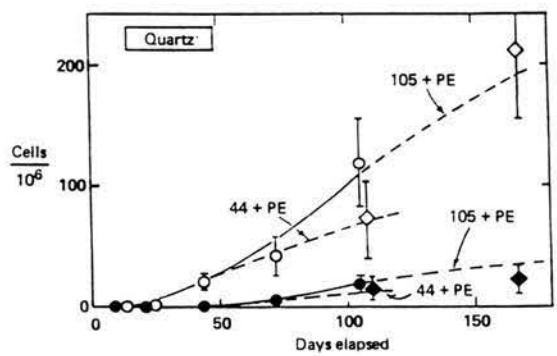


Figure 2 Neutrophil numbers as a function of time elapsed for quartz dust at exposure concentrations of 10 and 50 mg/m^3 (solid and open symbols, respectively). Circles refer to animals killed immediately at end of exposure and diamonds to those killed at indicated time postexposure. Error bars define experimental uncertainty. Curves relate to neutrophil numbers predicted by dosimetric model, under working assumptions stated in text; solid lines refer to continuous exposure, broken lines to postexposure beyond exposure period indicated—for instance, 105 + PE).

We now set out to examine the nature of integrated dose, as a means of relating the cellular response of the lung to the long term accumulation of particulate material. Here, the contrasting responses to titanium dioxide and quartz, especially postexposure, provide valuable insight into how to proceed. Qualitatively, the results show that the response to titanium dioxide falls postexposure by an amount that is greater *pro rata* than the estimated fall in lung burden due to clearance. On the other hand, for quartz, the response carries on increasing even though we know that the the lung burden cannot increase further. It is therefore clear that the instantaneous burden of material in the lung is not, on its own, a meaningful descriptor of the biological outcome.

There are three essential components of a dosimetric hypothesis on which to base the assessment of risk associated with inhaling airborne particles. The first is the exposure history, which may be expressed in terms of the exposure function, E_n. This is the time weighted average exposure concentration (or intensity) during the n^{th} day since the start of exposure. The second component is the time dependent retention of particles in the lung, where R_m, the retention function, is the proportion remaining of a particular "packet") of material at the end of the m^{th} day since it was deposited (where m can take all values up to and including n). This may be based on pharmacokinetic models such as the one shown in fig 1. The third component relates to the ability of the particles to transmit "harmfulness" to tissue. For a given material at the end of the m^{th} day since a packet has been deposited, this is expressed as G_m. This may be referred to as the harmfulness function and is a function of the particulate material itself and of the time since the particle arrived in the lung. Thus it embodies both the instantaneous potency of the material (which in turn is responsible for driving the corresponding instantaneous biological response) and its persistence.

To illustrate the concept of cumulative dose based on these concepts, consider the simple case of three days of exposure followed by two days postexposure. For this we may construct the array

Day	Incremental dose received
1	$E_1 R_1 G_1$
2	$E_1 R_2 G_2 + E_2 R_1 G_1$
3	$E_1 R_3 G_3 + E_2 R_2 G_2 + E_3 R_1 G_1$
4	$E_1 R_4 G_4 + E_2 R_3 G_3 + E_3 R_2 G_2$
5	$E_1 R_5 G_5 + E_2 R_4 G_4 + E_3 R_3 G_3$

where the overall cumulative dose is the sum of all these individual contributions. In general, for N days of exposure and ΔN days of postexposure, this is given by

$$D_{N,\Delta} = \sum_{n=1}^{N+\Delta N} E_n \sum_{m=1}^{N+\Delta N-n+1} R_m G_m \qquad (1)$$

where, for all $n > N$, E_n obviously must be zero. It should be noted that, mathematically, this solution is not strictly rigorous (N A Esmen, personal communication). This stems from the fact that E, by virtue of the way in which it must be measured and recorded, is a time averaged, and hence discrete, quantity. On the other hand, both R and G are mathematically continuous functions. It is therefore possible to envisage problems in using equation (1) in certain extreme situations—for example with rapidly varying exposure levels coupled with comparably rapid changes in pharmacokinetic response. Hence equation (1) may not be entirely general. Nevertheless, it may be shown to be adequate for computational purposes in potential applications of dosimetry in relation to long term health effects associated with insoluble particulate matter.

To link all this to the actual biological outcome, we need a working hypothesis linking integrated dose and response. Perhaps the simplest starting point is to assume that: (*a*) the relevant biological response parameter is the neutrophil population; (*b*) this is directly proportional to integrated dose of harmfulness, and (*c*) the rate at which the particulate material stimulates neutrophil influx does not change with the time the material spends in the lung—that is, G is constant. Based on the comments made at the beginning of this section about the role of lymphatic clearance, a further qualifying assumption is justified—namely, that effective dose only starts to accumulate once the lung burden has passed the threshold for which transfer of material to lymph nodes takes off.

In relating the dosimetric model to the experimental animal inhalation data, it may be taken that E is constant (at 10 or 50 mg/m^3), as this is a reasonably good approximation of the experimental conditions that prevailed. R as a function of time is derived from the pharmacokinetic model in fig 1, using the coefficients for quartz and titanium dioxide as determined by Vincent *et al.*[3] The results are shown in fig 2 for quartz, where experimental points represent the experimental results for neutrophil numbers and the drawn curves represent predicted numbers values (based on the working assumptions stated above). The solid curves represent what happens during continuous exposure (for the two exposure levels) and the broken curves represent what happens during postexposure. Figure 2 exhibits quite good agreement between experiment and theory for a single fitted value of the harmfulness function, G (= 110, in arbitrary units), for the two contrasting exposure levels (10 and 50 mg/m^3), and for both the exposure and the postexposure phases. Thus we have confirmation that, in biological terms, this material is highly persistent. This means that harmfulness continues to be delivered to the lung for as long as the particles are in contact with the tissue. There is apparently no decay in potency—nor, indeed, any increase.

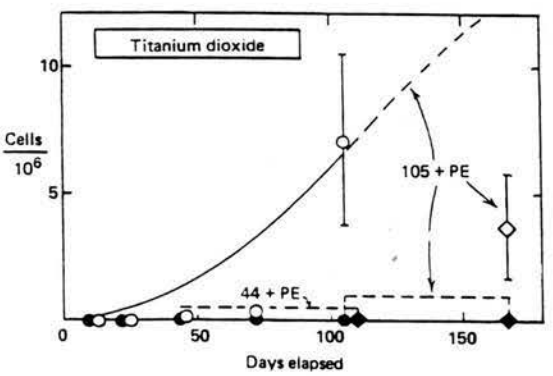


Figure 3 Neutrophil numbers as function of time elapsed for titanium dioxide at exposure concentrations of 10 and 50 mg/m^3. (Legend same as for figure 2).

The results for titanium dioxide are less easy to interpret. As shown in fig 3, whereas we can choose a value of G (= 5, much smaller than for quartz) that gives rough agreement for the exposure phase, the simple set of starting assumptions cannot account for the fall in response postexposure. A modification so that G is a rapidly decaying function of time would improve the situation somewhat in that it would restrict the continued rise in integrated dose. This would seem to be plausible for titanium dioxide in the light of its well known innocuousness. This single adjustment is not, on its own, sufficient to explain the fall in neutrophil counts. It would appear necessary also to build in negative contributions arising from the lung's ability to recover from the insult and return to normalcy. This too seems plausible. Unfortunately, at this stage, there are insufficient experimental data to enable us to develop and quantitatively test such a hypothesis. This therefore is an important area for further work.

Discussion

FINDINGS FROM THE RESEARCH

The principal aim of this paper has been to link changes in the patterns of the build up of mineral dusts in the lung and associated lymph nodes with concomitant changes in the alveolar neutrophil population.

The first, but not entirely surprising, observation is that the two dusts exhibit a remarkable difference in their estimated initial magnitudes for the harmfulness function (G), reflecting their relative abilities to deliver harmfulness to lung tissue—110 for quartz compared with only five for titanium dioxide. This appears to be one major factor in the differences in ability to cause lung injury and inflammation since the other likely factors (such as exposure intensity and the rates of deposition and clearance) are broadly similar for the two dusts. It is particularly interesting that quartz continues to deliver injury to the lung (as reflected in the continuing increase in neutrophil population) even during the postexposure period. By contrast, the corresponding postexposure response for titanium dioxide actually declines. A plausible explanation for this is that titanium dioxide is not persistent in its potency and that the lung is able to recover relatively rapidly from this particular insult. This therefore confirms what is already well known, that quartz presents the greater potential problem.

COMPARISON WITH OTHER DUSTS

Our findings may be compared with those derived from similar experiments carried out in our laboratory to investigate cellular responses in rats exposed to coaline dusts.[2] Broadly, the overall results show that, so far as inflammation is concerned, the magnitude of the response may be ranked:

titanium dioxide < coalmine dusts < quartz.

On the other hand, the ability of the lung to recover postexposure may be ranked in reverse order, relating to the magnitude of the inflammation present at the end of the exposure period:

quartz < coalmine dusts < titanium dioxide.

More specifically, lungs exposed to titanium dioxide exhibited sharply decreasing inflammation postexposure, lungs exposed to coalmine dusts showed persistence of inflammation but no further progression, and, as we have seen, lungs exposed to quartz showed pronounced progression.

IMPLICATIONS TO OCCUPATIONAL DUST RELATED LUNG DISEASE

Applying this whole scenario to workers exposed to such dusts, then the implications for occupational health may be highly significant, especially in relation to workers who have left an industry where they have been exposed to quartz. From the sequestration hypothesis, it is reasonable to deduce that for a man who has spent a lifetime working in such an industry, the vast majority of the dust particles present in his lung will have been sequestrated and so will not be available for clearance. This, together with the evidence that quartz continues to deliver considerable levels of harmfulness to the lung postexposure, may be a clue to understanding the progressive nature of silicosis in man. Thus removing a silicotic individual from the workplace so that his exposure is curtailed may not prevent progression of the disease and the development of subsequent disease. This is consistent with what has been reported epidemiologically.[11] The problem is brought out particularly clearly in the results described in this paper, opening the door to the possibility that quantitative prediction of progression in workers exposed to quartz might be achieved.

The observation that the onset of inflammation is dependent on the exposure intensity is important in relation to the establishment of occupational exposure limits, since such inflammation is generally accepted as being a precursor to pathological changes in the lung tissue. The fact that inflammation occurred in response to titanium dioxide albeit only significantly at higher exposure levels, suggests that no dust should be considered as totally innocuous so long as a sufficient quantity can be deposited in the lung. Recently, Lee *et al* have shown, in experiments with rats, that pathological change can indeed be induced by chronic exposure to high airborne concentrations of titanium dioxide.[12] So the widely held view that dusts such as titanium dioxide are "inert" needs to be treated with caution. The methods described here provide the opportunity to quantify the hazard associated with such dusts.

Finally, the above findings may provide some useful ideas for epidemiological research into dust related health effects. The integrated dose of harmfulness as expressed in equation (1) differs considerably from the cumulative exposure index.

$$C_N = \sum_{n=1}^{N} E_n \qquad (2)$$

which has been the foundation of much previous epidemiological inquiry. Equation (2) is convenient since it does not require any knowledge of, or imply any importance to, the time dependent shape of the exposure history. But, on the evidence of our findings presented in this paper, equation (1) would appear in general to be the more appropriate. To apply equation (1) raises the difficulty of how to quantify both E (the time weighted average exposure concentration) and G (the harmfulness function) in a manner relevant to the human exposure situation. At present we have only a preliminary grasp of the problem based on experiments with animals. So substantial further work is required before the desired goal can actually be achieved. In the meantime, it is interesting to note that equation (1) becomes equivalent to equation (2) for particulate materials that are either cleared rapidly from the lung or, as in the case of titanium dioxide, rapidly become innocuous after they have been deposited. For coal

dust which, as we have seen, provides a range of responses somewhere between that for titanium dioxide and quartz, it may be possible to argue that equation (2) is adequate. But we do not believe this to be the case for quartz.

Conclusions

The study was based on the assumption that bronchoalveolar lavage neutrophil populations are a general measure of tissue injury and inflammation. We have related these populations to the dose of harmfulness delivered to the lungs of rats for inhaled airborne dusts of contrasting type and known ability to produce disease—namely, quartz and titanium dioxide. The dosimetric estimates were based on a mathematical model for describing the kinetics of the retention of dust in the lung and pulmonary lymph nodes. The main conclusions are that:

(1) Inhalation of quartz, known to be a relatively toxic dust, gives rise to a substantial level of inflammation that is highly persistent and progresses even after exposure has ceased. The latter is associated with the long term retention and sequestration of a material whose potency to stimulate injury remains high throughout its residency in the lung.

(2) Inhalation of sufficiently large quantities of even a relatively non-toxic dust such as titanium dioxide dust can elicit a short term inflammatory response. This suggests the possibility that pathological changes may ensue if exposure to any inert dust is long enough and intense enough. The magnitude of such a response, however, is much less than for more toxic dusts such as quartz. Furthermore, it is much less persistent, decaying rapidly post-exposure.

(3) The development of inflammation, for both types of dust, is closely associated with transfer of dust to the lymph nodes and this could be an important factor in the pathological changes that occur in the lungs after chronic inflammation, in particular fibrosis and emphysema.

This picture points to the importance of determining whether improved exposure management, based on a proper appreciation of the kinetics of the various biological processes governing the true integrated dose to tissue, can further reduce the likelihood of the development of lung disease. Furthermore, acceptance of the importance of the role of the inflammatory response in the aetiology of dust related lung fibrotic diseases suggests the possibility of a test for individual susceptibility. Clearly much further work is required and justified.

We thank the many colleagues at the Institute of Occupational Medicine for their contributions to this research and also the Commission of European Communities and British Coal Corporation for their financial support.

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RATIONALE FOR ASSESSING EXPOSURE-DOSE-RESPONSE ELATIONSHIPS FOR OCCUPATIONAL DUST-RELATED LUNG DISEASE

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TRODUCTION

e complex chain of processes linking occupational exsure to airborne particles with the occurrence of related g disease is summarized in Figure 1. Epidemiology is ually concerned with relating the two ends since it is the ease on the one hand which is the 'problem' and exposure the other which can be monitored and controlled. wever, it has long been held that, by a proper understand- of the intermediate processes and its incorporation into epidemiological framework, substantial further advances l be made possible in epidemiology and risk assessment. om multidisciplinary studies carried out worldwide into the physical nature of the aerodynamic transport of airne particles in the respiratory tract and their deposition the lung, (b) the kinetics of their redistribution, clearance d storage, (c) the cellular and pathological responses to e presence of particles in the lung, and (d) epidemiology elf, such understanding is now available. The task now o bring together and apply the knowledge which has been quired.

is paper reviews the factors to be considered, including t only the level of initial challenge (i.e., involving conderations of the intensity of exposure, rate of deposition the lung) but also the time-dependent history of exposure volving considerations of sampling strategy), chemical mposition and indices of biological response. The ultimate jective is a dosimetric approach to the problem. What is esented here is a hypothesis upon which such an approach n be built.

HE CONCEPT OF 'DOSE'

ne concept of 'dose' is a fundamental issue. In the first inance, it involves the mass rate of deposition in the spiratory tract. The usual approach to this is to assume a nventionalized deposition fraction of the airborne parculate and to measure exposure in terms of that fraction. or the alveolar fraction, a number of quantitative definions have been widely used, notably that recommended by e British Medical Research Council.[1] In risk assessment, wever, it is worth noting that such an approach does not low for possible differences in deposition for workers gaged in different levels of physical activity (where eathing parameters might vary). Some of our estimates for derground mineworkers in different occupational groups ased on previous measurements of breathing patterns for similar groups of workers and on published lung deposition data) suggest that such effects could lead to differences in alveolar deposition by as much as x2, as compared with exposure measured according to a conventionalized deposition fraction. This suggests in turn that, at least in some epidemiological research, a more flexible approach to dust sampling may be desirable using instruments capable of providing a wider range of information (including particle size distribution and composition). Instruments suitable for this purpose, including dust 'spectrometers', are now available. Some have been the subjects of recent comparative studies carried out in several European laboratories, as reported elsewhere at this Conference by Vincent.

As far as 'dose' is concerned, however, the relationship between exposure and the rate of mass deposition in the lung is just the first stage in the process. The next step is to consider what happens after material has been deposited. In order to express dose in the context of potentially-hazardous inhaled particulate material, a useful starting point is the approach which is widely used for dealing with the dosimetry of inhaled radioactive particles.[2] Thus the hazard-related dose received by lung tissue is equivalent to the integral over time of the amount of particulate material present combined with some modifying 'harmfulness' (or 'damage') function. The latter describes the rate at which the intrinsic property associated with the hazard is transmitted from the material to the tissue and how it changes with the time during which the material is in contact.

In setting out to construct a quantitative dosimetric model, consider first the exposure history. This may be expressed as E_n, reflecting the mass deposited in the lung during the N^{th} day since exposure began. From this, cumulative exposure (C) at the n^{th} day is

$$C(N) = \sum_{n=1}^{N} E_n \qquad (1)$$

which is the form widely employed in epidemiological studies (where E_n is usually obtained in terms of the measured concentration of an appropriate dust fraction, time weighted over the working shift).

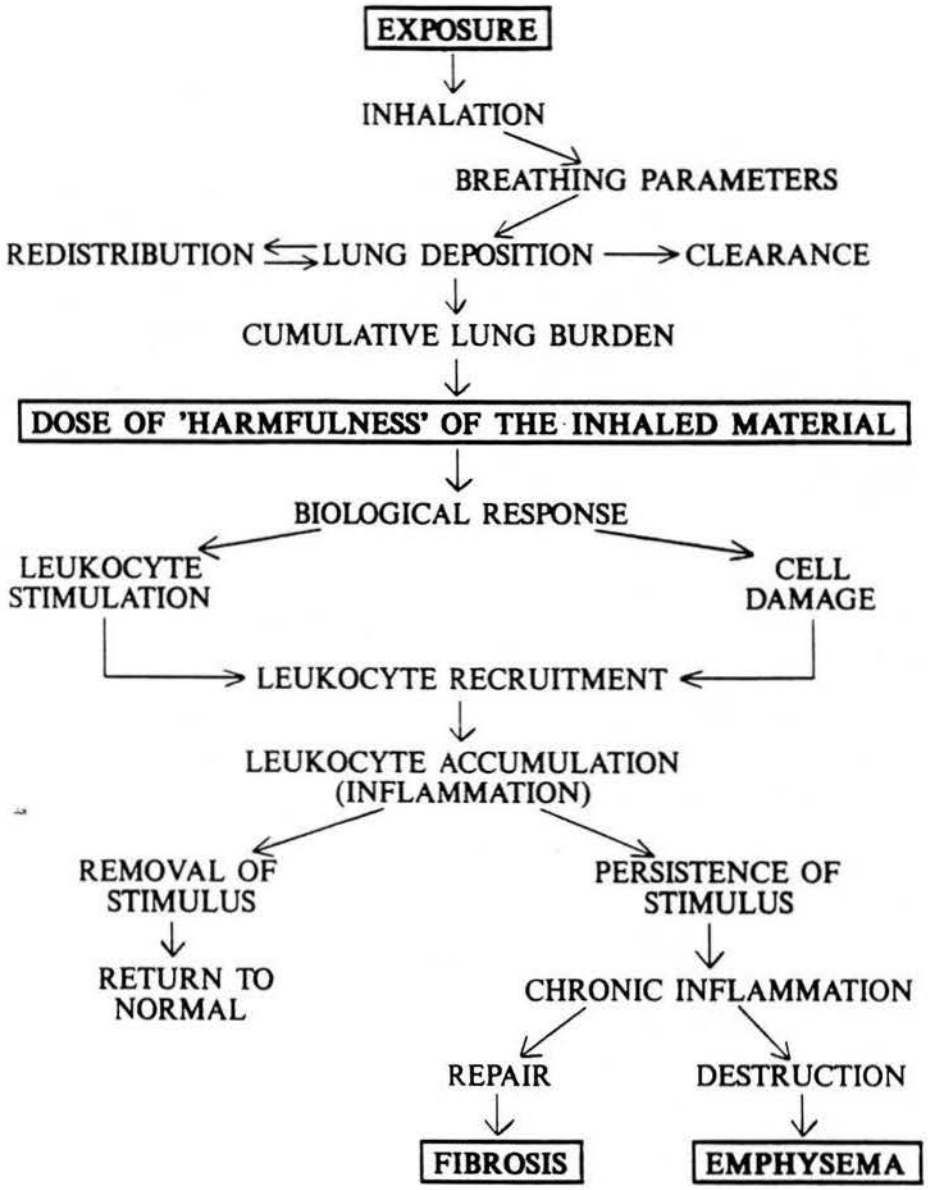


Figure 1. Processes linking exposure, dose and response associated with health effects due to mineral dusts in the deep lung.

xt consider the time-dependent retention of particulate terial in the lung. The function R_m describes the proportion remaining of a particular 'packet' of material at the m^{th} y after it has been deposited. The distinction is drawn between n (which refers to the overall time elapsed since exposure began) and m (which refers to the time elapsed since articular packet of material has been deposited in the lung). e value of R_m (between 0 and 1) is determined by the netics of redistribution, clearance and storage of the posited particles. By combining E_n and R_m, the accumulated mass (M) after N days have elapsed may be shown be

$$M(N) = \sum_{n=1}^{N} E_n R_{N-n+1} \qquad (2)$$

e now introduce the damage function G_m, which defines e rate per unit mass at which harmfulness is being transmitted to the tissue at the m^{th} day after is has been deposited. may thus be regarded as a hazard-related 'fingerprint' for e material in question. The transferred property which is sponsible for initiating the cell damage may be physical, ineralogical or biochemical.

e now have the three essential elements for constructing dosimetric model. Hypothetical examples are given in gure 2. These may be combined as follows:

Day	Dose received
1	$E_1R_1G_1$
2	$E_1R_2G_2 + E_2R_1G_1$
3	$E_1R_3G_3 + E_2R_2G_2 + E_3R_1G_1$

d so on. The cumulative dose of harmfulness is equivalent the sum of all the contributions indicated. Thus at the N^{th} y, we have

$$H(N) = \sum_{n=1}^{N} E_n \left\{ \sum_{m=1}^{N-n+1} R_m C_m \right\} \qquad (3)$$

relation to the epidemiology of dust-related lung disease, is suggested that Equation (3) should replace Equation (1) nd other simplistic forms of dose assessment.

RACTICAL CONSIDERATIONS

ractical implementation of the proposed rationale involves uantitative description of the three key elements; E, R nd G.

The first of these is derived from measurements of dust concentration in a way such that the life-time dust exposures of individual workers may be described. This is a complex task. In the first place, it involves choosing a sampling instrument that provides a measurement of the airborne concentration of a dust fraction relevant to the disease in question. In the case of pneumoconiosis, this is the respirable fraction (although there may still be some debate about the particular quantitative criterion by which this should be defined). In turn, there are many instruments available which can provide the required information. In choosing the instrument, considerations of how best to make the measurement relevant to the true exposure of the individual worker raises questions of personal versus static (fixed point) sampling which have been discussed elsewhere.[3] Both types provide the time-weighted shift average of the exposure concentration. The frequency of sampling and its relevance to the assessment of long-term exposure are a matter of sampling strategy, involving considerations of the 'smoothing' that takes place in the body after particles have been deposited (which, in turn, is dependent on R).[4] Furthermore, since the exposure history, if it is to be useful, must reflect the life-time experience of the individual worker, and since he (or she) may move around the workplace from time to time, a record of time worked in particular occupations is an important ingredient towards construction of exposure history. Finally, since it is likely that epidemiology will be desirable for workers for whom exposure records in the early years are either non-existent or imperfect, it may be necessary in many cases to retrospectively estimate exposure histories on the basis of intelligent extrapolations backwards, taking into account more recent measurements and engineering histories of the industries in question.[5,6]

As far as R is concerned, substantial progress in understanding has been made in recent years, mostly based on inhalation studies with animals.[7,8,9] Therefore we now have pharmacokinetic models which are applicable to various toxic and non-toxic, fibrous and non-fibrous materials over wide ranges of exposure level. It is, however, important to note, that such models are relevant strictly only to the animals in question, and need to be validated with respect to humans. Data obtained during epidemiological research in the British coal industry, in particular information from autopsy studies on the lung burdens of mineworkers for whom exposure histories are known, are at present being examined in order to explore the feasibility of establishing such a link.

Although the third quantity, G, is just as important in relation to dose, it is still more difficult to quantify. In the case of radioactive particulate matter (the starting point for the dosimetric hypothesis), the harmful property which is transferred between the particulate matter and the lung tissue is relatively easy to identify (e.g., ionizing radiation of a well-defined type). For mineral dusts, however, like those encountered in many industrial workplaces, the nature of the property is not known. Quartz is one example where, although there are well-known health hazards associated with inhaling respirable particles, somewhat inconsistent epidemiological findings have emerged, especially when other materials are present. As a result, attempts to determine the basic nature of the harmfulness of quartz have not

et provided definitive answers. European research, involv-
ng several laboratories, is presently in progress to address
his question, as described elsewhere at this Conference by
obock.

n setting out to quantify G, mineralogical assessment alone
oes not provide all that is required. Neither (necessarily)
oes toxicity evaluation based on in vitro cell viability tests.
n our own Institute, we are at present exploring how pro-
ress might be achieved by direct reference to the cellular re-
ponse in the lung itself.[10] Bronchoalveolar lavage studies in
ats exposed to dusts known to produce contrasting health effects (relatively-innocuous titanium dioxide and highly toxic quartz, for example) have been carried out. These have involved measurements of responses reflecting lung injury (e.g., leukocyte recruitment). Some of the results are particularly relevant in the present context—although the conclusions are preliminary at this stage. Some examples are shown in Figure 3, where the dusts were delivered into the lungs of the rats by inhalation and the leukocyte recruitment assessed subsequently (in terms of neutrophil counts). For the titanium dioxide, the results suggest a biological response is provoked which falls after the cessation of exposure. This in turn suggests that the intrinsic 'harmfulness' of the ma-

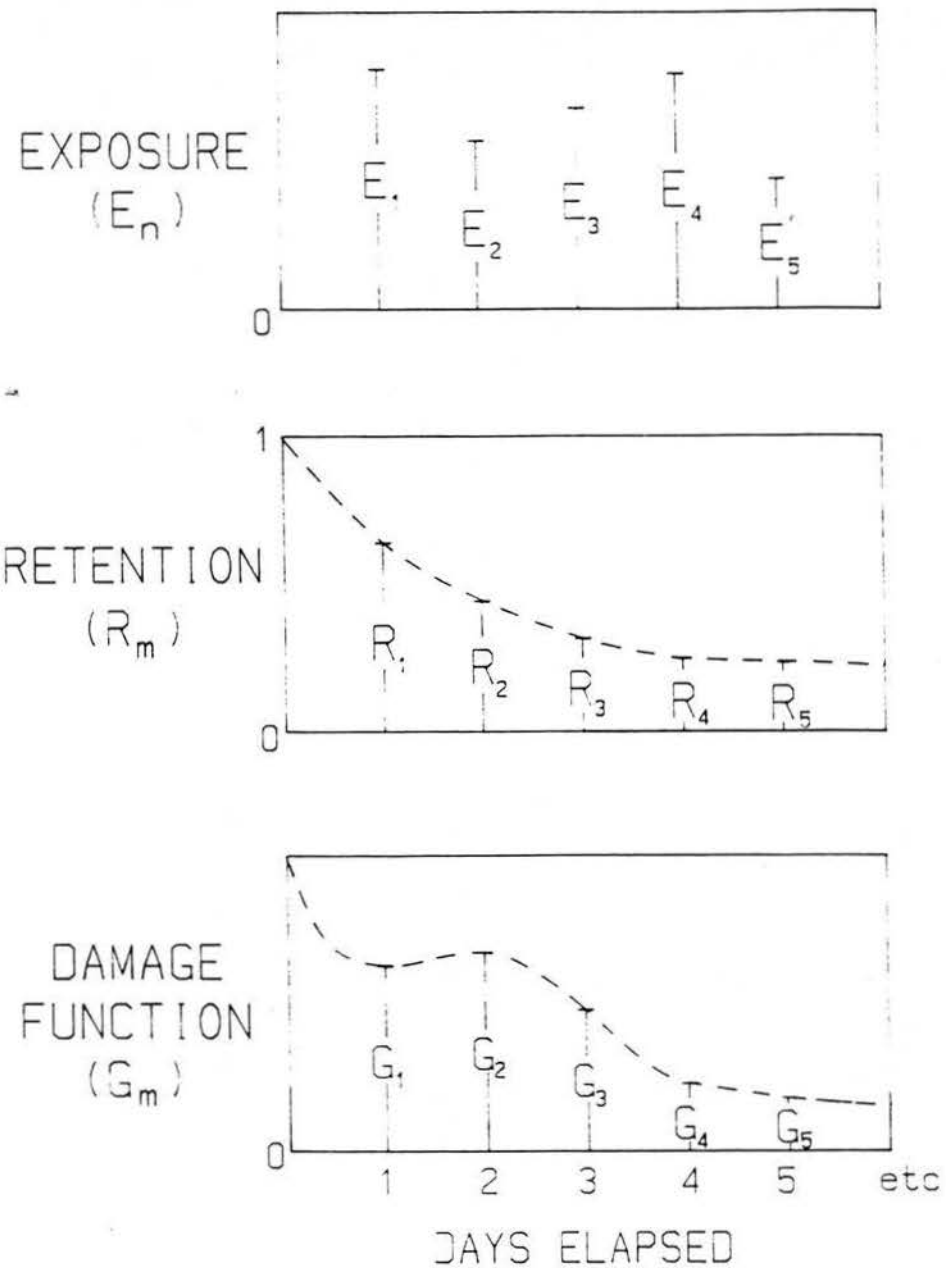


Figure 2. Hypothetical examples to illustrate the quantitative nature of exposure (E), retention (R) and damage function (G).

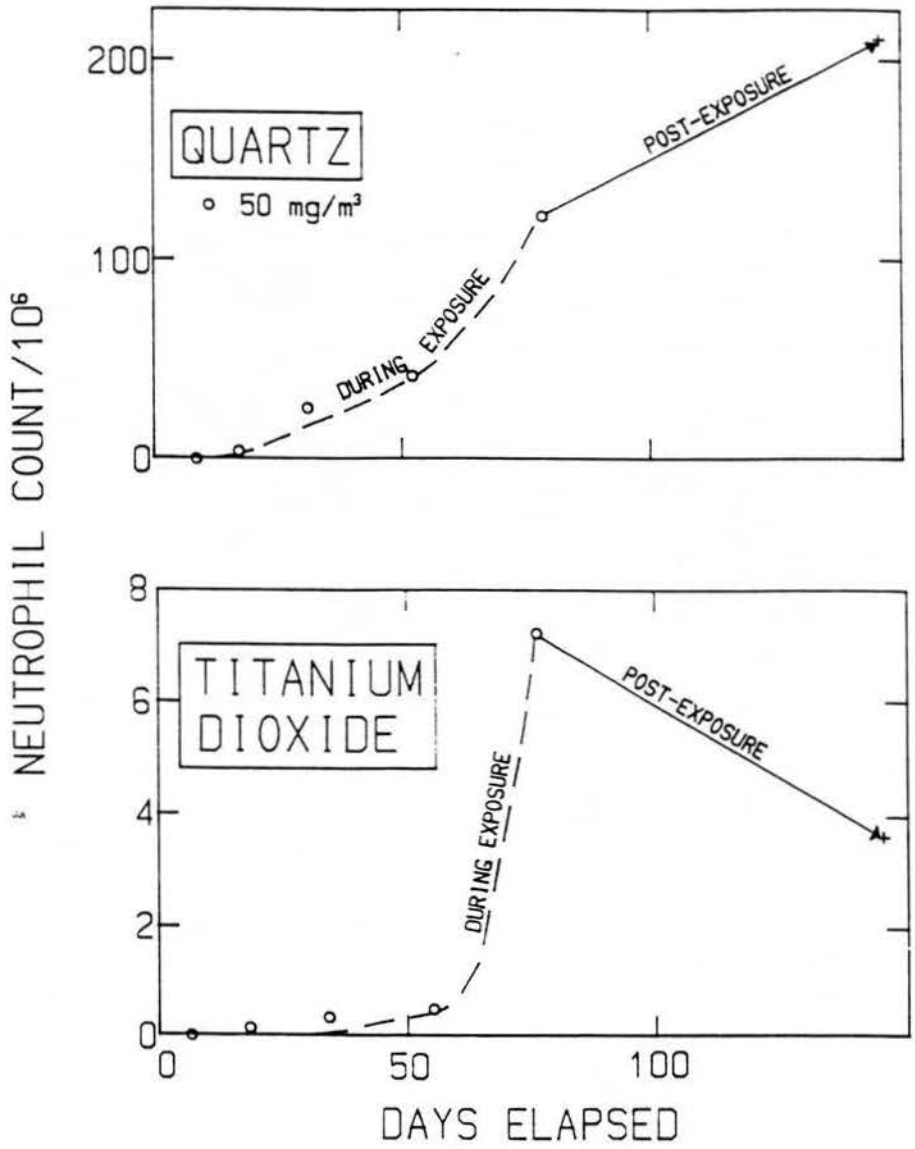


Figure 3. Typical results of cell-lavage study, showing neutrophil response during exposure- and post-exposure for quartz and titanium dioxide inhaled by rats at 50 mg/m³ (respirable). The results shown are means for four rats.

terial is not persistent but rather the damage function, G, decays with time. Throughout, its magnitude is relatively small. In contrast, the biological response to the inhaled quartz is much greater in magnitude and is much more persistent. That is, G is high upon arrival in the lung, and—unlike titanium dioxide—does not decline, even post-exposure. From these findings, the dosimetric implications are clearly consistent with what is known about the contrasting hazards associated with inhaling each of these two materials. Further work is now needed to place such ideas on a more quantitative footing, and to extend them to other, more-realistic mineral dusts.

CONCLUDING REMARKS

In the preceding, we have discussed the main ingredients of a dosimetric model for assessing the risk associated with inhaling airborne particles. The rationale for its development is summarised in Figure 4. At this stage, it is no more than an initial hypothesis. Before it can be proposed as a working model, it is necessary, (a) to establish the validity of pharmacokinetic models, derived originally from the results of animal inhalation studies, for describing retention in humans, and (b) to establish the validity of (and extend) the biological assays aimed at quantifying G for dusts relevant to work-

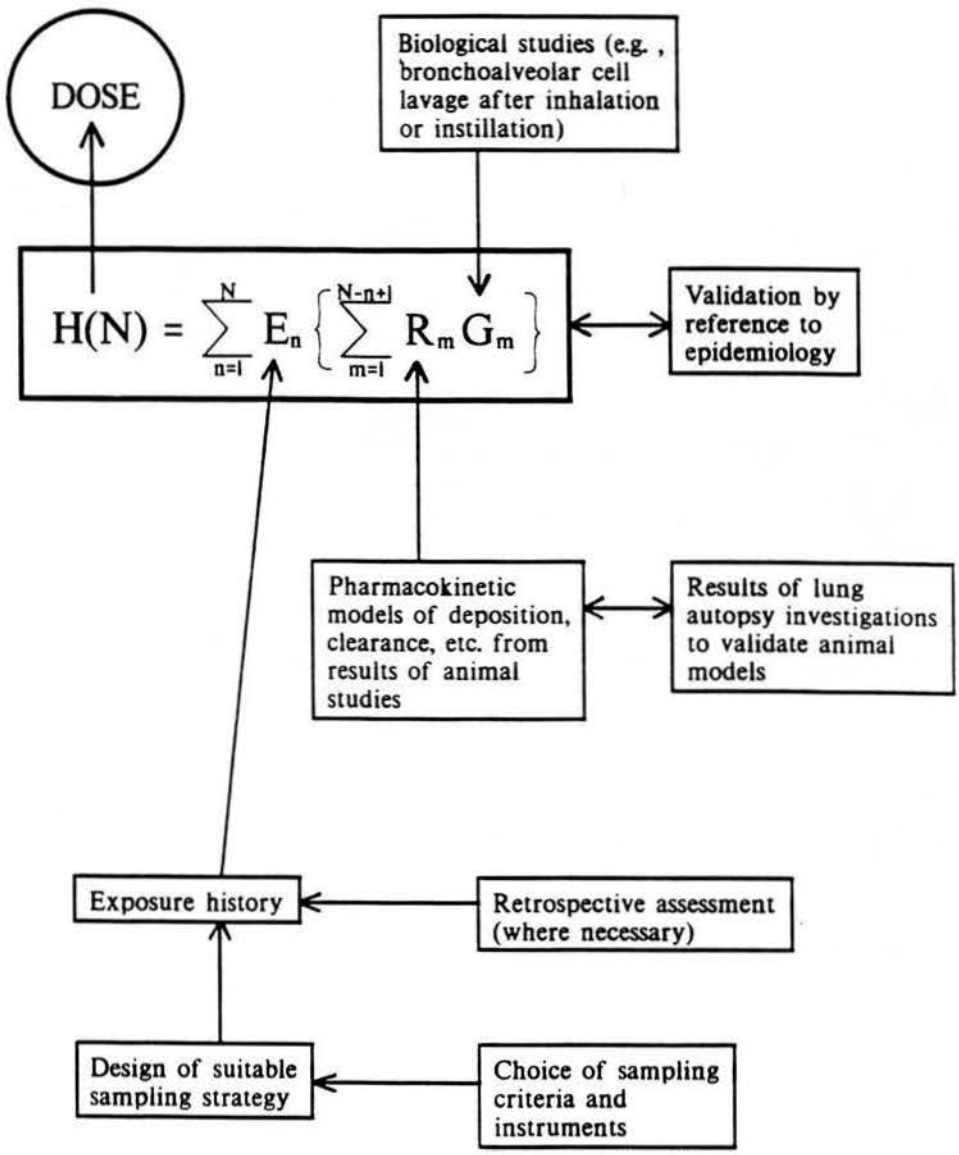


Figure 4. Summary of the rationale for the development of a dosimetric model.

place exposures. Some such studies are in progress. Having once established the working hypothesis, the next step is to validate it with respect to epidemiology for working populations whose exposure and occupational histories are sufficiently well-known. From this scenario, it may therefore be assumed that the emergence of an actual working dosimetric model is still some years away.

The broad benefits of the dosimetric approach to epidemiology have already been mentioned. Notably, as far as epidemiology is concerned, it is anticipated that improved sensitivity (and specificity) and reduced variability in explaining the relationships between the environment and health will be achieved. In turn, improved standards setting, more representative dust sampling strategies, and more effective control procedures (through appropriate worker deployment strategies, technical measures, etc.) will be made possible.

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THE FATE AND EFFECT OF INHALED CHRYSOTILE ASBESTOS FIBRES

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Abstract—An effective assessment of the relative toxicity of inhaled fibres requires an understanding of the rates of deposition and clearance, and of chemical or morphological changes which occur in fibres under the biochemical conditions of the lung. Although fibres of amphibole asbestos remain virtually unchanged after many years of residence in the lung, chrysotile fibres split longitudinally and leaching of both structural and trace elements occurs. In the experiments described, rats were exposed to airborne UICC chrysotile asbestos at 10 mg m^{-3} for 7 h per day, 5 days per week for periods of up to 18 months. During the exposure, groups of rats were killed at predetermined times, their lungs digested, the retained chrysotile fibres recovered, then counted and sized by scanning electron microscopy. The results showed that splitting of chrysotile fibres led to the number of long thin fibres in the lung increasing with time. The data describing the accumulation of chrysotile fibres, combined with the available evidence on the effects of the leaching of structural elements, provide an improved basis for interpreting the pathogenic effects of this fibre.

INTRODUCTION

EPIDEMIOLOGICAL studies have shown that amphibole asbestos minerals are more fibrogenic and carcinogenic than chrysotile, but chrysotile has proved more tumourigenic to rats when inhaled than either crocidolite or amosite (DAVIS *et al.*, 1978). The purpose of this paper is to assess whether this apparent inconsistency can be explained by a better understanding of the fate of fibres in the lung.

Amphibole asbestos fibres are largely unaffected by conditions in the lung, whereas chrysotile fibres can undergo extensive mineralogical and morphological changes. For example, JAURAND *et al.* (1977) found that more than 80% of the magnesium (Mg) normally present had been leached from chrysotile fibres recovered from the lungs of asbestos workers; similar results have been reported by other researchers. Recently, however, CHURG and DE PAOLI (1988) reported that fibres recovered from lungs of asbestos workers had compositions similar to that of intact chrysotile and concluded that some of the inhaled chrysotile fibres must escape significant compositional change. The carcinogenicity of chrysotile fibres, depleted in structural magnesium by acid treatment, has been studied in rats (MORGAN *et al.*, 1977a; MONCHAUX *et al.*, 1981). The carcinogenicity was unchanged by magnesium depletion up to 50%, but fibres with 80–90% depletion produced many fewer mesotheliomas.

Magnesium is leached rapidly from chrysotile by acids; slow and slight leaching occurs even in distilled water. The pH which an inhaled fibre encounters *in vivo* ranges from about 7 on the alveolar surface, or in epithelial cells, to about 5 in the phagolysosomes of macrophages. JAURAND *et al.* (1984) demonstrated that magnesium leaching of chrysotile in alveolar macrophages *in vitro* occurs at a similar rate to that in

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acids at pH 4, whereas leaching in mesothelial cells is very much slower, comparable with pH 7.

The rates of deposition and clearance of amphibole and chrysotile asbestos fibres have been compared in rats exposed to these materials, but the results have not been entirely consistent. Very short (30 min) nose-only exposures to radioactively-labelled fibres showed similar deposition patterns for amphibole and chrysotile fibres (MORGAN *et al.*, 1977b). In that study, the half-times of alveolar clearance ranged from 46 to 76 days with no significant difference between the amphibole and chrysotile fibres. Indeed, the alveolar clearance half-times of amosite (56 days) and chrysotile (chrysotile A 62 days, chrysotile B 46 days) were remarkably similar. ROGGLI *et al.* (1987) also observed similar depositions for crocidolite and chrysotile following 60-min nose-only exposures; the lung burdens were estimated using electron microscopy. However, during chronic exposure of rats to amphibole and chrysotile fibres, large differences in the rates of accumulation of fibres in the lung have been reported. Whereas the lung burdens of amphibole increased steadily throughout the exposure period, those of chrysotile reached a point after which no further increase occurred (WAGNER *et al.*, 1974).

Examination by electron microscopy of fibres recovered from rat lungs after either instillation (BELLMAN *et al.*, 1986) or inhalation (ROGGLI *et al.*, 1987) has shown that chrysotile fibres split longitudinally; consequently the mean diameters of the remaining fibres were reduced whilst mean lengths did not alter. Changes in composition might well be expected to affect clearance, and this was confirmed by BELLMAN *et al.* (1986) who instilled a sample of chrysotile, pretreated with acid to leach 90% of its cations, into rat lungs and found rapid clearance.

In order to improve our understanding about how chrysotile fibres may change within the lung, and to develop a model for the appropriate assessment of dose, further information is needed on the amount and condition of chrysotile fibre which accumulates in the lung during chronic exposure. This was the purpose of the experiments described in this paper.

EXPERIMENTAL METHODS

Male SPF rats of the AF/HAN strain were exposed for 7 h per day, 5 days per week to UICC chrysotile asbestos at a target respirable dust concentration, as measured with the Casella MRE 113A sampler, of 10 mg m^{-3} for up to 22 months. Groups of six rats were removed from the exposure chamber after periods of 1 day or 4, 13, 26, 52, 65 and 95 weeks and killed in subgroups of three at 3 and 38 days after removal. The lungs were resected and the right lobes taken for analysis. Based on previous studies, the ratio of the fibre burdens in the left and combined right lobes is 0.6:1. The right lobes were dried, rinsed in ether–ethanol mixtures to dissolve fat, and ashed in a low-temperature plasma incinerator. From the dry ash, which was kept in a sealed glass vial, a small portion was taken, rinsed in water and recovered by filtration onto a polycarbonate filter. This filter was prepared for scanning transmission electron microscopy (STEM) and the fibres were sized at a magnification of $\times 10\,000$. Initially, 100 randomly-selected fibres of all sizes were measured, and then 100 fibres longer than 5 μm. A computer program was used to calculate the volume of each fibre and hence the total mass (M) of fibres in each lung, assuming cylindrical geometry and a density of 2550 kg

m^{-3}. On a selection of the samples, the relative amounts of Mg and Si were determined for 20 fibres using energy dispersive X-ray analysis (EDXA) of diameter 0.3 μm. Since the analytical technique can be affected by particle size, especially if surface chemistry of a fibre differs from the interior, the decision to standardize on a fixed fibre diameter was made in order to achieve improved comparability of the analyses on different samples. Tests of the recovery procedures showed that the original chrysotile could be mixed with lung tissue and then processed without producing detectable changes in fibre composition. However, it was observed that chrysotile fibres in lung ash underwent mineralogical changes when kept for several months in sealed glass vials; in particular, leaching of magnesium from the chrysotile fibres occurred together with the formation of rhomboidal magnesium salt crystals in the lung ash. Consequently, it was decided to accept only results based on samples on electron microscope grids which had been prepared within 1 month from the start of sample preparation.

For standardization purposes, chrysotile fibres were leached in 0.1 M HCl at 60°C for 2, 4, 6, 8, 16 and 24 h, to produce samples with a range of Mg depletions. These were analysed individually by EDXA, and in bulk by the infra red (i.r) spectrophotometry technique used previously to assess the amount of chrysotile in rat lungs.

RESULTS

Exposure concentrations

The mean airborne mass concentrations of respirable fibres, averaged over 5 days per week to allow for minor interruptions in the longer exposures due to maintenance, etc., were within the range 9.2–10.1 mg m^{-3}, except for the 1-day exposure group (7.7 mg m^{-3}). Airborne fibre number concentrations (length ≥ 5 μm, diameter < 3.0 μm, aspect ratio $> 3{:}1$), estimated from 42 snatch samples, showed that 10 mg m^{-3} corresponded to about 2600 fibres ml^{-1}.

Quantity of asbestos in the lung

In Fig. 1 the mass of fibre in the lung (computed from the number–size data) is plotted against time of exposure. Each data point is the result for an individual rat lung. Also included are results from previous studies in which the mass of fibre retained was based on i.r. spectrophotometry (MIDDLETON *et al.*, 1979; DAVIS *et al.*, 1988). These are seen to be consistent with the new results. If the earlier data are increased by about 20%, to compensate for the likely effect of Mg depletion on the i.r. absorption coefficients, the agreement is even closer.

Although the results based on electron microscopy are in agreement with those from i.r. spectrophotometry, caution has to be exercised in fitting a curve to such scattered data. In addition to the large spread of data points, there is an anomaly in the data in that after some of the longer exposure periods ($T = 362$ or 453 days) the results indicate that more dust is retained in lungs of rats killed at 38 days after removal from the exposure chamber than in those killed at 3 days. This anomaly is attributable partly to the biological variation between animals and partly to the imprecision of the fibre counting procedure.

As far as the trend in lung fibre burden is concerned, two models are considered as shown in Fig. 1. In the first, the lung burden follows simple, linear, first-order clearance kinetics, reaching a plateau at about 200 days. The second model follows the apparent

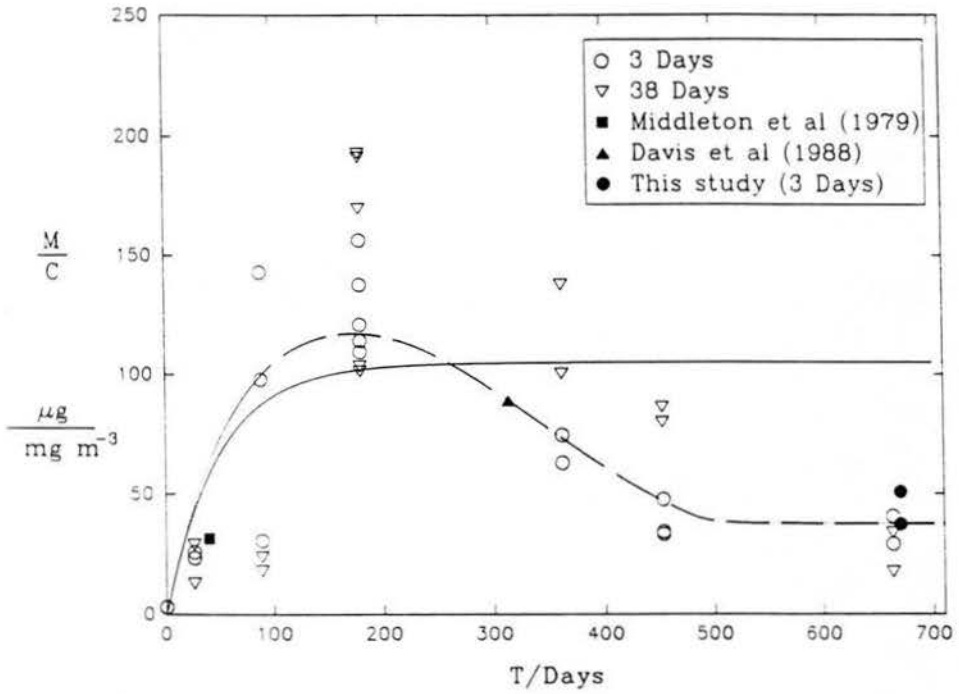


FIG. 1. Mass M of chrysotile asbestos in the lung after chronic exposure for time T at a concentration C of approximately 10 mg m^{-3}. The standardizrd lung burden M/C is plotted to allow for slight differences from the target concentration. Lung burdens measured at post-exposure times of 3 (○) or 38 (▽) days. Dark symbols are for results obtained by i.r. spectrophotometry in the present and previous studies. The two curves are calculated from clearance models which are described in the text.

fall in lung burden after about 180 days and is more consistent with the observed trend. Interestingly, the latter resembles that obtained for the burden in lavaged samples of the lung content taken during the course of repeated instillations of chrysotile into the sheep lung (SÉBASTIEN *et al.*, 1994).

In Fig. 2 the numbers of fibres with length ≥ 5 μm which had accumulated in the lung are plotted against time of exposure. This graph reflects three processes: continuing deposition, splitting, and clearance of fibres. The fibre numbers rise initially and then, after approximately 180 days of exposure, appear to decline.

Composition and size distribution of the asbestos fibres

Analyses by EDXA of individual fibres (diameter 0.3 μm) recovered from rat lungs are shown in Table 1, which indicates a magnesium depletion of between 10 and 40%. The fibre size results followed the expected pattern, with the mean length remaining unchanged whilst, as shown in Fig. 3, the mean diameter decreased with exposure time. Consequently, the proportion of long thin fibres increased during chronic exposure and the number of long thin fibres in the lung increased faster than the rate of deposition, as shown earlier (JONES *et al.*, 1989).

DISCUSSION

Comparison with durable fibres

The most interesting feature of the results in Fig. 1 is that during chronic exposure to chrysotile asbestos the mass of fibre in the lung rises initially and then appears to

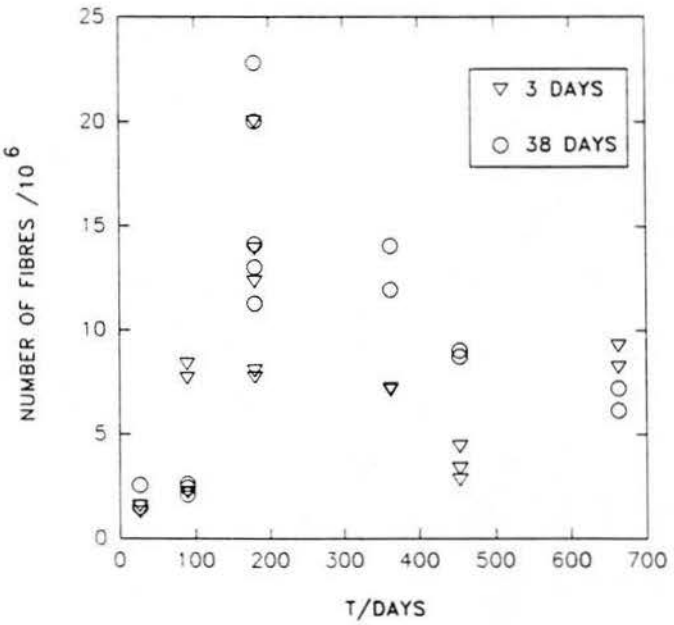


FIG. 2. The number of fibres recovered from rat lungs after exposure at 10 mg m^{-3} (2600 fibres ml^{-1}) for T days. Data for fibres with length greater than or equal to 5 μm.

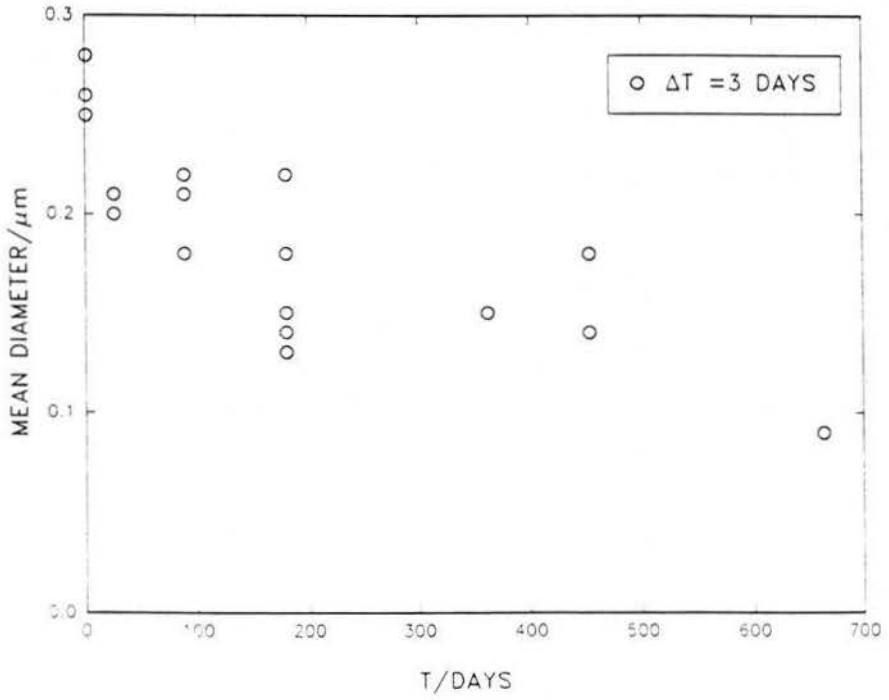


FIG. 3. Mean diameter of fibres recovered from rat lungs after chronic exposure for T days.

TABLE 1. SEMI-QUANTITATIVE RESULTS FOR THE CHANGE IN THE AVERAGE RATIO OF MAGNESIUM TO SILICON AS INDICATED BY EDXA FOR INDIVIDUAL CHRYSOTILE FIBRES OF 0.3 μm DIAMETER RECOVERED FROM RAT LUNGS AFTER EXPOSURE TIMES T AND POST-EXPOSURE TIMES ΔT

Number of lungs	Total number of fibres analysed	T (days)	ΔT (days)	Mg:Si relative decrease in ratio of X-ray counts (%)
3	48	1	3	13
2	40	26	3	15
2	40	26	28	20
2	41	89	3	15
2	19	89	38	39
3	60	89	122	22
2	40	180	3	23
5	99	180	38	17
2	40	361	3	9

decrease. For chronic exposure to amphibole asbestos a very different trend has been observed (WAGNER *et al.*, 1974; VINCENT *et al.*, 1985; JONES *et al.*, 1988). This is consistent with the results of CHURG *et al.* (1988) who compared the rates of clearance of chrysotile and amosite asbestos, instilled as a mixture into the lungs of guinea pigs, and showed that the rate of clearance of chrysotile was significantly greater than for amosite.

It is known that peribronchiolar lesions develop by the end of 1 year of exposure to chrysotile at 10 mg m^{-3} (DAVIS *et al.*, 1988). It is possible that the resulting morphological changes might alter the distribution of airflow and consequently shift the pattern of regional deposition, this accounting for the results in Fig. 1. However, lesions of the same type also occur following exposure to amphibole asbestos (e.g. DAVIS *et al.*, 1986). So, it is unlikely that these changes can account for the marked differences observed between the accumulation of amphibole and chrysotile in the lung. Since the trends observed for lung burdens of amphibole asbestos (BOLTON *et al.*, 1983) and of other dusts (VINCENT *et al.*, 1985) are all consistent with approximately constant rates of deposition, we have applied the same working hypothesis in modelling the results for chrysotile deposition.

Assessment of dose to lung tissue

As has been argued in preceding papers (e.g. VINCENT *et al.*, 1990) the cumulative harmful dose imparted to vulnerable lung tissue by the presence of inhaled particles can be expressed terms of the subject's exposure history and the kinetics of both the retention of the particles and of the changes which may take place in their toxicity during residence in the lung. Such considerations are especially relevant—although difficult to apply quantitatively—to chrysotile.

The composition of the recovered fibres showed an average level of depletion of magnesium of the order of 20%, which is similar to that found by BELLMAN *et al.* (1986) during the 18 months following intratracheal instillation of chrysotile fibres. Since the change in the structure of the chrysotile has a major effect on both its biological activity, and on the rate at which it can be cleared from the lung, it is noteworthy that in the present study the average level of Mg depletion in the retained fibres in the lung is

only about 20%. This suggests that the fibres remain highly biologically active throughout the study.

The measurements of the amount of asbestos fibre retained in the lung show considerable scatter but, despite such limitations, they are sufficient to enable us to estimate how the difference in retention between chrysotile and other fibres might influence the harmful dose to the lung.

Two simple models of the clearance kinetics are used to describe the lung retention of chrysotile. The first is a single exponential clearance model with constant rates of deposition and clearance. The second has the same constant rate of deposition, but the clearance rate increases with time up to a maximum value; this produces the second curve shown as a broken line in Fig. 1. Changes in clearance rate might arise from the alterations to the retained fibres. As the chrysotile fibres undergo leaching most rapidly in the phagolysosomes of macrophages, it may also be significant that an influx of macrophages into the lung occurs in response to exposure to chrysotile (DONALDSON *et al.*, 1988). The influx of phagocytic cells is not, on its own, likely to be a sufficient explanation of an increased alveolar clearance rate because similar inflammatory responses are produced by several other dusts for which accumulation in the lung continues unabated during chronic exposure (VINCENT *et al.*, 1985).

Starting with the simpler model with constant rates of deposition and clearance, we note from our earlier results for amosite (VINCENT *et al.*, 1985) that the rate (k) of linear increase in lung burden was approximately 2.1 μg per day (averaged over the 7 days of the week) for each mg m^{-3} of exposure concentration. To a first approximation, this is also a reasonable estimate for describing the build-up of chrysotile since exposure for 1 day gave lung burdens which were equivalent to a (7 days per week) average deposition rate of approximately 2.2 μg per day per mg m^{-3}. If we then postulate that the chrysotile is being cleared at a fractional rate (λ) of 0.02 per day (i.e. 2% of lung burden cleared per day), this yields a curve (see Fig. 1) which reaches a plateau. This simplification of the clearance kinetics for chrysotile and amosite leads to two contrasting situations; one where there is a single rate of clearance, and the other where there is none. Appropriate indices of dose relating to these two situations have already been applied in epidemiological studies (FINKELSTEIN, 1985).

The cumulative lung burden after N days of exposure, where the concentration and duration of exposure on day i were C_i and Δt:, respectively, is:

$$M = \sum_{i=1}^{N} kC_i(1-\lambda)^{N-i}\,\Delta t_i, \tag{1}$$

where k and λ are the rates of deposition and clearance respectively. If there is zero clearance, this becomes:

$$M = kCt, \tag{2}$$

where the product Ct is the index of cumulative exposure which is frequently used in epidemiological studies for exposure at a mean concentration C over a total time t.

For continuous exposure at a constant concentration, Equation (1) may be written as a differential equation which can be solved for the model with a constant value of λ, to yield:

$$M = \frac{kC}{\lambda}(1 - e^{-\lambda t}) \tag{3}$$

The cumulative harmful effect of the interaction between the lung burden and the lung may be assumed to depend on some toxicological (or damage) function H, embodying the effects of changing morphology and composition, which describes the mean harmfulness of the fibres in the lung on a given day. After N days of exposure this would yield a cumulative dose, $D(t)$, which can be approximated, following our original model (VINCENT *et al.*, 1990), as:

$$D(t) = \sum_{i=1}^{N} M_i H_i \, \Delta t, \tag{4}$$

where M_i and H_i are the lung burden and damage function on day i. For the present, we will assume that H remains relatively constant, since the change in average magnesium content during the course of the 1-year study is not sufficient to alter the tumourigenicity of the fibres and because the trends for fibre number (Fig. 2) are broadly similar to those for fibre mass (Fig. 1). Then (for constant exposure concentration C) Equations (4) and (3) yield:

$$D(t) = l \int_0^t \frac{kC}{\lambda}(1 - e^{-\lambda t}) \, dt \tag{5}$$

which on integration, gives:

$$D(t) = \frac{KC}{\lambda}\left[t - \frac{1}{\lambda}(1 - e^{-\lambda t}) \right]. \tag{6}$$

This contrasts with the equivalent expression, applicable when M is estimated by Equation (2), which is:

$$D(t) = \frac{1}{2} kCt^2. \tag{7}$$

Equation (7) appears to be the more relevant to durable fibres, whereas Equation (6) appears more relevant for non-durable fibres such as chrysotile. Over the 1-year inhalation study with rats, Equation (7), for durable fibres gives $D(t)$ as approximately 1.4×10^6 μg days^2. The corresponding dose for the non-durable fibres is 3.3×10^5 μg days^2. The difference in the retention of the fibres affects the dose over 1 year by a factor of about 4. Over 40 years, relevant to human exposure, a similar difference in retention would reduce the dose by a factor of about 140, i.e. a 35-fold greater difference in dose.

In attempting to draw inferences for the consequences of exposure for humans, we also need to take into account as far as possible interspecies differences in clearance rates. The mechanical clearance of insoluble particles is generally an order of magnitude slower in humans than in rats (KREYLING, 1990). In contrast, it is believed that fibre dissolution probably proceeds at very similar rates in the lungs of most animals. Therefore, it appears likely that fibre durability will play an even more prominent part in the fate and effect of fibres in the human lung. However, the mechanism of the interaction between the leaching of magnesium from chrysotile fibres and the enhancement of mechanical clearance is not yet clear and consequently further information is needed in order to take the difference between species fully into account.

The calculations of cumulative dose were also applied to the second curve (which reaches a peak at $T=174$ days) in Fig. 1. This curve corresponds to a clearance rate λ given by:

$$\lambda=0.02\ (0.8+2\ (T/480)^3)\ \text{for}\ T<480$$

$$\lambda=0.0560\ \text{for}\ T>480. \tag{8}$$

Computation using Equations (1), (4) and (8) yielded a cumulative dose of 3.4×10^5 μg days^2 for the exposure to chrysotile over 1 year, a value which differs only slightly from that (3.3×10^5 μg days^2) obtained previously from Equation (6). However, the estimates of dose for 40 years from the two curves in Fig. 1 differ more. The value from the second curve suggests that the difference in retention (between durable and non-durable fibres) would make approximately a 97-fold greater difference in dose over 40 years as compared to over 1 year.

CONCLUSIONS

The main conclusions of this research relate to the fate of inhaled chrysotile fibres during the course of chronic exposure, and how these events affect the effective harmful dose. They are listed below.

(1) During chronic exposure inhaled chrysotile fibres undergo splitting which leads to a reduction in mean fibre diameter while the mean fibre length remains approximately constant.

(2) The mean depletion of magnesium from the chrysotile fibres was approximately 20% for fibres of 0.3 μm diameter retained in the rat lungs during the chronic exposure, which means that the fibres probably remained tumorigenic over a time scale relevant to the life-span of rats.

(3) The lung burden of chrysotile increases initially and then after 150 days of exposure ceases to increase and, indeed, may even decrease although exposure continues.

(4) The difference in the durability and clearance between chrysotile and amphibole asbestos affects the harmful dose to an extent which is sufficiently different over chronic exposure for 1 year (for the rat) and over 40 years (relevant to the human) to explain apparent anomalies which have arisen from pathogenicity studies with chrysotile and amphibole asbestos at similar concentrations.

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Bromo-deoxyuridine (BRDU) uptake in the lungs of rats inhaling amosite asbestos or vitreous fibres at equal airborne fibre concentrations*)

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With 2 figures and 2 tables

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Abstract

Rats were exposed, by inhalation, to target airborne fibre concentrations of 1000 f/ml (PCOM fibres by WHO criteria) of a long amosite asbestos sample and a vitreous fibre sample; the target was closely attained for both fibre samples. The size distributions of the two fibre samples was closely similar. Rats were placed in the chambers for 7 hours and then, following a further 16 hours in room air, were injected with bromo-deoxyuridine (BRDU). The presence of BRDU-positive cells in terminal bronchioles/alveolar ducts was assessed in blocks taken from various parts of the left lung, from apex to base. There were significant differences in the proliferative responses between animals but there were also significant differences between the treatments. Lungs from rats exposed to vitreous fibres showed no greater response than the controls, but there was a markedly greater proliferative response in the lungs of rats inhaling long amosite. There was a decreasing gradient of proliferative response from the apex of the lung to the base with all treatments. This could be explained by different degrees of deposition in different areas of the lung. Similar amounts of fibre accumulated in the lungs of rats exposed to the two fibre types and it is unlikely that dissolution could be important over the timescale used here. We conclude that, when amosite asbestos deposits in the lungs of rats it stimulates a proliferative response and that deposition of an equal number of similar-sized vitreous fibres has no effect.

Introduction

The association between the deposition of asbestos in the lung and the development of lung disease is well recognised but the pathogenic potential of the various forms of vitreous fibre is uncertain. Increases in the proliferative activity of cells in the lungs of rats exposed to a range of fibres and dusts has been described (eg MCGAVRAN and BRODY 1989). Increased proliferation may underlie fibrosis, where increased numbers of fibroblasts are present in the interstitium, or could be involved in 'promoting' effects leading to neoplastic change. Increased proliferation in lungs exposed to dust could be an attempt to replace damaged cells or could result from stimulation of division in response to raised levels of growth factor.

We previously reported differences in the ability of equal airborne fibre concentrations of amosite asbestos or vitreous fibre to elicit inflammation in rat lungs (DONALDSON et al. 1993). In this study, short-term exposures to the two different fibre types caused inflammation in the case of amosite and no response above control in the case of vitreous fibre.

We report here on the cellular proliferative response, measured by bromo-deoxyuridine (BRDU) uptake, in the lungs of rats inhaling these two different fibre types for 7 hours.

Material and methods

Rats: Wistar-derived rats of the HAN strain 12 weeks of age at the start of exposure, were used throughout.

*) Presented as a poster at the 4th International Inhalation Symposium, Hannover, Germany, 1–5 March 1993.

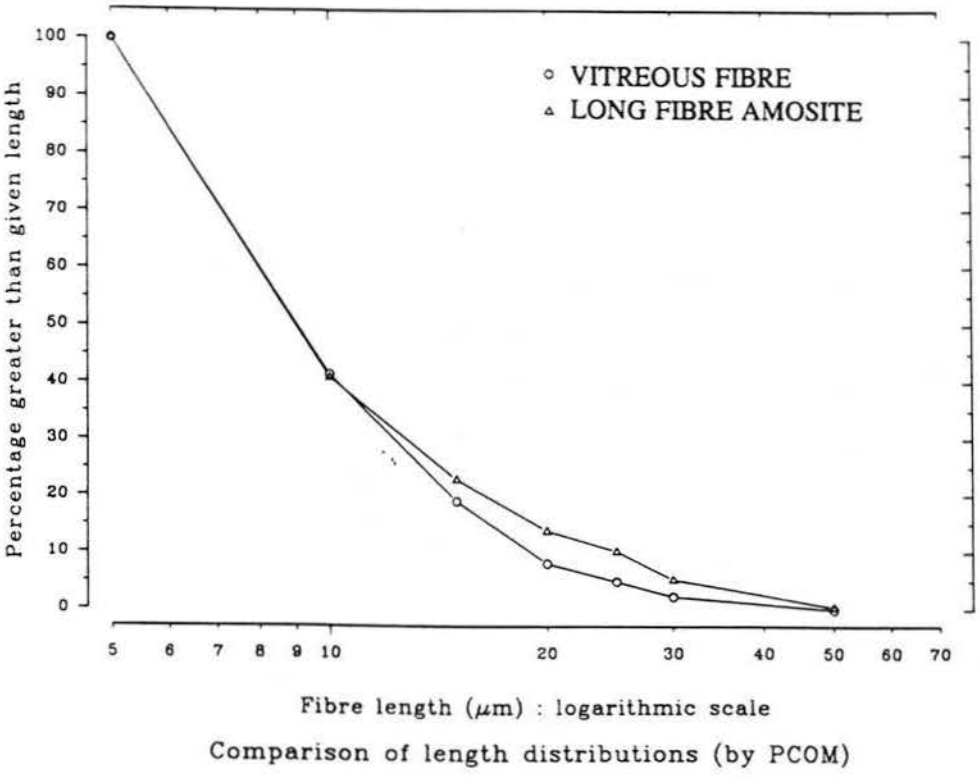


Fig. 1. Length distribution of 100/475 vitreous fibre and long amosite. Measurements by Phase contrast optical microscopy for 400 fibres > 5 um long, < 3 um in diameter and with an aspect ratio > 3:1.

Inhalation exposure: Rats were placed in 1 m^3 whole-body inhalation chambers and exposed to fibres produced using Timbrell dust dispensers as described extensively previously (DAVIS et al. 1986). Rats had access to food and water ad libitum and were exposed for 7 hours. The fibres used were a long fibre amosite asbestos sample (long amosite), which we have reported on extensively (DAVIS et al. 1986; DONALDSON et al. 1989) and a special purpose glass microfibre sample-Johns Manville glass microfibre Code 104/475 (Code 100/475).

Fibre cloud characteristics: The ratio of the fibre number concentration to fibre-mass concentration was estimated at the start of the exposure by taking 8 short period samples for fibre counting during a day (7 hours) of exposure. The mean fibre number concentration was estimated from these short period samples, and the number to mass ratio calculated as the ratio of these mean concentrations. The consistency of the result was checked by repeating the exercise on at least two further days. This number to mass ratio was used to estimate the mass concentrations which would correspond to the target number concentration of 1000 fibres/ml as measured by the standard phase contrast optical microscope method. The concentration in each chamber was monitored daily for mass concentration using the Casella MRE 113A respirable dust sampler, and the flow rates though the chamber adjusted to achieve the target mass concentration.

Measurement of BRDU incorporation: Following a 7 hour exposure, rats were kept overnight and injected with BRDU 2 hours prior to sacrifice. Left lungs were sliced transversely into 6 equal thickness blocks as shown in figure 1. The blocks were processed to paraffin wax and sections cut from each block. Conventional immunocytochemistry, using a monoclonal antibody to BRDU, was used to demonstrate BRDU-positive nuclei.

All terminal bronchioles which opened out into alveolar ducts (hereafter referred to as TB/alv duct units) were counted in a section from each of the 6 levels. This was carried out in 3 control rats, 3 vitreous fibre-exposed rats and 4 amosite-exposed rats. Similar numbers of TB/alv ducts were counted for all 3 treatments: – means – control 69; vitreous fibre 59; long amosite 80. The perimeter of each TB/alv duct unit was ascertained using an image analysis system based on an Apple Mac. The number of BRDU-positive cells was then recorded. Results were expressed as number of BRDU-positive cells per mm of airway surface.

Statistical analysis: The data were counts of BRDU-positive cells, and a suitable starting assumption for the analysis was that these were likely to exhibit random variation described by the Poisson distribution. However, these counts were made on perimeters of different lengths, and were scaled to rates per unit length prior to analysis. They were therefore analysed under a log-linear model for scaled Poisson variates which has had application e.g. in fibre counting (MILLER 1984). This is one case of a generalised linear model (MCCULLAGH and NELDER 1989), a family of models which extend analysis of variance and regression analysis to allow the use of non-linear models and error distributions other than the Normal. In these models, variation is quantified by the quantity deviance (based on likelihood ratios) which is an extension of the concept of a variance.

Results

Attained fibre concentrations: The target fibre concentration was 1000 fibres/ml and the attained fibre concentration was 912 f/ml for vitreous fibre and 908 f/ml for the long amosite. The PCOM size distributions of the two fibre clouds were closely similar as shown in figure 1.

Side-by-side samples for PCOM and Scanning Electron Microscopy counting were taken on a single day for both fibre clouds. These revealed that, for fibres longer than 5 um, the PCOM counts closely reflected the actual number of fibres for long amosite but under-estimated the Code 100/475 by about 60 %. For all fibres longer than 0.4 um the SEM counts on that day showed a mean figure of 1748 for long amosite and 3396 for Code 100/475. The increased pulmonary cell proliferative response seen with long amosite inhalation was therefore produced in the face of a higher airborne fibre number of the Code 100/475; both clouds were > 80 % respirable.

BRDU-positive cells: The observed rates of BRDU positive cell counts plus smoothed averages are shown in table 1 and the analysis of deviance is shown in table 2. The residual mean deviance is close to its expected value of 1.0, suggesting that the assumption of scaled Poisson variation was appropriate for these data. Therefore, it is legitimate to test the deviances for the individual terms as chi-squared test statistics. The most significant sources of variation were between levels in the lung, between fibre types, and between animals within fibre type. There was some evidence of an interaction between levels and animals within fibre type, suggesting that the differences between levels were not uniform from animal to animal, but the effect was not very strong. The term for differences between fibre types was highly significant, but that for differences between animals receiving the same treatment was also highly significant. The data are summarised in figure 2, to assist interpretation of the results. These graphical summaries show the decrease in response from top to bottom of the lung. In addition, figure 2 shows that the means for the controls and the code 100/475 vitreous fibre were very close, but that for the long fibre amosite was considerably higher. (Note that the data are plotted on a logarithmic scale). Figure 2 demonstrates the variation between animals treated with the same fibre type: as it happened, within each type, one animal produced a lower response than the others treated with the same fibre. It seems unlikely that this represents a real effect, but suggests some caution regarding estimation of the positions of the mean for the fibre types. The fact that all animals treated with long fibre amosite produced higher responses than either of the other two fibre types, reinforces the belief that the increased response is indeed due to the long fibre amosite.

Discussion

In the present study, the airborne fibre number in the amosite asbestos and vitreous fibre clouds were very si-

Table 1. Observed rates of BRDU cell counts per mm of alveolar perimeter, plus soothed averages from an analysis model containing terms for fibre type and for level.

Fibre Type	Lung Level	Animal C1	C2	C3	Smoothed Average
Controls	1	0.00	1.71	0.00	1.44
	2	0.71	1.16	0.71	1.12
	3	0.00	1.95	2.33	1.05
	4	1.12	1.22	1.48	0.81
	5	0.00	0.73	1.13	0.85
	6	0.00	1.11	0.91	0.49
	Lung Level	Animal V1	V2	V3	
Vitreous fibre	1	0.77	2.67	1.50	1.37
	2	0.80	1.63	0.99	1.06
	3	0.00	1.17	2.61	0.99
	4	0.00	0.91	0.00	0.76
	5	0.00	0.75	0.78	0.81
	6	0.00	0.63	1.94	0.46
	Lung Level	Animal A1	A2	A3	A4	
Long fibre amosite	1	9.86	2.92	0.00	5.41	5.61
	2	6.52	4.40	2.13	4.35	4.35
	3	5.18	6.31	2.21	3.33	4.07
	4	5.73	3.86	1.46	1.53	3.14
	5	3.60				

Table 2. Summary analysis of variance of BRDU data; analysis is weighted by length of lung perimeter assessed and assumes Poisson variation in observed counts.

Source of variation	Deviation	Degrees of freedom	Mean deviance
Between levels in the lung	35.98	5	7.20
Between fibre types	179.46	2	89.73
Between animals within type	84.89	7	12.13
Interaction level. type	13.63	10	1.36
Interaction level. (animal w type)	63.66	35	1.82
Residual (replicate counts)	633.79	645	0.983

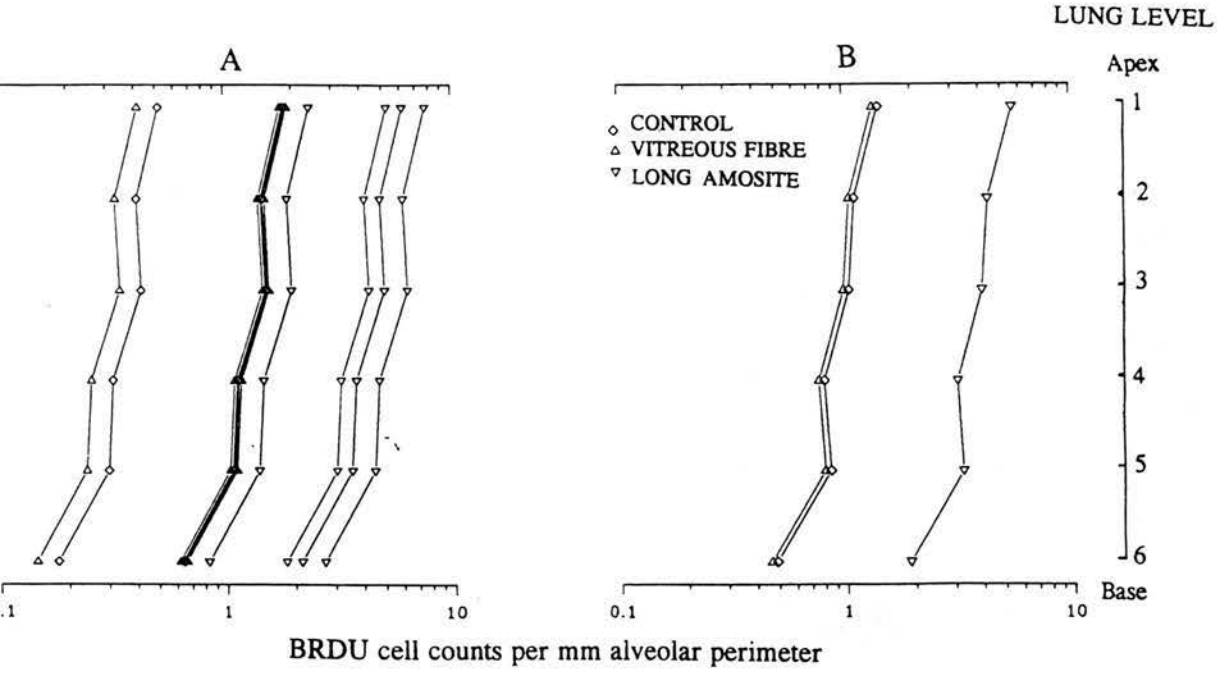


Fig. 2. Numbers of BRDU-positive cells at various levels in the lungs of control rats and rats inhaling either vitreous fibres or amosite asbestos. Panel A shows the mean data from individual animals and panel B shows the data averaged across all the animals in each of the 3 treatment groups.

milar. In other studies on the same fibres we show that the fibre types accumulate in rat lungs at similar rates. Within the 24 hour time span used in this experiment, dissolution is not a likely factor. The differences in proliferative response between the two fibre types was therefore unlikely to be a result of different doses being delivered. Thus an alternative explanation must be sought for the increased proliferative response seen with amosite asbestos. Differences in the surface chemistry of the two fibres seems likely to be the basis of the difference.

We previously reported that macrophages exposed *in vitro* to equal numbers of respirable fibres of different chemical composition but similar size distribution have very different abilities to stimulate macrophages to release the cytokine tumour necrosis factor (BROWN and DONALDSON, in press). A similar differential release of other cytokines such as platelet-derived growth factor (BAUMANN et al. 1990) by macrophages phagocytosing the two different fibre types used here could underlie the different response elicited by the two fibre types.

We previously described inflammatory responses, characterised by neutrophil recruitment into the BAL, with amosite asbestos but not vitreous fibres, under the same conditions of exposure (DONALDSON et al., 1993). This lends support to the contention that inflammatory cell-derived growth factors could be involved.

The lack of inflammatory response and proliferative response seen with the vitreous fibre is in agreement with the preliminary results from long term pathology studies which we are in the process of conducting with this fibre. These suggest that the vitreous fibre is non-pathogenic under the exposure conditions used here, although there is a considerable lung burden of fibres retained in the lung following exposure, as noted in the companion paper by Brown mentioned above. We have previously reported the amosite asbestos sample to be highly pathogenic (DAVIS et al. 1986) and the ability to cause proliferation demonstrated here could be involved in the pathogenic process.

PINKERTON et al. (1986) have reported increased deposition of fibre in the apical parts of the lung compared to the base. This could be an explanation for the increased proliferation in the apical parts of the lung. In these areas of maximal deposition of the amosite fibres there would be greatest stimulation of macrophages or damage to lining cells. There is a gradient of proliferation in the control lung which could be caused by selective deposition of background levels of respirable animal house dust in the apices of the control animals.

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Section 2

Section 2 Effects of particles on cells *in vitro*

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Release of Superoxide Anion and Hydrogen Peroxide by Macrophages in Response to Asbestos

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Introduction

The multiple roles of the macrophage in the inflammatory response are well documented (Nathan et al. 1980). In particular the importance of reactive oxygen intermediates (ROI) during inflammatory defence against microbes has been recognized (Klebanoff 1980). However, it has also been suggested that inappropriate ROI release by phagocytes during non microbially induced inflammation may, under some circumstances, prolong and exacerbate tissue damage and inflammation (Fantone and Ward 1982). The possibility that the deposition of pathogenic dusts in tissue could result in just such a build up of potentially toxic ROI has been suggested (Gee and Walker-Smith 1984). Using mouse peritoneal macrophages elicited with asbestos we have found support for this contention in the raised oxidative status of asbestos activated macrophages as measured by chemiluminescence (Donaldson and Cullen 1984). In the present paper we report on the levels of superoxide anion and hydrogen peroxide released by asbestos-activated macrophages. We also describe the effect of hydrogen peroxide and superoxide anion on the functional activity of lymphocytes as an indicator cell population.

Materials and Methods

Animals and Treatment

The animals used were inbred male C57BL6 mice, 8 - 12 weeks old at the time of use. Mice were injected intraperitoneally with 0.5 ml sterile Dulbecco A (Dul A); up to 2.5 mg chrysotile asbestos (Union Internationale Contre Cancer. Sample A); up to 2.5 mg DUL A, or 0.4 mg Corynebacterium parvum (Wellcome).

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Cells

Peritoneal exudate cells were harvested 5 days after injection with 5 ml of DUL A + 10 U/ml Heparin (Leo Laboratories) and kept on ice. The cells were washed and resuspended in F10 medium + 10% Heat Inactivated Foetal Calf Serum (Gibco) plus antibiotics at 10^6 cells/ml. One ml of this suspension was added to 30 mm plastic petri dishes (Sterilin) and allowed to adhere for one hour at 37° C in 5% CO_2. Just prior to assay the cells were washed three times with DUL A to memove non-adherent cells.

ROI Measurement

Superoxide anion was assayed according to the method of Johnston (1981) with phorbol myristrate acetate (PMA: Sigma) present at 1 µg/ml as trigger; superoxide dismutase controls were always included. Hydrogen peroxide was measured according to the method of Pick and Keisari (1980) with PMA (1 µg/ml) present as trigger.

Cellular Toxicity of ROI

To test for toxic effects of ROI at levels similar to those produced by the asbestos elicited macrophages, exogenous hydrogen peroxide, and an enzymic superoxide generating system were used. These were added to splenocytes proliferating in response to mitogen and the effect of ROI on thymidine uptake was measured by liquid scintillometry. The splenocyte proliferation assay used was that described by Donaldson et al. (1984) except that phytohaemagglutinin (PHA: Sigma) at 10 and 50 1g/ml, was used as mitogen. Hydrogen peroxide (Sigma) was added to cells to concentration of 500, 100, 20, and 5 µM. To generate superoxide anion a xanthine (Sigma: 50 µg/ml) / xanthine oxidase (Sigma: 10 mU/ml) mixture was used; by cytochrome C reduction this system generated 11.6 n.moles of superoxide anion/0.5 h.

Statistics

Data were examined by analysis of variance and paired t' tests.

Results

ROI Release by Asbestos-Primed Macrophages

Table 1 shows that macrophages from asbestos treated mice release significantly (see legend for details) more hydrogen peroxide and superoxide than control macrophages, on treatment with the secretagogue PMA.

Table 1. ROI release by macrophages from variously treated mice

Treatment in vivo	Superoxide Anion[a]	Hydrogen Peroxide[b]
Saline	5.0 ± 1.0[b]	0.5 ± 0.9
C. parvum	14.1 ± 3.6	13.6 ± 7.2
Chrysotile asbestos	11.4 ± 1.3[c]	14.7 ± 6.4[d]

(a) n.moles/10^6 cells/0.5 h

(b) Figures represent $\bar{x} \pm$ sd of means from 3 separate experiments. Significant difference from saline: -

(c) $P < 0.05$.

(d) $P < 0.1$

The ROI release by asbestos elicited macrophages in comparable with the levels released by a conventional macrophage activating (priming) agent C. parvum. Fig. 1. shows that the ROI release by asbestos-primed macrophages is approximately linear over the first hour of culture.

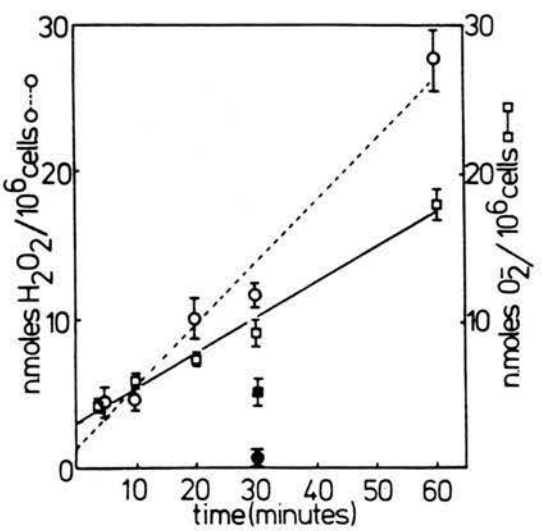


Fig. 1. Time course for release of superoxide anion and hydrogen peroxide by asbestos elicited peritoneal macrophages in the presence of 1 µg/ml phorbol myristate acetate. Closed symbols represent release by control macrophages. Symbols denote $\bar{x} \pm$ SD of 3 plates

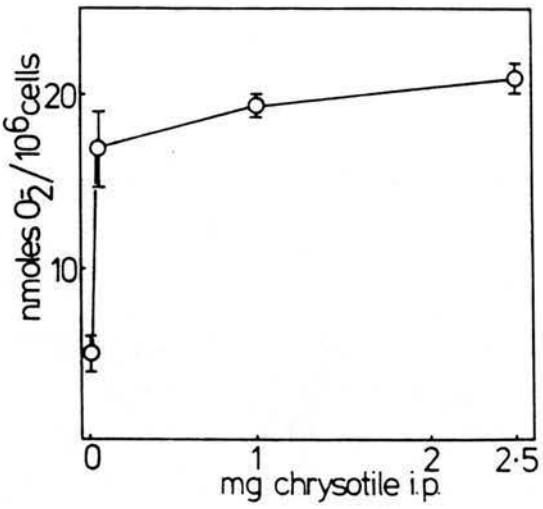


Fig. 2. Superoxide anion release by macrophages from mice treated with 0, 100 µg, 1 mg, or 2.5 mg of chrysotile asbestos intraperitoneally.
Each point represents $\bar{x} \pm$ SD of 3 mice

Although the dose of chrysotile used routinely in these experiments was 2.5 mg Fig. 2. reveals that the same priming effect is produced down to 100 µg per mouse.

Toxicity of ROI

Exogenous hydrogen peroxide was added in a concentration range which spanned the levels produced by 10^6 asbestos-primed macrophages in 1 h. Hydrogen peroxide caused inhibition of mitogen-induced lymphocyte proliferation only at very high concentration (500 µM) as shown in Fig. 3.

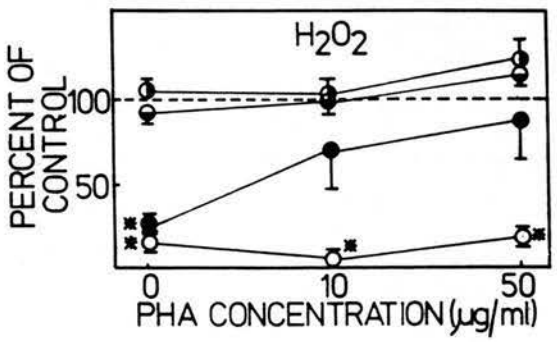


Fig. 3. Effect of exogenous hydrogen peroxide on the uptake of thymidine by splenic lymphocytes proliferating in response to phytohaemagglutinin (PHA). Hydrogen peroxide concentrations o = 500 µM; ● = 100 µM; ◒ = 20 µM; ◑ = 5 µM. Results expressed as percentage of thymidine uptake by controls (no hydrogen peroxide present) and is $\bar{x}$ - SEM of 3 separate experiments. *= significantly different from control $p < 0.001$

Superoxide anion was used at concentrations similar to those generated by 10^6 asbestos primed macrophages in 0.5 h. The effects of this level of enzy-

matically generated superoxide anion on lymphocyte mitogenesis is shown in Fig. 4 and is obviously more variable between experiments than hydrogen peroxide.

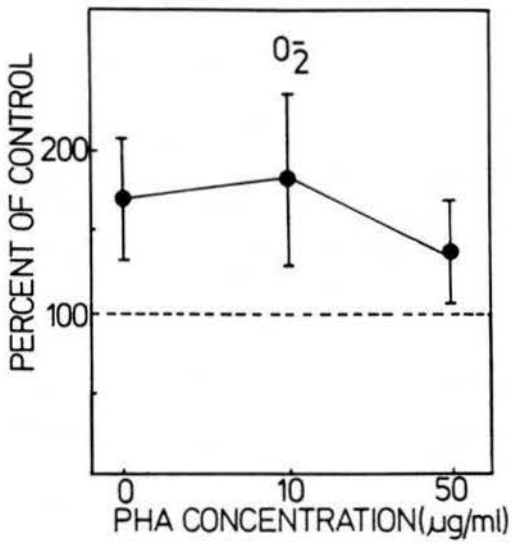


Fig. 4. Effect of xanthine/xanthine oxidase on the uptake of thymidine by splenic lymphocytes proliferating in response to phytohaemagglutinin (PHA). Results expressed as percentage of controls (no xanthine/xanthine oxidase) and is $\bar{x} \pm$ SEM of 3 separate experiments. No significant effects of superoxide anion on proliferation of the indicator cell population were present.

Discussion

In this paper we present support for our previous indirect evidence that, in peritoneal macrophages at least, asbestos in vivo acts as a priming agent (Donaldson and Cullen 1984). Primed macrophages, as extensively studied by Nathan and co-workers (Nathan 1982), are capacitated to release large amounts of ROI when suitably stimulated, or triggered, in vitro. The ability to prime in a property shared by macrophage activating agents in general, which includes asbestos as shown by ourselves and other workers. Although a 2.5 mg intraperitoneal dose of chrysotile asbestos was the routine one used in these experiments a 100 μg dose was equally effective in priming macrophages for increased ROI release.

In view of the above findings it is appropriate to ask whether the levels of superoxide anion and hydrogen peroxide produced are sufficient to cause oxidative damage to cells. In addressing this question we chose mitogen-transforming lymphocytes as a convenient indicator cell system for detecting oxidative damage. It was necessary, however, to take into account our previous findings of both high and low molecular weight inhibitors in supernatants of asbestos activated macrophages (Donaldson et al. in press[a]). To circumvent the problem of the high molecular weight inhibitors in the supernatant, which

were clearly not ROI, we chose, in the first instance, not to use the supernatants themselves. We therefore tested the effect of exogenous ROI, at appropriate concentrations, on proliferation of the indicator cell population. The exogenous ROI were tested against 10^5 lymphocytes and the levels used were chosen to include those produced by 10^6 asbestos primed macrophages in 1 h. It was evident that at these levels (< 30 μM) of both superoxide anion and hydrogen peroxide there were no effects on the functional activity of the indicator cells. Only at 500 μM hydrogen peroxide was there evidence of oxidant damage while the one concentration of superoxide used had no effect. It seems unlikely that in vivo a general continued build up of ROI would occur beyond that produced after 1 h in vitro without this being ameliorated by the normal antioxidant defence. However, local accumulation of ROI between closely apposed cells has been reported and remains a possiblity (Seim and Espevik 1983), In this type of localized accumulation the toxic levels of hydrogen peroxide indicated above (500 μM) could be obtained.

Although the oxidant levels released by asbestos primed macrophages do not appear to be sufficient to cause direct oxidative damage to the target cells, the levels of hydrogen peroxide were sufficient to impair the functional activity of α protease inhibitor in a previous study. (Donaldson et al. in press[b]). The possibility exists, therefore, for proteolytic tissue damage to arise as an indirect consequence of the observed oxidant release.

In future work it is intended to study the whole supernatants for toxic levels of ROI. The ability to prime alveolar macrophages for increased ROI release will also be studied.

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INCREASED RELEASE OF HYDROGEN PEROXIDE AND SUPEROXIDE ANION FROM ASBESTOS-PRIMED MACROPHAGES

Effect of Hydrogen Peroxide on the Functional Activity of α_1-Protease Inhibitor

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Abstract—The ability of asbestos-elicited murine peritoneal macrophages to release superoxide anion and hydrogen peroxide, following *in vitro* triggering, has been investigated. The asbestos-elicited macrophages produced increased levels of superoxide and hydrogen peroxide compared to control macrophages and similar levels to those produced by *Corynebacterium parvum* elicited macrophages. The supernatants from asbestos-elicited macrophages which had been triggered *in vitro* were capable of impairing the ability of α_1-protease inhibitor to inhibit elastase function. The catalase sensitivity of this effect showed it to be due to hydrogen peroxide.

INTRODUCTION

Asbestos, in common with other industrial dusts, is known to cause pulmonary fibrosis in exposed individuals (1). Fibrosis constitutes an essential part of the formative phase of the chronic inflammatory response and several studies have provided evidence that asbestos can affect the host defence in ways which could prolong and exacerbate inflammation. A major target in these studies has been the inflammatory mediating role of the activated macrophage since alveolar macrophages come into direct contact with, and phagocytose, inhaled asbestos in the lung (2).

The reactive oxygen intermediates (ROI) are a group of toxic, leukocyte-derived compounds which have received particular attention with regard to their microbicidal (3), tumoricidal (4), and tissue-damaging (5) activity. A potential role for these oxidants in lung damage following asbestos exposure has been postulated (6, 7), and we have reported provisional evidence that supports this

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suggestion (8). Thus, using asbestos-elicited murine peritoneal macrophages a a model, we reported some evidence of raised oxidative status in asbestos-ac tivated macrophages as measured by lucigenin-amplified chemiluminescence.

In the present study we extend these findings and report on the release o the specific components of the ROI cascade, hydrogen peroxide and superoxid anion, by asbestos-activated macrophages. We also report on the effects of as bestos-activated macrophage-derived ROI on the ability of α_1-protease inhibito to inactivate elastase.

MATERIALS AND METHODS

Mice. C57BL/6 mice, 12–16 weeks old at the time of injection, were used.

Treatment. Mice were injected intraperitoneally with sterile Dulbecco A (Dul A) 0.5 m chrysotile asbestos [Union Internationale Contre Cancer (UICC) sample A] 2.5 mg in 0.5 ml of D A or *Corynebacterium parvum* (Wellcome, heat killed) 0.2 ml (0.4 mg).

Harvesting of Peritoneal Exudate Cells. Peritoneal exudate cells were collected by lava with a total of 5 ml of Dul A + 10 units/ml of heparin (Leo Laboratories) and kept on ice. Followi washing, the cells were resuspended in F10 medium + 10% heat-inactivated fetal calf serum (Gibc at 10^6 cells/ml. One milliliter of this suspension was plated onto 30 mm plastic petri dishes (Steril and allowed to adhere for 1 h at 37°C in 5% CO_2. Before assay, the plates were washed three tim with Dul A to remove nonadherent cells.

Superoxide Assay. Superoxide anion was assayed according to the method of Johnstone (1 Briefly 1.5 ml of 80 μM cytochrome *c* solution (Sigma) in Dul A, containing 2 mg/ml dextro was added to each plate and 1 μg/ml phorbol myristate acetate (PMA; Sigma) was added a trigger at zero time; identical cultures also received 25 μg/ml superoxide dismutase (SOD; Sig type IV). After 30 min of incubation at 37°C in 5% CO_2, the supernatant was harvested and absorption at 550 nm of each sample was determined in a scanning spectrophotometer (Pye Unic SP1800). Absorbance was converted to nanomoles of cytochrome *c* reduced (10).

Hydrogen Peroxide Assay. Hydrogen peroxide was assayed according to the method of P and Kiesari (11). Briefly, 1 ml of prewarmed 10 nM phenol red solution (Sigma), containing Na (140 mM), dextrose (5.5 mM), and horseradish peroxidase (50 μg/ml; Sigma type II) in 10 m potassium phosphate buffer, was added to each plate. Triplicate plates for each condition recei PMA (1 μg/ml) or no PMA. Plates were incubated for 30 min at 37°C in 5% CO_2. The supernata were then harvested and alkalized by the addition of 30 μl of 1 N NaOH. The absorbance of solution at 610 nm was determined in a spectrophotometer (Pye Unicam SP30) and converted H_2O_2 equivalent using a standard curve.

Triggers. Different triggers of ROI release were prepared in some assays to the following fi concentrations: concanavalin A (Sigma) 15 μg/ml; zymosan (Sigma) 1 mg/ml; chrysotile asbes (UICC sample A) 1 and 0.1 mg/ml; tuftsin (Sigma) 50 μg/ml.

Effects of Macrophage Supernatants on Functional Activity of α_1-Protease Inhibitor. T functional activity of α_1-protease inhibitor was assessed by its capacity to inhibit porcine pancre elastase. Enzyme activity was measured by the rate of increase in absorbance at 410 nm using chromogenic substrate *N*-succinyl-(L-ala)$_3$-*p*-nitroanilide (SLAPN: Calbiochem) as described Bieth (12). Briefly, 1 ml of unstimulated or PMA-stimulated (1 μg/ml; 1 h 37°C) macroph culture supernatant was incubated with 1 ml (250 μg/ml) purified α_1-protease inhibitor (Sigma) 10 min at room temperature. Sixty μl of this mixture was then incubated with 570 μl of por

pancreatic elastase (0.3 units; 1 unit solubilizes 1.0 mg elastin in 20 min at 37°C; pH 8.8) for 30 min at room temperature. Residual elastase activity was then measured by adding 1.2 ml of SLAPN solution (1.25 mM in 0.2 M Tris HCl, pH 8.0) and the increase in absorbance at 410 nm/minute was recorded.

In some experiments supernatants were preincubated with catalase (Sigma) to a final concentration of 62.5 μg/ml before inclusion in the above assays. In some experiments H_2O_2 (Sigma), up to a final concentration of 20 μM, was used to pretreat α_1-protease inhibitor before its inclusion in the assay system.

Data was examined using analysis of variance of pooled results and, where differences in means were evident, the significance was tested using paired *t* tests.

RESULTS

ROI Release from Macrophages. In the first instance we set out to compare superoxide and hydrogen peroxide release between saline (control), *C. parvum*-, and chrysotile asbestos-activated peritoneal macrophages. As shown in Table 1 there were clear differences in the PMA-triggered release of both superoxide and hydrogen peroxide from peritoneal macrophages elicited with both *C. parvum* and asbestos compared to control macrophages. In the case of superoxide release, these differences were significant at the 5% level for *C. parvum* versus saline macrophages; the difference was significant at the 0.2% level for chrysotile asbestos versus saline macrophages. With H_2O_2 release, the significance of the differences between saline and *C. parvum*- or chrysotile-elicited macrophages only attained the 10% level due to the less sensitive assay system.

Figures 1 and 2 show the time course for the release of hydrogen peroxide and superoxide from chrysotile asbestos-activated macrophages. The release of both ROIs is seen to be linear over the 1 h of the time course following addition of the PMA trigger.

Figure 3 shows the relative activities of various agents in bringing about the release of superoxide and hydrogen peroxide from asbestos-elicited perito-

Table 1. Release of Superoxide and Hydrogen Peroxide by Peritoneal Macrophages Elicited with Saline, *C. parvum*, or Chrysotile Asbestos; Phorbol Myristate Acetate as Trigger

PEC induced with	Superoxide[a]	Hydrogen peroxide[a]
Saline	5.0 ± 1.0	0.5 ± 0.9
C. parvum	14.1 ± 3.6*	13.6 ± 7.2**
Chrysotile asbestos	11.4 ± 1.3*	14.7 ± 6.4**

[a]Nanomoles/10^6 cells/0.5 h. Figures represent the mean ± standard deviation of the mean from three separate experiments (three dishes in each experiment). Significance of differences from saline: *$P < 0.05$, **$P < 0.1$.

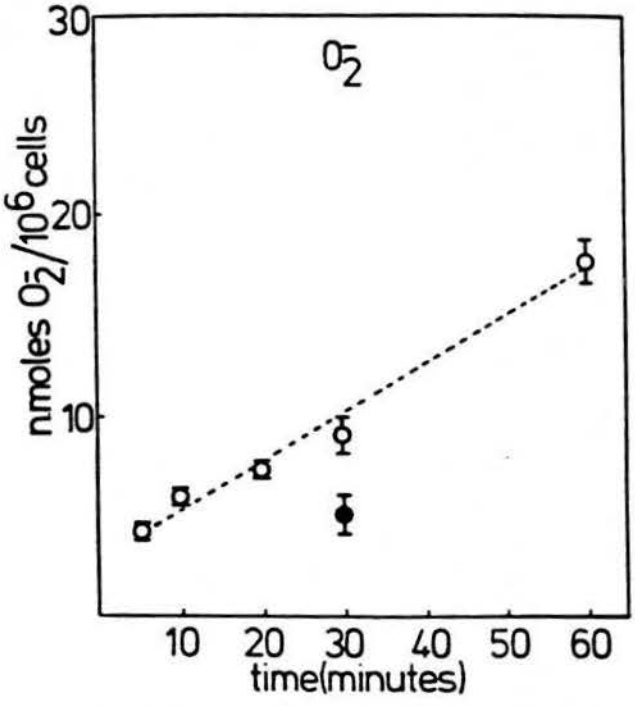


Fig. 1. Open circles show the time course for the release of superoxide anion (O_2^-) by asbestos-activated macrophages. Phorbol myristate acetate (1 μg/ml) added at time 0. Closed circle shows release of O_2^- by control macrophages at 30 min. Mean ± standard deviation of three separate dishes.

neal macrophages. It is clear that PMA produced the biggest release of both superoxide and hydrogen peroxide compared to unstimulated cells ($P < 0.1$), although it failed to trigger release of hydrogen peroxide. Concanavalin A, chrysotile asbestos at two doses, and the naturally occurring peptide tuftsin, all failed to act as triggers for ROI release.

Effect of Macrophage Supernatants on Functional Activity of α_1-Protease Inhibitor. Having established that the supernatants from chrysotile asbestos-elicited macrophages triggered with PMA contained higher concentrations of hydrogen peroxide and superoxide anion than a similar supernatant from saline-elicited macrophages (Table 1), we went on to examine the effect of these supernatants on the functional activity of α_1-protease inhibitor. Experiments revealed (Table 2) that both the saline- and the asbestos-elicited macrophage supernatants could significantly suppress the ability of α_1-protease inhibitor to inhibit elastase (saline $P < 0.01$; asbestos $P < 0.001$); it was also notable that the asbestos macrophage supernatants caused significantly more inhibition of

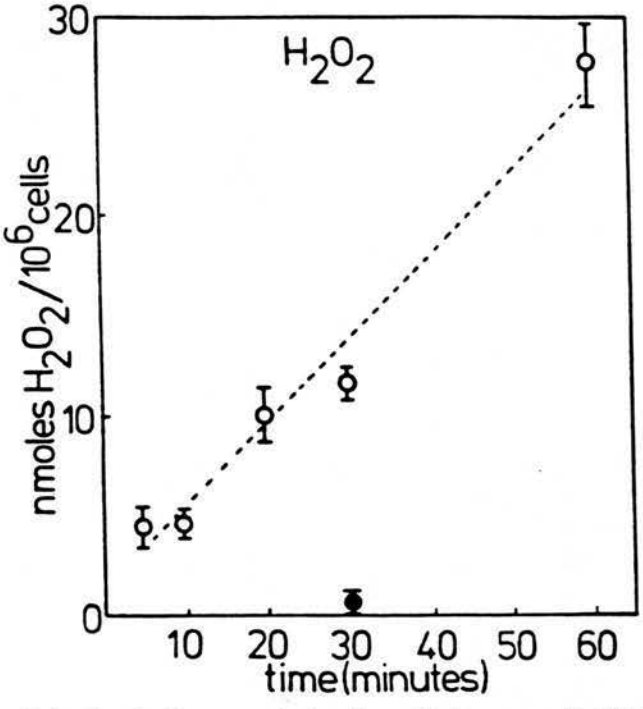


Fig. 2. Open circles show the time course for the release of hydrogen peroxide (H_2O_2) by asbestos-activated macrophages. Phorbol myristate acetate (1 μg/ml) added at O time. Closed circle shows H_2O_2 release by control macrophages at 30 min. Mean ± standard deviation of three separate dishes.

α_1-protease inhibitor than the saline macrophage supernatants ($P < 0.001$). The pretreatment of these supernatants with catalase caused significant reductions in their ability to impair the elastase-inhibiting capacity of α_1-protease inhibitor. ($P < 0.001$). Experiments with exogenous, reagent grade H_2O_2 confirmed that H_2O_2 was indeed highly effective in this respect and the specificity was confirmed using catalase (Table 3).

DISCUSSION

The central role of the macrophage in inflammation has been extensively described (15, 16). The effects of asbestos in causing macrophage activation and the release of inflammatory mediators has also been described (17–20). It has been suggested that leukocyte ROI could play a part in asbestos-induced inflam-

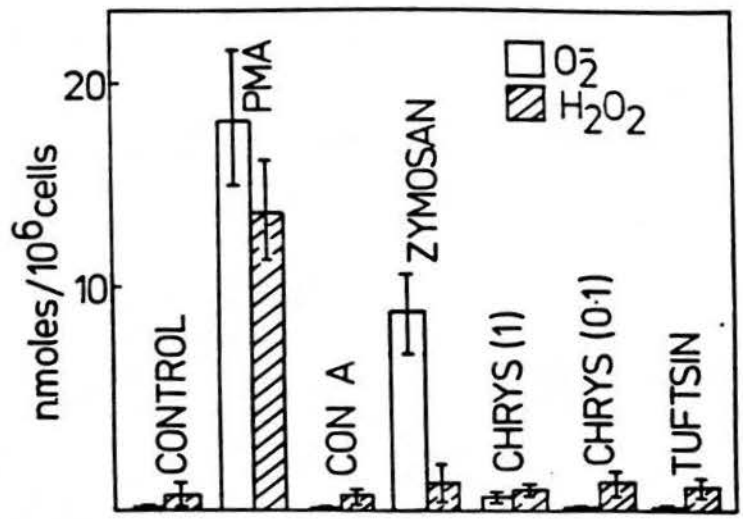


Fig. 3. Release of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) by asbestos-activated macrophages in response to various triggers: PMA = phorbol myristate acetate; CON A = concanavalin A; zymosan; CHRYS = chrysotile asbestos (1 mg/ml and 0.1 mg/ml); tuftsin. Mean ± standard deviation of six separate dishes from two separate experiments.

mation (6–8) in a manner similar to that shown for inflammation due to other causes (5).

In the present study we have shown that asbestos-activated inflammatory macrophages are primed to release increased levels of superoxide anion and hydrogen peroxide in vitro in response to the secretagogue phorbol myristate acetate. Although concanavalin A (12) and tuftsin (13) have both been reported to trigger ROI release from macrophages, we found neither of them to affect

Table 2. Effects of Saline- and Asbestos-Elicited Macrophage Supernatants on Ability of α_1-Protease Inhibitor to Inhibit Elastase and Effect of Pretreatment of Supernatants with Catalase (representative experiment)

	Residual elastase activity[a]	
Macrophage-eliciting agent	Supernatant only	Supernatant + catalase
Saline	0.6 ± 0.1^a	0 ± 0^b
Asbestos	1.4 ± 0.1^c	0.6 ± 0.2^d
No Supernatant	0.2 ± 0.2^e	

[a]Units = Δ absorbance per min at 410 nm × 10^2. Figures represent the mean ± the standard deviation of nine tubes. Differences analyzed using paired t tests: a vs. b, $P < 0.001$; a vs. c, $P < 0.001$; c vs. d, $P < 0.001$; a vs. e, $P < 0.01$; c vs. e, $P < 0.001$.

Table 3. Effect of Hydrogen Peroxide on Ability of α_1-Protease Inhibitor to Block Elastase Activity and Abolition of Effect with Catalase (appropriate controls included)

Elastase	α_1-Protease inhibitor	Hydrogen peroxide	Catalase	Residual elastase activity[a]
+	–	–	–	10.2 ± 1.0^a
+	+	–	–	0.2 ± 0.2^b
+	+	+	–	9.9 ± 0
+	+	+	+	0.9 ± 0.4^d
+	+	–	+	0.6 ± 0.4^e

[a]Units = Δ absorbance per minute at 410 nm × 10^2. Figures represent the mean ± the standard deviation of nine tubes. Differences analyzed using paired t tests: a vs. b, $P < 0.01$; a vs. c, no significant difference; c vs. d, $P < 0.001$; d vs. e, no significant difference.

ROI release by asbestos-activated macrophages. Zymosan, a particulate stimulus was, however, capable of eliciting superoxide anion release, although it had no effect on hydrogen peroxide release. We have previously reported the ability of zymosan to trigger lucigenin-amplified chemiluminescence, a superoxide-dependent phenomenon, by asbestos-activated macrophages (8).

In the present experiments chrysotile asbestos failed to trigger the release of superoxide and hydrogen peroxide from asbestos-primed macrophages. This contrasts with our previous report that such macrophages are capable of a small but significant chemiluminescent response on treatment with chrysotile (8). A likely explanation for this apparent contradiction lies in the fact that the chemiluminescence assay is carried out on cells in suspension, while the ROI assays reported in the present study rely on adherent macrophages. Monolayer formation is reported to modulate the respiratory burst, leading to a diminution of response to conventional triggers (9); this could lead to the observed failure to respond significantly to chrysotile.

In the second series of experiments described here, the supernatants from asbestos-activated macrophages treated with PMA in vitro were shown to be capable of impairing the ability of α_1-protease inhibitor to inhibit elastase. Previous studies showing similar activity released by neutrophils revealed hydrogen peroxide to be the active agent (21). Pretreatment of the supernatants from asbestos-activated macrophages with catalase resulted in a marked reduction in their ability to impair the functional activity of α_1-protease inhibitor; hydrogen peroxide was therefore also an effector molecule in our studies.

A series of experiments using exogenous H_2O_2 confirmed the ability of H_2O_2 to impair the functional activity of α_1-protease inhibitor in our system.

In the experiments described above, peritoneal macrophages have been used as a model for pulmonary macrophages as previously described (22). If these results hold true for pulmonary macrophages, then pulmonary inflammation fol-

lowing asbestos deposition in the lung could be prolonged and exacerbated b oxidant from asbestos-primed macrophages in two separate ways: (1) direct o> idant damage to membranes and (2) heightened protease damage due to in paired functional activity of α_1-protease inhibitor.

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In vitro fibrinolytic activity and viability of rat alveolar macrophages treated with inflammation generating mineral dusts

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Abstract

Rat alvolar macrophages demonstrated plasminogen dependent fibrinolysis *in vitro* which was inhibited to varying degrees by the addition of zymosan, the non-toxic particulate titanium dioxide, and the toxic dusts quartz and chrysotile asbestos. Assessment of viability suggested that the inhibition produced by zymosan and titanium dioxide could be accounted for by cytotoxic effects but in the case o quartz and chrysotile asbestos there was evidence that stimulation of fibrinolysis preceded cell death Zymosan, which caused no observeable enhancement of alveolar macrophage fibrinolysis was found t markedly stimulate peritoneal macrophage fibrinolysis. The choice of assays of cell function to asses the action of toxic dusts are discussed.

Introduction

Exposure to mineral dusts such as quartz and asbestos, is associated with pulmonary inflammation [1–2] and fibrosis [3]. Experimental studies have indicated that such pathogenic dusts have the ability to initiate or prolong inflammation by activation of macrophages [4], recruitment of inflammatory cells [5], increased release of mediators [6–7] and activation of the complement cascade [8].

The fibrinolytic, clotting, kinin-forming and complement pathways of inflammation interact in a complex series of reactions [9] which are central to the initiation, persistence and resolution of inflammation. Cells of the macrophage series are involved in these pathways, via secretion of coagulation factors, complement and proteases. In particular, plasminogen dependent fibrinolysis by inflammatory macrophages, and its control, have been extensively studied [10–11].

In the present study we set out to examine the effect on alveolar macrophage plasminogen dependent fibrinolysis, of two inflammatory generating mineral dusts – quartz and chrysotile asbestos, one non-inflammatory generating particulate-titanium dioxide [5] and the conventiona particulate trigger of leukocyte secretion, zymosan.

Materials and methods

Animals

Male PVG rats inbred at the Institute of Occupational Medicine Laboratory Animals Unit were used throughout.

Broncho-alveolar lavage

The lungs were removed from the thoracic cavity of Nembutal (Ceva Ltd)-killed control rats and

lavaged four times with 10 ml aliquots of saline at 37 °C. The pooled cells were > 95% macrophages by morphology and will be referred to hereafter as alveolar macrophages.

Peritoneal lavage

In experiments using peritoneal macrophages the peritoneal cavity of normal rats was lavaged with 30 ml of Dulbecco's phosphate buffered saline containing 10 U/ml of Heparin (Leo Labs.) and treated as for alveolar cells.

Fibrinolysis assay

Alveolar macrophage, plasminogen dependent fibrinolysis was measured using the assay of Unkeless et al. [11]. Briefly, fibrinogen (Sigma) was labelled with ^{125}Iodine (Amersham International), diluted with 0.1% unlabelled fibrinogen and dried onto flat bottomed microtitre plate wells (1×10^5 cpm/well/40 µg fibrinogen).
Fresh foetal calf serum (Gibco) (2.5%) was used to convert the fibrinogen to fibrin and the plates were washed before addition of macrophages (1.5×10^5) and plasminogen (Kabi) (600 ng/well) in a 100 µl volume. Plates were incubated for 24 hours in 5% CO_2 before assessing the solubilised ^{125}I-Fibrin present in 80 µl of supernatant using a gamma counter. Four replicate wells were used for each condition and in each plate one set of wells was set up without plasminogen to assess plasminogen independent fibrinolysis.

Particulates

The particulates used were rutile titanium dioxide (TiO_2: Tioxide Ltd.) median volume diameter 0.2 µm, quartz (DQ_{12}) median volume diameter 1.4 µm and UICC chrysotile asbestos sample 'A' median length 2.4 µm median diameter 0.3 µm and zymosan (Sigma). For use unopsonised the particulates were suspended in Neumann & Tytell medium and 20 µl added to individual wells to produce final concentrations of 1000, 100, 10 and 1 µg/ml. To prepare opsonized dusts they were pre-incubated for 30 mins. at 37 °C in 10% fresh rat serum in Neumann & Tytell medium. The dusts were then spin-washed 3 times (3000 G : 10 mins.) and suspended in Neumann & Tytell medium: twenty microlitre aliquots were then added to individual wells to obtain final concentrations of 1000, 100, 10 or 1 µg/ml.

Cytotoxicity of dusts

A series of experiments were undertaken to determine whether cytotoxicity could be a factor in the observed differences in fibrinolysis found between particulates. The degree of cytotoxicity produced by opsonised particulates at a dose of 100 µg/ml, was therefore determined by conventional dye exclusion for comparison with fibrinolysis at that dose. The viability of alveolar macrophages cultured on fibrin mats, assessed by ability to exclude 0.85% Trypan Blue (Flow Laboratories), was determined at zero time and at 24 hours and expressed as mean number of live cells in 10 randomly selected high power fields.

Statistical analysis

All experiments were repeated 2–4 times. The data was examined using analysis of variance and the differences in means was assessed for significance using a 't' test. The statistical significance of dose effects was tested as described by Cochrane and Cox (1957).

Results

Validity of the assay system

As a model plasminogen activator, urokinase was used in preliminary studies at various dilutions in the presence of plasminogen to generate the fibrinolytic protease plasmin which was found to cause fibrinolysis in the assay system in a dose-dependent manner. Serum which is known to contain inhibitors of plasmin abolished fibrinolysis as did soybean trypsin inhibitor.

Rat alveolar macrophage fibrinolysis

Addition of rat alveolar macrophages to ^{125}I Fibrin plates in the presence of plasminogen resulted in sustained plasminogen dependent fibrinolysis over a 48 hour period (Figure 1). A 24 hour time point was chosen to represent fibrinolysis in subsequent experiments. The controls revealed that there was some fibrinolytic activity due to

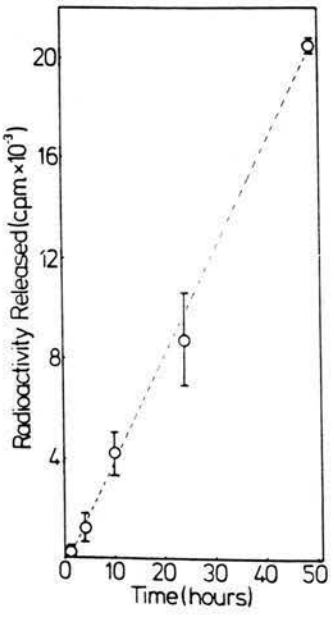


Figure 1
Time response of plasminogen dependent fibrinolysis produced by control rat alveolar macrophages; mean ± standard deviation.

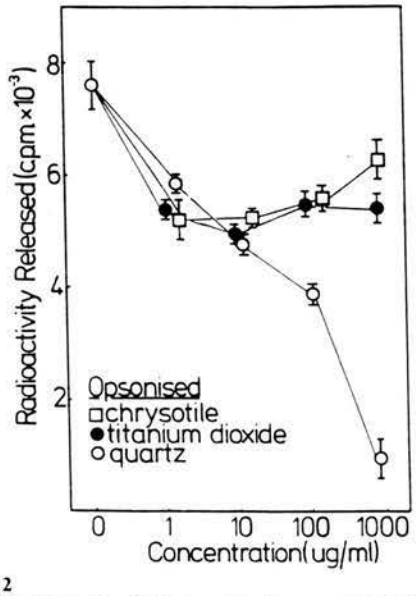


Figure 2
The effect of opsonised titanium dioxide, chrysotile asbestos and quartz on plasminogen dependent fibrinolysis by rat alveolar macrophages; mean ± standard error.

non-specific proteolysis of the ^{125}I-Fibrin n
The proportion of the total fibrinolysis due
plasminogen-independent activity ranged fr
10 to 40% of the total and had the same varia
as that found with plasminogen dependent
brinolysis. In the present paper this backgrou
was always subtracted to obtain the cpm due
plasminogen dependent fibrinolysis. Nit
guanido benzoate is an active site serine prote
inhibitor which has been reported to abolish pl
minogen activator activity [11]. When nit
guanido benzoate was present (10 µg/ml) it
tally inhibited alveolar macrophage dependent
brinolysis. It was noteable that nitroguani
benzoate did not abolish plasminogen-
dependent fibrinolysis suggesting that it was n
due to serine protease.

Effect of particulates on plasminogen dependent fibrinolysis by rat alveolar macrophages

All opsonised mineral dusts caused highly signi
cant ($t = 7.26$ to 13.67; $P < 0.001$) reductions in
brinolysis by alveolar macrophages (Figure
Only quartz however produced a highly signi
cant ($t = 16.05$; $P < 0.001$) dose dependent inh
bition of fibrinolysis (Figure 3). The effect fou
with dust which had not been opsonised was d
ferent to that found with opsonised dust. On
again all the dusts were significantly inhibit
($t = 11.7$ to 13.90; $P < 0.001$) to alveolar mac
phage fibrinolysis (Figure 3). There was hig
significant dose related inhibition of fibrinoly
with chrysotile ($t = 14.2$; $P < 0.001$) and qua
($t = 18.3$; $P < 0.001$). With TiO_2 the whole dose
sponse data does not fit a linear trend.
The yeast cell wall preparation zymosan was
cluded as a particulate which was not a mine
dust but which has been used frequently to
duce macrophage secretion. Opsonised and un
sonised zymosan produced similar highly sign
cant ($t = 29.97$, 29.47; $P < 0.001$) dose-related
ductions in plasminogen dependent fibrinol
by rat alveolar macrophages (Figure 4).

Peritoneal macrophages

In contrast to the response of alveolar ma
phages, peritoneal macrophages produced a h
ly significant ($t = 2.64$; $P < 0.001$) dose depend

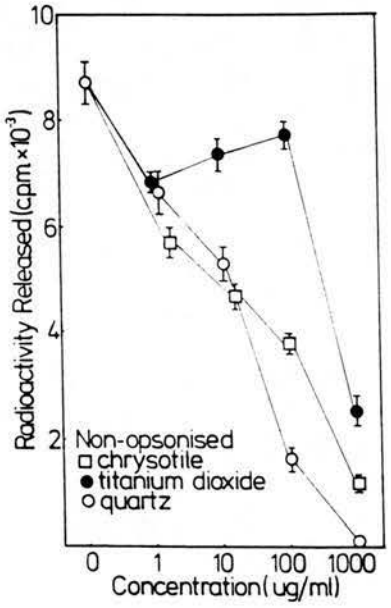


Figure 3
The effect of non-opsonised titanium dioxide, chrysotile asbestos and quartz on plasminogen dependent fibrinolysis by rat alveolar macrophages; mean ± standard error.

increase in plasminogen dependent fibrinolysis (Figure 5).

Cytotoxic effect of mineral dusts

Experiments were undertaken to determine whether toxic effects of the mineral dusts could explain the particle induced reduction in fibrinolysis by alveolar macrophages. The results (Figure 6) showed that after 24 hours there was slight but significant cell death amongst control cells. All the opsonised particulates produced significant toxic effects. Zymosan and TiO_2 both produced approximately 30% reduction in viability and were not significantly different from each other in this respect ($t = 0.63$; NSD). Both chrysotile asbestos and quartz, however, were significantly more toxic than TiO_2 (chrysotile $t = 3.08$; $P < 0.02$, quartz $t = 8.96$; $P < 0.001$), with quartz proving to be highly active and decreasing the viability by 88%.

In the inset to Figure 6 are shown viability and fibrinolysis following 24 hours exposure to the opsonised particulates at 0.1 mg/ml and expressed as a percent of untreated macrophages. For TiO_2

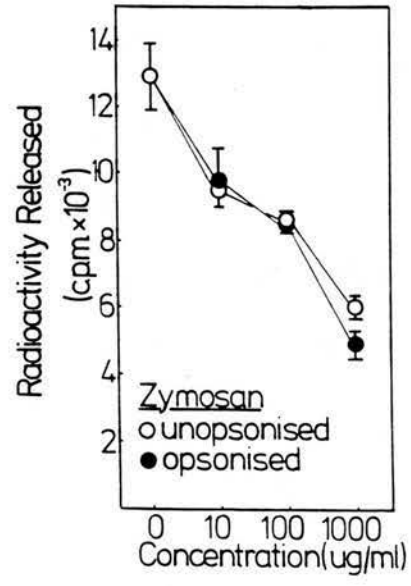


Figure 4
The effect of opsonised and unopsonised zymosan on plasminogen dependent fibrinolysis by control rat alveolar macrophages; mean ± standard error.

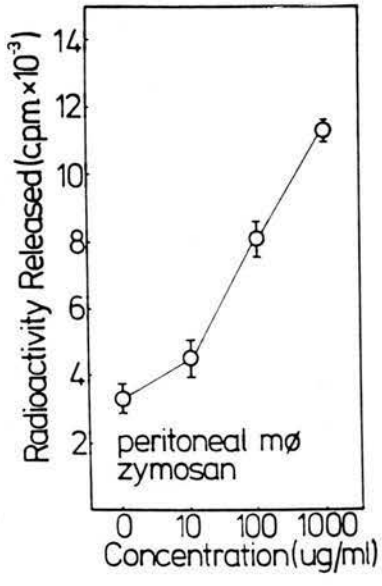


Figure 5
The effect of zymosan on plasminogen dependent fibrinolysis by control rat peritoneal macrophages.

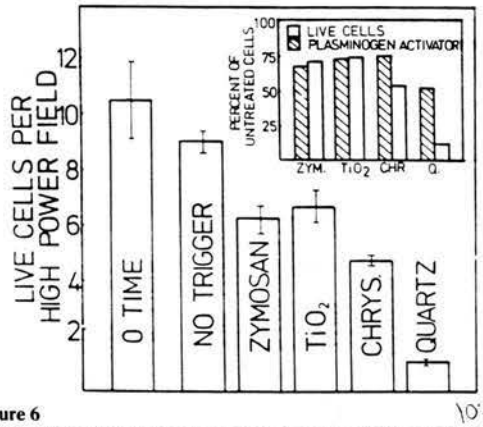


Figure 6
The number of viable cells per high power field remaining 24 hours after treatment of alveolar macrophages with 0.1 mg/ml of opsonised zymosan, titanium dioxide, chrysotile or quartz; mean ± standard error. Inset: Comparison of viability and fibrinolysis following 24 hours of exposure to the opsonised particulates; both expressed as percent of untreated macrophages.

and zymosan there is good agreement between live cells remaining and fibrinolysis but with quartz and chrysotile there is more fibrinolysis than might be expected from the number of viable cells present.

Discussion

Rat alveolar macrophages were found to cause plasminogen dependent fibrinolysis for up to 48 hours after plating on to ^{125}I-fibrin mats as found in alveolar macrophages from other species and considered to be due to plasminogen activator release [13–15]. This fibrinolysis was inhibited to varying degrees by all of the particulates tested which included, opsonised and unopsonised, the non toxic particles zymosan and titanium dioxide, and also the cytotoxic dusts quartz and chrysotile asbestos. Experiments were therefore carried out to study the role of cytotoxic effects in the inhibition of alveolar macrophage fibrinolysis. These experiments revealed that the number of viable cells and degree of fibrinolysis at 24 hours compared to controls, were in good agreement for TiO_2 and zymosan. For chrysotile asbestos, however, there was a similar amount of plasminogen activator release to that found with TiO_2 and zymosan but fewer viable cells were present. This suggests that some stimulation of plasminogen dependent fibrinolysis precedes cell death due to chrysotile. In the case of quartz, both plasm ogen activator release and the number of viat cells fell but expressed in comparison with cont cells there was much more plasminogen activa release than would be expected for the number live cells present; this could again indicate sor degree of stimulation preceding cell death wi quartz. A close examination of the data reve that the release of fibrinolytic activity from de cells is not a likely explanation of the results. Tl is because chrysotile caused decreases in viabili with no effect on fibrinolysis while quartz caus decreases in viability and decreases in fibrin lysis. Such data are more consistent with di ferential stimulation of fibrinolysis precedir death.

When rat peritoneal macrophages were used i the assay system instead of alveolar macrophag there was no evidence of inhibition of fibrinolys caused by phagocytosis of zymosan particl although zymosan was toxic and inhibitory t macrophages from the alveolar site; zymosa particles in fact caused dose-dependent stimu lation of fibrinolysis by peritoneal macrophages. The difference in the fibrinolytic response of a veolar and peritoneal macrophages to treatme with phagocytosable particles may be attributab to the sensitivity of alveolar macrophages to dan age incurred during phagocytosis *in vitro* [16 This is consistent with the increased degree activation reported for alveolar macrophag compared to resting peritoneal macrophages [1 and evidence that activated macrophages a more susceptible to damage by toxic particles tha resting macrophages [18]. However, increased fr gility to all particles, as an explanation for t inhibition of alveolar macrophage fibrinolysis, precluded by the marked differences in the d gree of inhibition found with different dusts a with opsonisation. These are more likely to be reflection of the well documented toxic potent of some mineral dusts, particularly quartz a chrysotile asbestos, for macrophages in vitro [1 These toxic effects were particularly evident in t case of the unopsonised dusts as might be expe ed since there would be no protective prote coating on the active surface of the minerals [1 Following opsonisation the inhibition of brinolysis was less marked particularly in the ca of chrysotile asbestos although there were s toxic effects.

The study has highlighted the problems of choosing and interpreting an assay of the functional inflammatory status of alveolar macrophages when the modulating agents under study are mineral dusts with differing cytotoxicities. In the assessment of macrophage fibrinolysis and its modulation by asbestos, previous studies have utilised peritoneal macrophages as models for effects on alveolar macrophages [6]. These studies and our own results (unpublished) where we also used mouse peritoneal macrophages, have demonstrated unequivocal stimulation of peritoneal macrophage fibrinolysis by asbestos. The use of the rat alveolar macrophage, as described in the present study showed inhibition of fibrinolysis by asbestos and other particles. The inhibition appeared to be a product of conflicting toxic effects and stimulation, which was only revealed by assessment of viability. The study has pointed out the difference between peritoneal and alveolar macrophages in their functional response to mineral dust treatment and highlights the problem of interpreting the results of assays of cell function when comparing the effects of dusts with different toxicities. The study has also re-emphasised the need for taking into consideration toxic effects as well as the stimulation of inflammatory mediator release, in studying mineral dust induced inflammation. These factors must be taken into consideration in further work aimed at assessing the role, in mineral dust induced inflammation, of leukocyte products which affect the coagulation/fibrinolysis pathways of the inflammatory response.

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OXIDANT PRODUCTION BY CONTROL AND INFLAMMATORY BRONCHOALVEOLAR LEUKOCYTE POPULATIONS TREATED WITH MINERAL DUSTS IN VITRO

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Abstract—Using a rat model we set out to determine whether exposure of bronchoalveolar-derived leukocytes to pathogenic mineral dusts in vitro caused them to undergo an oxidative burst and release potentially harmful oxidants. Three different populations, obtained by bronchoalveolar lavage, were chosen: control cells, cells obtained following instillation of heat-killed *Corynebacterium parvum* into the lung, and cells obtained following instillation of quartz. None of the populations showed any evidence of superoxide anion or hydrogen peroxide production when treated in vitro with the pathogenic dusts quartz and chrysotile asbestos, or the inert particulate titanium dioxide. Zymosan caused modest release of superoxide anion with all three populations, indicating that a respiratory burst was being provoked, while PMA, a soluble inducer of leukocyte oxidative burst, caused large-scale production of both oxidants. Preopsonization of mineral dust in rat serum did not render them capable of provoking an oxidative burst from lung-derived leukocytes.

INTRODUCTION

Occupational exposure to certain mineral dusts is associated with the development of pulmonary inflammation (1–4) and fibrosis (5). Experimental deposition of pathogenic mineral dusts, principally quartz and asbestos, in the lungs of laboratory animals has confirmed their ability to cause alveolitis (6, 7) and fibrosis if exposure is persistent (8, 9). This dust-induced parenchymal fibrosis has resulted in the inclusion of the pneumoconioses among the interstitial lung diseases which are typified by persistent alveolar inflammation and, at the end stage, fibrous tissue accumulation in the interstitium (10). The cell biology of the interstitial lung diseases has been extensively studied, and the leukocytic inflammatory cells present in the lung have been implicated in the alveolar dam-

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age which leads to fibrosis (11, 12). Oxidants are one class of leukocyte product which are considered to be likely arbiters of tissue damage during persistent inflammation (13, 14). However, release of oxidants by lung-derived leukocytes on exposure to mineral dusts has not been systematically studied.

The present study was undertaken to assess, using a rat model, the oxidant produced by three different populations of bronchoalveolar cells in response to mineral dust. The three populations constitute bronchoalveolar cells relevant to the study of mineral dust alveolitis. These populations were: control, 16 h after intratracheal instillation of heat-killed *Corynebacterium parvum*, five days after intratracheal instillation of quartz dust. The release of superoxide anion and hydrogen peroxide, produced during the respiratory burst of the leukocytes, was measured following in vitro exposure to the non-inflammation-generating dust titanium dioxide (15) and two dusts known to cause alveolitis, quartz and chrysotile asbestos. Standard particulate and soluble triggers of the respiratory burst were included for comparison.

MATERIALS AND METHODS

Materials. Cytochrome *c*, dextrose, phorbol myristate acetate (PMA), horseradish peroxidase, hydrogen peroxide, superoxide dismutase, and zymosan were obtained from the Sigma Chemical Company, Poole, Dorset. *Corynebacterium parvum* (heat-killed) was obtained from Wellcome Reagents, London. Dulbecco's phosphate-buffered saline (PBS) and F10 medium were obtained from Gibco Limited, Paisley, Renfrewshire. Nembutal was purchased from Ceva Limited, Watford, Hertfordshire. Plastics were obtained from Sterilin, Feltham, Middlesex.

Animals. PVG rats, inbred under SPF conditions at the Institute of Occupational Medicine Animal Unit, were used throughout.

Dusts. The following dusts were used: titanium dioxide (rutile; Tioxide Limited, Stockton on Tees, Cleveland, England), DQ_{12} quartz, chrysotile asbestos (UICC standard sample A).

Intratracheal Instillation. Rats were anesthetized with ether and the trachea exposed by blunt dissection. A blunt-ended needle was introduced into the trachea down to the carina, through a small incision. A volume of 0.2 ml containing 1.4 mg of heat-killed *Corynebacterium parvum* or 1 mg of DQ_{12} quartz was injected, and the skin closed with metal clips. To obtain an acute neutrophil-rich exudate, the rats injected with *C. parvum* were killed 16 h later; to obtain the dusted macrophage/neutrophil population, the DQ_{12} rats were killed five days later.

Bronchoalveolar Lavage. Rats were killed by intraperitoneal injection of Nembutal. The lungs were dissected free from the thoracic cavity and lavaged with 3 × 10 ml sequential volumes of saline at 37°C. The lavaged cells were kept in plastic tubes on ice during counting and preparation of cytocentrifuge smears.

Superoxide Anion Assay. Superoxide anion was measured according to the method of Johnstone (16). The reaction buffer (phosphate-buffered saline, PBS), containing 1 mg/ml cytochrome *c* and 2 mg/ml dextrose) was prepared and 1.5 ml were added to 30-mm petri dishes. Bronchoalveolar lavage cells were prepared at 5×10^6/ml in PBS and 50 μl of cells (0.25×10^6) were added to the reaction buffer in dishes. The particulates quartz, chrysotile asbestos, titanium dioxide, and zymosan were prepared in PBS at 2 mg/ml and diluted so that the addition of 50 μl to each petri

dish yielded a final concentration of 10 or 100 μg/ml. Phorbol myristate acetate was freshly prepared and added as 10 μl to yield 1 μg/ml. Following 2 h incubation, the supernatant was obtained and centrifuged to remove particulates and then read at 550 nm in a scanning spectrophotometer against a reagent blank; this reading was converted to superoxide anion concentration (16). Superoxide dismutase (SOD) controls were always included, and superoxide anion was measured as SOD-inhibitable cytochrome *c* reduction expressed as nanomoles superoxide anion per 2.5×10^5 cells per 2 h.

Hydrogen Peroxide Assay. Hydrogen peroxide was assayed according to the method of Pick and Keisari (17). The reaction mixture (10 mM phosphate buffer containing 1 mg/ml dextrose, 8.4 mg/ml NaCl, and 0.5 mg horseradish peroxidase) was freshly prepared, and 1 ml of this solution was added to 30-mm petri dishes; 50 μl of cells (0.25×10^6 cells) were added as in the superoxide assay. Particulates and PMA were prepared and added exactly as in the superoxide anion assay. Plates were incubated for 2 h, the supernatant centrifuged to clarity, and 30 μl of 1 N NaOH were added to each sample prior to reading at 610 nm against a reagent blank. Standards were prepared from reagent H_2O_2 and a standard line constructed from which the unknowns were obtained, expressed as nanomoles per 2.5×10^5 cells per 2 h.

Opsonization. To test the effect of opsonization of dusts, quartz, chrysotile, and titanium dioxide were incubated at 1 mg/ml in F10 medium plus 10% rat serum for 30 min at 37°C in a shaking water bath. Dusts were washed three times with PBS using high-speed centrifugation and then used in the assays as for unopsonized dusts.

Statistical Analysis. Results were analyzed using two-way analysis of variance and difference in means were assessed for statistical significance using the *t* test.

RESULTS

Composition of Different Bronchoalveolar Lavage Populations

The composition of the three different lavage cell populations is shown in Table 1. The control cells were composed almost entirely of macrophages while the *C. parvum*-treated lung yielded 90% neutrophils; in the quartz-treated bronchoalveolar lavage *population* there were approximately equal numbers of neutrophils and dust-exposed macrophages. In both of the inflammatory populations there were large scale increases in the number of cells obtainable by bronchoalveolar lavage (Table 1).

The Effect of Mineral Dust on Different Bronchoalveolar Lavage Populations

Control Bronchoalveolar Lavage Cells. The effect of the mineral dusts on superoxide anion and hydrogen peroxide production by control alveolar macrophages is shown in Figure 1. It is clear that superoxide production by the cells was largely unaffected by treatment with the three mineral dusts. Only with

Table 1. Composition of Bronchoalveolar Cells from Control Rats and Rats Injected Intratracheally with *C. parvum* and Quartz

Bronchoalveolar lavage population	Percent			Total number of cells retrieved ($\times 10^6$ per rat)
	Macrophages	Neutrophils	Lymphocytes	
Control	98.5 ± 1.9[a]	0		6.6 ±2.3
16-h *C. parvum*	11.0 ± 8.2	89.8 ± 6.7	<2	43.5 ±11.9
5-day quartz	55.0 ± 2.6	45.0 ± 6.7		22.7 ±7.6

[a] Results represent mean ±*SEM* of at least five separate rats.

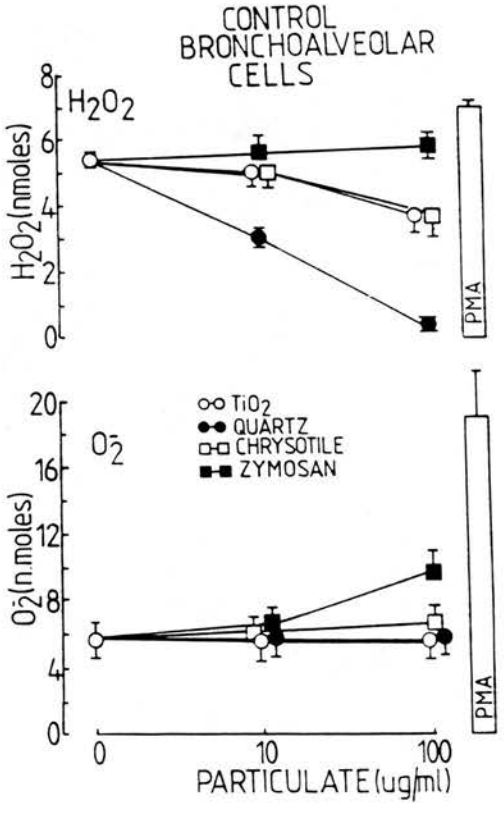


Fig. 1 Effect of mineral dusts, zymosan, and PMA on production of oxidants by control bronchoalveolar cells. Significant ($P < 0.001$) reduction in hydrogen peroxide (H_2O_2) production by chrysotile and titanium dioxide (TiO_2) at 10 and 100 μg/ml. Significant ($P < 0.001$) stimulation of superoxide anion (O_2^-) by zymosan at 100 μg/ml. Results represent mean ± *SEM* of duplicate plates in three separate experiments.

zymosan and PMA was there obvious stimulation ($P < 0.001$). In the case of H_2O_2, the dust caused inhibition of release ($P < 0.001$) with quartz being particularly active in this respect.

Sixteen-hour C. parvum Bronchoalveolar Lavage Cells. These acute inflammatory cells, approximately 90% neutrophils, were not stimulated by the dusts, and only in the case of zymosan, with superoxide anion release, was there evidence of particulate stimulation of the oxidative burst ($P < 0.001$) (Figure 2). PMA was a potent stimulant for release of both superoxide anion and hydrogen peroxide. Inhibition of hydrogen peroxide release was caused by both quartz and chrysotile ($P < 0.001$).

Five-Day Quartz Bronchoalveolar Lavage Cells. As with the other lavage

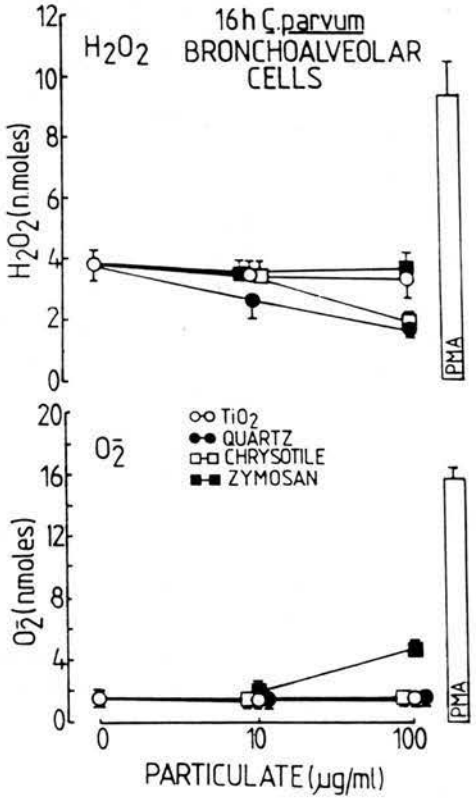


Fig. 2. Effect of mineral dusts, zymosan, and PMA on production of oxidants by 16-h *C. parvum* bronchoalveolar cells. Significant reduction in production of hydrogen peroxide (H_2O_2) by chrysotile at 100 µg/ml and quartz at 10 and 100 µg/ml. Significant increase ($P < 0.001$) in H_2O_2 production by PMA. Significant increase in superoxide anion (O_2^-) production by 100 µg/ml zymosan and PMA. Results represent mean ± *SEM* of duplicate plates in three separate experiments.

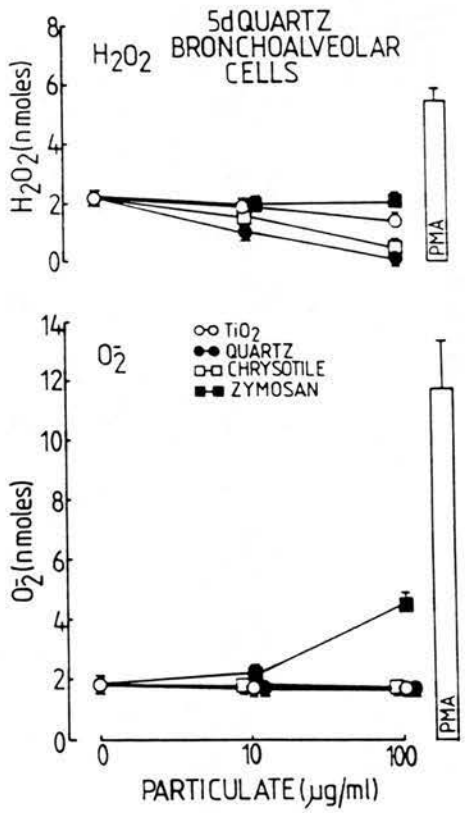


Fig. 3. Production of oxidants by 5-day quartz bronchoalveolar cells in response to mineral dusts, zymosan, and PMA. Significant ($P < 0.001$) reductions in hydrogen peroxide (H_2O_2) production by chrysotile asbestos and quartz at 10 and 100 μg/ml and significant ($P < 0.001$) increase with PMA. Significant ($P < 0.001$) increase in superoxide and anion (O_2^-) production by zymosan at 100 μg/ml and PMA. Results represent mean ± *SEM* of duplicate plates in three separate experiments.

populations, the three mineral dusts caused no stimulation of oxidative burst products, while zymosan caused stimulation of superoxide anion ($P < 0.001$) and PMA caused an increase in both superoxide anion and hydrogen peroxide (Figure 3). Both chrysotile and quartz caused inhibition of hydrogen peroxide release ($P < 0.001$).

Effect of Opsonizing the Mineral Dusts

Figure 4 shows the effect of opsonizing the mineral dust in autologous serum on ROI release by macrophages, represented by the control bronchoal-

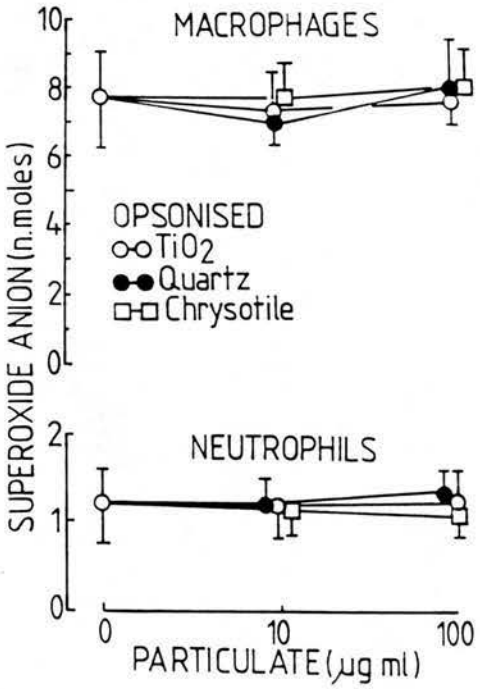


Fig. 4. Production of superoxide anion by macrophages (control bronchoalveolar cells) and PMN (16 h *C. parvum* bronchoalveolar cells) in response to mineral dusts pre-opsonised by incubation in serum. Results represent mean ± *SEM* of duplicate wells in two separate experiments.

veolar lavage cells, and neutrophils, represented by the *C. parvum* cells. The opsonized dust was as inactive as the unopsonized dusts in stimulating oxidant production.

Oxidant Production with PMA Stimulation

The oxidant production profile following treatment with PMA differed between the three bronchoalveolar populations used in the study, and the PMA results from Figures 1–3 are summarized in Table 2. The control population was high in superoxide production and moderate in hydrogen peroxide production. The acute neutrophil-rich *C. parvum* population produced significantly ($P < 0.001$) less superoxide than control cells and more ($P < 0.001$) hydrogen peroxide. The quartz population was low in superoxide production ($P < 0.001$) compared to control but similar in hydrogen peroxide production.

Table 2. Oxidant Production by the Three Bronchoalveolar Cell Populations in Response to PMA

Bronchoalveolar lavage population	Response to PMA[a]	
	O_2^- [b]	H_2O_2[b]
Control	19.1 ± 2.9	5.4 ± 0.8
C. parvum	15.6 ± 0.8	9.3 ± 1.1
Quartz	11.8 ± 1.6	5.5 ± 0.4

[a] PMA 1 μg/ml.
[b] Nanomoles/10^5 cells/2 h.

DISCUSSION

We report here on the failure of pathogenic mineral dusts to stimulate an oxidative burst in leukocytes derived, by bronchoalveolar lavage, from control and inflamed rat lung. Oxidative burst was assessed by release, into the medium, of superoxide anion and hydrogen peroxide (18). Two different bronchoalveolar lavage populations were used to represent inflammatory cells found in dust-induced alveolitis. The *C. parvum* population, which was 90% neutrophils, was used to gain information on the response of acutely exudated neutrophils to mineral dusts, and this almost pure population minimized the contribution of machrophages. Bronchoalveolar lavage cells obtained after injection of quartz into the lung were used to represent a population of dust-activated macrophages and neutrophils present in approximately equal numbers.

The results obtained show that all three populations were capable of eliciting an oxidative burst in response to PMA, a soluble trigger of leukocyte metabolism. The particulate trigger of leukocyte metabolism, zymosan, also stimulated superoxide anion production, but not hydrogen peroxide production, in all three populations. However, all three mineral dusts failed to elicit an oxidative burst as shown by failure to detect release of superoxide anion or hydrogen peroxide into the supernatant. In the case of hydrogen peroxide, the levels produced on addition of the mineral dusts were significantly less than those produced by untreated leukocytes, while zymosan had no such effect. It seems likely that these effects are due to the acute cytotoxic effects of mineral dust and quartz in particular which we (data not included) and others (19) have demonstrated.

Mineral dust surfaces can be highly active in view of their chemical structure, and this has been implicated in their toxicity and in their biologic activity in general, particularly in the case of quartz and chrysotile asbestos (19). Therefore the dusts were opsonized by preincubation in autologous serum and then

tested for their ability to trigger oxidant release from macrophages (control bronchoalveolar lavage cells) and neutrophils (*C. parvum* bronchoalveolar lavage cells). As was the case with the unopsonized dusts, opsonized dusts failed to cause oxidant release.

The three lung cell populations utilized in the present study were chosen for their relevance to mineral dust-induced lung inflammation. Control alveolar macrophages are among the first cells that encounter dust when normal lung is exposed (20), while inflammatory neutrophils and macrophages are found in increased numbers in the bronchoalveolar cells populations from lungs following deposition of pathogenic mineral dusts in occupationally exposed humans (3, 4) and experimentally exposed animals (6, 7).

In experiments aimed at understanding the inflammation caused by mineral dusts, the release of inflammatory mediators from leukocytes following treatment with mineral dusts in vitro has been extensively demonstrated. Using various leukocyte indicator populations, asbestos and quartz have been shown to stimulate release of acid protease (21, 22) neutral protease (23), arachidonic acid metabolites (24–26), and chemotaxins (27, 28). Several studies have suggested that oxidant produced during the oxidative burst of inflammatory leukocytes may be of particular importance in causing damage to the alveolar structure during lung inflammation under experimental conditions (13, 14). Subsequently a range of studies have provided indirect evidence that leukocyte-derived oxidants could be generated in mineral dust-induced inflammation. Using the technique of chemiluminescence for indirect measurement of phagocyte oxidant production, Doll and coworkers observed stimulation of peripheral blood neutrophils (29) but inhibition of monocytes (30) using asbestos. In addition quartz has been shown to stimulate increased chemiluminescent responses of human neutrophils (31, 32) and rabbit alveolar macrophages (33). In a mouse peritoneal model of mineral dust inflammation, the cells elicited by asbestos showed increased chemiluminescent response to conventional triggers of phagocyte oxidative burst and asbestos (34), and a similar finding has been reported in bronchoalveolar lavage cells from patients with silicosis and coalworkers' pneumoconiosis (4).

However, in a study carried out by Myrvik et al. (35), direct measurements were made of hexose monophosphate shunt activity in rabbit alveolar macrophages following treatment with chrysotile asbestos and including zymosan as a positive control; chrysotile asbestos failed to stimulate an oxidative burst thus lending support to the findings of the present study.

Several reasons can be put forward for the stimulation of ROI production produced by mineral dusts in some studies, compared with the findings of Myrvik et al. (35) and those of the present study. These include the use of non-pulmonary-derived leukocytes in many studies where profound differences in oxidative metabolism are to be anticipated between pulmonary-derived and other

leukocytes (18); the use of rat, rabbit, and mouse leukocytes as well as human cells when species differences undoubtedly exist (18); and the use of chemiluminescence which, in the case of alveolar macrophages stimulated with a particulate, is the complex product of particle-cell, particle-oxidant interactions (36) which may present difficulties of interpretation given the highly active surface of some mineral dusts.

In macrophages, phagocytosis and generation of an oxidative burst can be dissociated from one another, and specific engagement of IgG Fc receptors has been found to be mandatory for an oxidative burst in one study using peritoneal macrophages (37). Such receptors would not be specifically engaged during phagocytosis of unopsonized dust but might be expected to be involved in phagocytosis of serum preopsonized dust. In the present study, however, there was no evidence of respiratory burst production on phagocytosis of opsonized dust either. Clearly other mechanisms for stimulation of leukocytes exist since nonspecific soluble triggers such as PMA are capable of eliciting an oxidative burst. Certainly secretion of other leukocyte inflammatory mediators can be stimulated by both unopsonized (26, 27, 29, 32, 33) and opsonized (21, 24, 29, 32) mineral dust, suggesting that specific leukocyte receptors are not involved, although a systematic study of opsonization in mineral dust-induced leukocyte secretion has never been carried out.

The present study places in question the ability of mineral dust to stimulate release of oxidants by leukocytes in the alveolar region of the resting or inflamed lung. It appears unlikely, therefore, that in the experimental model used here, this represents a source for accumulation of oxidants with sufficient potential to damage elements of the alveolus. However, PMA treatment of all three populations caused release of hydrogen peroxide, an oxidant with particular activity in causing damage to lung cells (38, 39). Compared on a cell-for-cell basis with control cells, the production of hydrogen peroxide was increased in the *C. parvum* population and remained unchanged in the quartz population. This was, however, in the face of approximately four- to sevenfold increases in the total number of cells lavaged from the inflamed lungs. Given that soluble triggers such as complement may well be present as part of the overall inflammatory response in *C. parvum*- and quartz-treated lung, but not in control lungs, oxidant accumulation could therefore still occur. The decreased production of superoxide anion shown by the two inflammatory populations on treatment with PMA can be explained to some extent by the previously noted decrease in superoxide anion production of neutrophils compared to macrophages (40). However, such was the extent of the increases in inflammatory cells recruited to the bronchoalveolar compartment that the cumulative effect of the whole population could again lead to build-up of this oxidant.

The present study suggests that rapid release of respiratory burst-derived oxidant following contact between pathogenic mineral dust and bronchoalveolar

leukocytes is not a mechanism for accumulation of oxidants in the lung parenchyma of dust-exposed individuals. However, several other mechanisms exist whereby an increased oxidant burden could exist in dust-exposed lung as a consequence of changes in the alveolar leukocyte populations and their interaction with humoral components of the inflammatory response, as recently reviewed by Repine (41).

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SECRETION OF INTERLEUKIN 1 AND TUMOUR NECROSIS FACTOR BY ALVEOLAR MACROPHAGES FOLLOWING EXPOSURE TO PARTICULATE AND FIBROUS DUSTS

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ABSTRACT

Inflammatory leukocytes have been implicated in the pathogenesis of lung disease in persons exposed to asbestos. Inflammatory leukocyte-derived cytokines may play an important part in the disease process through their ability to modulate the function of other cell types. We have previously shown that long and short fibre amosite asbestos samples have different pathological potential. In this study, we have therefore examined cytokine secretion by bronchoalveolar leukocytes exposed to long and short fibre amosite asbestos *in vivo* and *in vitro*. We have shown that both *in vivo* and *in vitro* exposure to amosite results in increased secretion of IL-1 and TNF which was greater with the long fibre sample. The relationship between long term pathology and increased secretion of TNF and IL-1 suggests that cytokines may play a part in the pathogenesis of lung disease in workers exposed to asbestos.

INTRODUCTION

A common finding in individuals occupationally exposed to asbestos is increased numbers of macrophages in the bronchoalveolar space (Begin *et al.*, 1986). These macrophages are likely to be activated due to phagocytosis of dust particles (Schnyder & Baggiolini, 1978) and perhaps also to interaction of the intracellular fibres with the cell membrane (Brown *et al.*, 1991). The cytokines interleukin 1 (IL-1) and tumour necrosis factor (TNF) are secreted by inflammatory macrophages (Billingham, 1987) and may play a role in the development of lung fibrosis and neoplasia in persons exposed to asbestos (Bignon & Jaurand, 1984) through their ability to cause proliferation and oxidant production in

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certain cell types. A major factor governing the harmfulness of fibrous dusts is thought to be their geometry although surface chemistry may also be important. Increasing fibre length has been shown to correlate with increasing carcinogenic potential *in vivo* (Stanton & Wrench, 1972) and with increasing toxicity *in vitro* (Brown *et al.*, 1986). We have also demonstrated experimentally that long asbestos fibres have significantly greater inflammatory potential than short fibres which will result in the recruitment of large numbers of inflammatory macrophages to the bronchoalveolar space (Donaldson *et al.*, 1989).

In the present study, we have investigated production of IL-1 and TNF by rat alveolar macrophages at various time points after intratracheal instillation of long and short amosite asbestos. In order to investigate the direct effects of asbestos on macrophage cytokine production, alveolar macrophages from untreated rats were exposed *in vitro* to amosite asbestos and then IL-1 and TNF secretion were measured.

Recent work in the Institute of Occupational Medicine has indicated that particulate dusts may potentiate the carcinogenic effects of fibres (Davis *et al.*, manuscript in preparation). We therefore also assessed production of TNF by alveolar macrophages exposed *in vitro* to mixed dusts of long and short amosite in combination with quartz or titanium dioxide.

MATERIALS AND METHODS

Dusts

Long and short fibre amosite were prepared from a commercially available South African amosite as previously described (Donaldson *et al.*, 1989). Titanium dioxide (TiO_2) was the rutile form (Tioxide Ltd., Stockton on Tees) and quartz was the DQ12 standard.

Exposure regimen and preparation of bronchoalveolar macrophage supernatants

Male HAN rats were exposed to dust by intratracheal instillation of 1 mg as a single bolus in 0.5 ml phosphate buffered saline (PBS); control rats were injected with PBS alone. At selected time points thereafter, the rats were killed and the bronchoalveolar (BAL) leukocytes retrieved by lavage as previously described (Brown & Donaldson, 1988). Total cell and differential counts were performed on the BAL leukocyte populations using Trypan blue and May Grunwald-Geimsa stains respectively.

For *in vitro* exposure, BAL leukocytes were obtained from untreated rats and these were set up in 24-well culture plates as 1 ml of Ham's F10 medium (Gibco, Paisley) plus 2% bovine serum albumin (BSA) containing $1x10^6$ cells/ml and 50, 100 or 500 μg dust/ml. Cultures exposed to mixed dusts received 50 μg/ml asbestos and 50 μg/ml TiO_2 or quartz. Supernatant medium from these cultures was obtained 24 h later and analysed for content of IL-1 and TNF. Supernatants from BAL leukocytes from rats exposed to dust *in vivo* were obtained under the same conditions but in the absence of dust.

Cytokine measurement

IL-1 in leukocyte supernatants was measured using the standard mouse thymocyte bioassay (Kusaka *et al.*, 1990). The presence of TNF in the supernatants was assessed by measuring their ability to cause lysis of the TNF-sensitive L929 cell line (Manson *et al.*, 1989).

RESULTS

Effect of *in vivo* exposure to long and short amosite asbestos on bronchoalveolar leukocyte numbers

Short-fibre amosite elicited an alveolitis which was evident as recruitment of PMN to the bronchoalveolar region by 3 days after intratracheal injection (Table 1). The greatest influx of inflammatory leukocytes was apparent at 7 days and was comprised largely of macrophages; inflammation in the short-fibre amosite-exposed rats persisted up to 14 days. Long fibre amosite did not elicit a measurable increase in numbers of leukocytes in the BAL at any time point and at 3 days there was a reduction in the number of lavageable leukocytes in these animals. However, the presence of PMN in the BAL at each time point with long fibre amosite was indicative of an inflammatory response

IL-1 and TNF secretion

Although there was evidence of an inflammatory response in both long and short amosite-exposed rats at 3 days post-injection, there was no evidence of increased secretion of TNF by BAL leukocytes at this time-point with either amosite sample (control-46.91($\pm$4.98); long-41.53($\pm$0); short-44.58 ($\pm$0) (mean (sd) units of TNF/ml in three rats per group). By 14 days, however, BAL leukocytes elicited by both amosite samples secreted more TNF than did control BAL macrophages; this was greater with long fibre than with short (control - 25.94 ($\pm$31.0); long - 54.48 ($\pm$18.8); short - 46.22 ($\pm$2.31).

TABLE 1. Total number and differential count of cells in the bronchoalveolar region of rats exposed by intratracheal injection to 1 mg long and short fibre amosite asbestos

Days exposed		Total($\times 10^6$)	Differential count (%)		
			[1]Mϕ	PMN	Lymph[3]
3	Control[2]	5.29(1.0)	99(0.6)	0	1(0.6)
	Short	7.34(2.5)	96(4.7)	3(4.2)	1(0.6)
	Long	2.03(1.13)	92(6.1)	4(2.0)	1(0.6)
7	Control	6.5(1.6)	98(5.5)	0	2(0.6)
	Short	24.7(18.8)	92(2.7)	7(2.9)	1(0.6)
	Long	6.2(1.8)	91(4.6)	5(5.7)	1(1)
14	Control	7.3(1.0)	99(0.6)	0	1(0.6)
	Short	12.1(6.9)	97(4.7)	2(4.3)	1(0.5)
	Long	5.8(2.5)	94(4.1)	5(3.5)	0

[1] Macrophages, [2] 3 rats/group at each time point.

IL-1 secretion by long fibre amosite-elicited BAL leukocytes was greater than that of control BAL macrophages 7 days post-injection (control - 4892 (±1291); long - 6819 (±1093) (mean (sd) cpm for 3 rats per group) but returned to near control levels by 14 days - 5731 (±1714). Short fibre was no different from the control at 7 days (4348 (±408)), or 14 days (4732 (±607)).

Bronchoalveolar leukocytes from untreated control rats comprised > 95% macrophages. When these cells were exposed to dust *in vitro* at 500 µg/ml, only the long fibre amosite induced increased IL-1 secretion compared with control BAL leukocytes (control -2260; long - 4038; short - 2513 cpm). TNF secretion by macrophages exposed to 500 µg/ml amosite *in vitro* was greater with long (31.48 (±22.4) units/ml) than with short fibre amosite (16.16 (±21.99) units/ml).

Exposing control bronchoalveolar leukocytes to mixed dusts composed of long and short fibre amosite and toxic or non-toxic dust *in vitro*, did not result in potentiation of TNF secretion with either asbestos sample (Table 2).

DISCUSSION

In this study we have investigated the ability of long and short fibre amosite preparations to elicit secretion of cytokines by bronchoalveolar leukocytes. We used *in vivo* and *in vitro* exposure to examine the effects of long and short fibre amosite asbestos on secretion of IL-1 and TNF by rat alveolar macrophages. We have previously demonstrated that long fibre amosite causes greater recruitment of inflammatory leukocytes into the mouse peritoneal cavity (Donaldson *et al.*, 1989) than does short fibre. In rats exposed by inhalation (Davis *et al.*, 1986), we have also shown that there is greater tumour production with long fibre amosite. In the present study, the bronchoalveolar lavage data showed more inflammation with short amosite than with long and although we did observe an inflammatory response in terms of PMN recruitment into the BAL with long fibre amosite, there was no measurable increase

TABLE 2. TNF secretion by control bronchoalveolar macrophages following *in vitro* exposure to long and short fibre amosite asbestos, either alone or in combination with quartz and TiO_2

	Amosite alone	Mixed dusts	
		+TiO_2	+quartz
Short	23.58	29.16	21.97*
Long	44.58	29.31	36.05

* Units of TNF/ml.

All dusts are at a concentration of 50 µg/ml which gives a final concentration of 100 µg/ml with the mixed dusts (mean of 3 wells/sample in a single experiment).

in total numbers of leukocytes. This apparently disagrees with the hypothesis that lung injury is related to the recruitment of inflammatory leukocytes into the bronchoalveolar region. The apparent lack of a macrophage response in the long fibre-exposed rats, however, is probably due to the problems of instilling a long fibre sample which may block the airways, so making
lavage difficult. We have previously shown that there is an inflammatory response in rats to inhaled asbestos (Donaldson *et al.*, 1988) that leads to long term pathology.

Those leukocytes which could be lavaged from the long fibre amosite-exposed animals were different from both control BAL leukocytes and the short-fibre amosite-elicited BAL leukocytes in terms of cytokine secretion. Both TNF and IL-1 secretion by long amosite-elicited leukocytes was greater than secretion by short fibre-elicited cells. The most likely source of these cytokines is the macrophage since the percentage of PMN was small and was similar in both short and long fibre- elicited BAL leukocytes. The finding of increased cytokine production with long fibre is in agreement with the study of (Bissonette & Rola-Pleszczynski, 1989) who demonstrated increased secretion of TNF in mice exposed to chrysotile asbestos but not silica.

In order to investigate the direct dust particles/leukocyte interaction in cytokine production, we exposed control bronchoalveolar leukocytes *in vitro* to amosite asbestos. Secretion of both TNF and IL-1 was greater with exposure to long fibre amosite than to short fibre. Thus, increased secretion of cytokines by BAL leukocytes exposed to dust *in vivo* may be due to direct interaction between fibres and leukocytes in the lung. Both TNF and IL-1 can act as chemotaxins and so the direct interaction of dust and alveolar macrophages to induce secretion of these cytokines, may be involved in initiation of the inflammatory response following deposition of dust in the lung.

In long term pathology studies, we have recently shown that exposure to non-fibrous dust in combination with asbestos potentiates the carcinogenic potential of the asbestos. In the present study therefore, we assessed the ability of non-toxic (TiO_2) and toxic (quartz) particulate dusts to potentiate secretion of TNF by leukocytes exposed to amosite *in vitro*. There was no evidence in the present study that the additional particulate burden could stimulate increased TNF secretion.

This study has shown that amosite asbestos can cause increased release of IL-1 and TNF from BAL leukocytes following *in vivo* and *in vitro* exposure. We have shown that long fibre amosite, which causes long term pathology in our rat model, induces greater secretion of both cytokines. The inflammogenic and immunostimulatory roles of TNF and IL-1 are central to normal host defence in the lung but they may also contribute to the pathogenesis of lung disease in situations where there is excessive secretion of these cytokines (Beissert *et al.*, 1989; Updyke *et al.*, 1989). In workers occupationally exposed to asbestos, increased secretion of cytokines in the bronchoalveolar region of the lung may occur in response to fibre deposition and thus may ultimately contribute to the pathogenesis of asbestosis and neoplasia.

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ASBESTOS-STIMULATED TUMOUR NECROSIS FACTOR RELEASE FROM ALVEOLAR MACROPHAGES DEPENDS ON FIBRE LENGTH AND OPSONIZATION

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SUMMARY

Fibre length has been shown to be an important factor in the ability of respirable fibres to cause lung fibrosis and cancer. We have reported that a long sample of amosite asbestos is more carcinogenic and fibrogenic than a short sample of similar diameter. These amosite asbestos samples were studied with regard to their ability to stimulate the release of the pro-inflammatory cytokine tumour necrosis factor (TNF) from rat alveolar macrophages *in vitro*. The long fibre sample was found to stimulate substantially greater release of the cytokine than the short sample. Furthermore, on treatment of the fibres with rat immunoglobulin G (IgG), there was an increase in the ability of both the long and the short sample to stimulate TNF secretion, although the long sample retained by far the greatest activity. Coating of the fibres with a range of other proteins had no substantial effect on their ability to stimulate TNF secretion. Quartz and titanium dioxide (TiO_2) were included as control particles and the TNF-stimulating activity of quartz was notably increased by opsonization with IgG. TiO_2 showed a similar low activity to that of the short fibre sample of amosite but this again could be modestly increased by opsonization with IgG. The simulation of TNF release caused by treatment with immunoglobulin-opsonized long fibre amosite could be inhibited by treatment of the macrophages with the protein kinase C-inhibitor staurosporine. The study demonstrates a fibre length-related ability to stimulate cytokine secretion by alveolar macrophages, and its enhancement by opsonization with IgG. This is likely to be relevant to the relationship between fibre length and pathogenic potential to the lung.

KEY WORDS—Asbestos, fibres, macrophages, quartz, opsonization, TNF, inflammation, pneumoconiosis.

INTRODUCTION

Exposure of workers to fibres of asbestos is associated with the development of pulmonary fibrosis and neoplasia of the lungs and mesothelial linings of the pleural and peritoneal cavities.[1,2] Bronchoalveolar lavage studies in asbestos-exposed workers have revealed the presence of inflammation[3,4] and experimental exposure of laboratory animals has revealed that the onset of inflammation is an early event[5] which may persist in the absence of further exposure.[6]

Substantial progress has been made in understanding the cellular and molecular basis of the asbestos-related lung disease but many questions remain to be answered.[7] Injurious proteases,[8] oxidants,[9] and growth factors[10] are produced by leukocytes exposed to a range of dusts. This suggests that the inflammation may be a major factor in tissue damage leading to fibrotic and neoplastic pathological change following exposure to asbestos or other pathogenic dusts and fibres.

Tumour necrosis factor (TNF) is a pro-inflammatory cytokine released by inflammatory leukocytes in increased amounts at sites of inflammation.[9] TNF has been implicated in the genesis and persistence of inflammation caused by pathogenic dusts.[10,11] In the previous study we demonstrated that a long fibre sample of amosite asbestos, which we had demonstrated to be more

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pathogenic,[12] caused more inflammation[13] than an otherwise identical short fibre sample. We hypothesized that these two fibre samples may have different abilities to stimulate the release of TNF from alveolar macrophages and that this may contribute to their different pathogenicities.

Particles that deposit in the lung are likely to encounter the lung-lining fluid, which is a complex mixture of various substances.[14] A previous study[7] demonstrated that incubation of chrysotile asbestos in IgG, a component of the lung-lining fluid, resulted in an increased ability of the fibre to stimulate the oxidative burst in alveolar macrophages. We therefore set out also to investigate whether the ability to release TNF from alveolar macrophages could be similarly modulated by opsonization of long and short fibre amosite with IgG; in addition, we included quartz (silica) and the inert particulate titanium dioxide (TiO_2) to control for any non-specific effects.

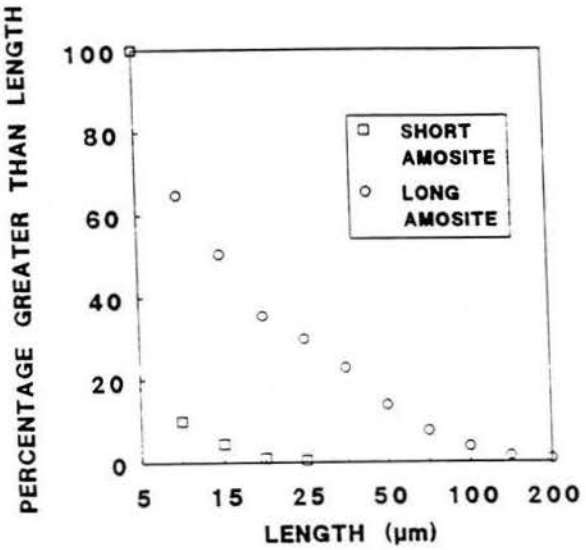


Fig. 1—Length distribution of the long- and short-fibre amosite samples. Phase contrast optical microscopy of over 400 fibres

MATERIALS AND METHODS

Rats

Wistar-derived rats of the HAN strain, approximately 12 weeks of age, were used throughout.

Alveolar macrophages

Alveolar macrophages were obtained by bronchoalveolar lavage from normal rats.[5]

Dusts

The dusts used were (1) a long- and short-fibre amosite asbestos sample whose size ranges are shown in Fig. 1 for fibres less than 3 μm in diameter, greater than 5 μm in length, and with an aspect ratio of more than 3:1. Previous electron microscope counts have shown the two samples to be very similar in diameter.[12] In the short sample (SFA), there are very few fibres longer than 10 μm, whilst in the long sample (LFA), there are more than 40 per cent of fibres greater than 10 μm and 15–20 per cent fibres greater than 20 μm; (2) the DQ12 standard quartz sample. This is a standard sample of respirable quartz (silica) known to cause silicosis experimentally following inhalation or instillation; and (3) a respirable-sized sample of rutile titanium dioxide (TiO_2; Tioxide Ltd., Stoke on Trent, U.K.).

Opsonization of dusts

Dusts were mixed with phosphate-buffered saline (PBS; unopsonized), rat serum, heat-inactivated rat serum (56°C, 1 h), bovine serum albumin (BSA; Sigma, 2 per cent in F10 medium), rat IgG (Sigma, 50 μg/ml in medium), or fibronectin (Sigma, 50 μg/ml in medium) at a concentration of 1 mg dust/ml. The mixtures were then incubated, with rotation for 30 min at 37°C. The suspensions were spun-washed three times in PBS, resuspended in PBS at a concentration of 1 mg/ml, and used to stimulate macrophage TNF production.

Preparation of macrophage supernatants

Alveolar macrophages were cultured in F10 medium (Gibco, Paisley, U.K.) plus 2 per cent BSA (Sigma, Poole, Dorset, U.K.) at 1×10^6 cells/ml in 12 well plates (Falcon, Oxnard, CA, U.S.A.). The dusts were added to a concentration of 50 μg/ml and the supernatants collected after 24 h culture at 37°C for 24 h; supernatants were stored frozen until they were assayed for TNF. To inhibit protein kinase C, 0·2 μg/ml of staurosporine (Sigma) was included during the preparation of the supernatants in some experiments.

TNF assay

The presence of TNF in supernatants from alveolar macrophages treated with the dusts was detected by cytotoxic activity in the cell-lytic assay using L-929 cells as targets standardized with TNF alpha (a gift kindly supplied by Dr J. Symonds). L-929

cells were cultured in minimum essential medium (MEM, Gibco) with 5 per cent fetal calf serum (FCS; Gibco).

The assay medium was MEM + 5 per cent FCS without antibiotic. Cells were detached from a semi-confluent status and resuspended in assay medium at 3×10^5/ml and dispensed into Falcon 96 well multiwell plates as 100 μl. The plates were incubated at 37°C, 5 per cent CO_2 for 24 h. The supernatant was then aspirated and discarded. One hundred μl of assay medium plus 1 μg/ml actinomycin D (Sigma) was then added to all wells. Fifty μl of test supernatants and 50 μl of 2 μg/ml actinomycin D (Sigma) were added to experimental wells in triplicate. They were then double-diluted using a multichannel pipette. The plates were then incubated for 18–20 h at 37°C, 5 per cent CO_2. The medium was then discarded and replaced with 100 μl of a crystal violet solution. The L-929 cells were left for a few minutes, washed vigorously with water, and allowed to dry. The plate was then read in an MR650 reader (Dynatech Laboratories Inc., U.S.A.) at 540 nm. The addition of medium alone to the L-929 cells represented the negative control, while maximal cell lysis was obtained from wells cultured without L-929 cells. The amount of TNF present in the pleural leukocyte supernatants was determined using a TNF alpha standard dilution curve. The specificity of the assay was confirmed using an antibody to TNF kindly supplied by Dr S. Kunkel and Dr J. Fantone, University of Michigan.

Statistical analysis

All experiments were repeated at least three times under identical conditions. Results from the separate experiments were logarithmically transformed and analysed by two-way analysis of variance, experiments versus treatment, using the Minitab statistical computer package. From the ANOVA table, *F*-tests were used to determine any significant treatment effects. Where there was a significant treatment effect, individual comparisons were tested for significance using *t*-tests with a pooled measure of standard error.

RESULTS

Unopsonized particles

When untreated particles were used, as Fig. 2, reveals, the production of TNF by macrophages exposed to the LFA exceeded ($P < 0.05$) that of the SFA sample, which produced the same amount as the TiO_2 control. Quartz produced slightly more than TiO_2 or SFA but this did not attain statistical significance in the small number of experiments carried out.

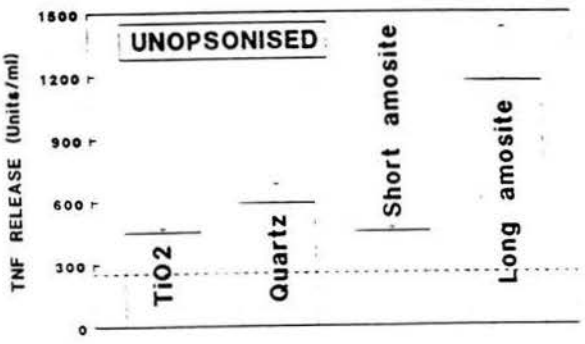


Fig. 2—Production of TNF by rat alveolar macrophages exposed to unopsonized long amosite, short amosite, titanium dioxide, or quartz. The dashed line indicates production of TNF by alveolar macrophages alone, without the addition of particulate

Opsonized particles

Figures 3a–3d are shown with the same scale of vertical axis, normalized to the production of TNF by control, unstimulated alveolar macrophages, to emphasize the differences between the different particles and treatments. They show the effect of a single dose of particles (50 μg/ml) opsonized in a range of solutions as indicated. It is clear that the long-fibre amosite sample was the most active in causing release of TNF. As can be seen within any one of the panels in Fig. 4, opsonization with IgG caused each of the different particle types to stimulate a significantly increased release of TNF compared with particles incubated in PBS, i.e., unopsonized ($P < 0.001$ for DQ12 and LFA: $P < 0.01$ for SFA and TiO_2).

In addition, as can be seen from comparing the furthest right bar in all of the panels in Fig. 3, opsonized LFA had the greatest activity in stimulating TNF secretion, being significantly increased over IgG-opsonized SFA and TiO_2 ($P < 0.01$). However, IgG-opsonized quartz was also more stimulatory to TNF secretion than IgG-opsonized TiO_2 ($P < 0.05$); IgG-opsonized SFA was not significantly different from IgG-opsonized TiO_2 in TNF-stimulating activity.

Increasing the opsonizing dose of IgG from the 50 μg/ml used in the experiments described above to

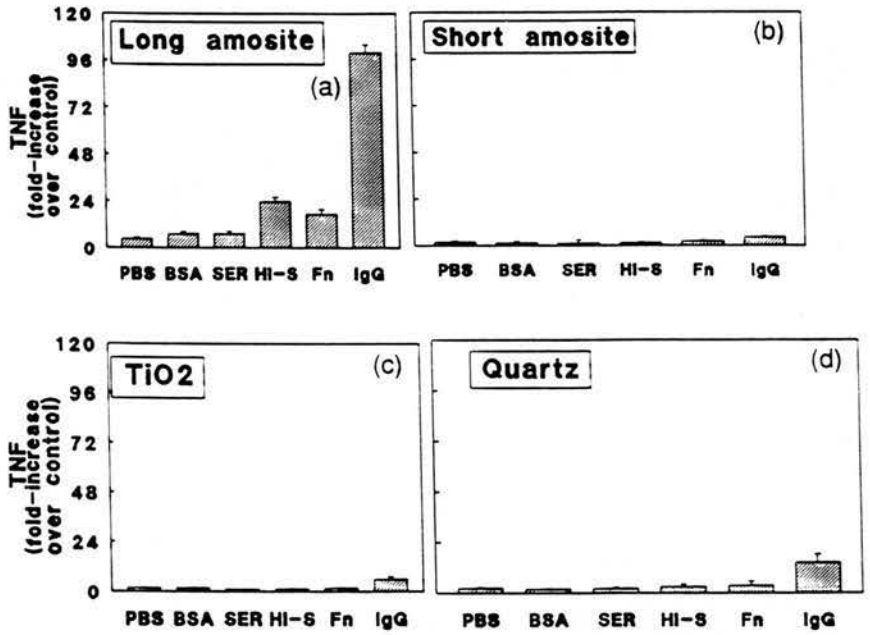


Fig. 3—Production of TNF by rat alveolar macrophages exposed to long amosite (a), short amosite (b), titanium dioxide (c), or quartz (d). Dusts were pre-incubated in the following: PBS = phosphate-buffered saline; BSA = bovine serum albumin; SER = rat serum; HI-S = heat-inactivated rat serum; Fn = fibronectin; IgG = rat immunoglobulin G. All data are shown as the fold-increase in production compared with the spontaneous release from untreated cells (260 units)

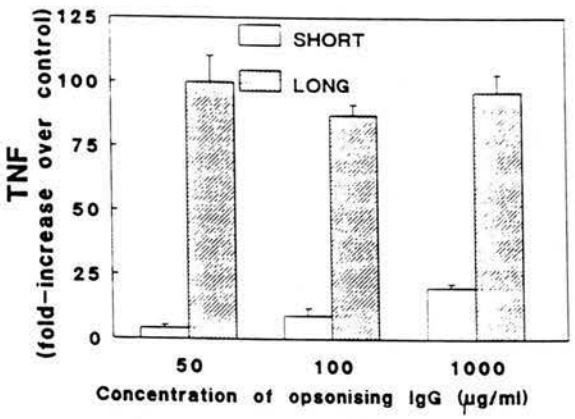


Fig. 4—Effect of increasing the opsonizing concentration of IgG on the ability of long and short fibre amosite to stimulate macrophages to release TNF. The dotted line indicates the production of TNF by control alveolar macrophages in the absence of stimulation with particulate. All data are shown as the fold-increase over production by control, untreated cells (260 units)

100 and 1000 μg/ml did not greatly affect the ability of either SFA or LFA to stimulate release of TNF (Fig. 4). Although there was an indication that the ability of SFA to cause release of TNF was enhanced with increasing dose of opsonizing IgG, this did not attain statistical significance ($P > 0{\cdot}1$) in the small number of experiments that were carried out.

Role of protein kinase C

Inclusion of the protein kinase C inhibitor staurosporine during incubation of macrophages with opsonized LFA caused a marked decrease in the levels of secreted TNF. There was not complete abolition with staurosporine treatment, but in three successive experiments with opsonized LFA staurosporine caused 43·8, 91·0, and 55·0 per cent inhibition of the TNF release caused by opsonized LFA in the absence of staurosporine.

DISCUSSION

The samples of long and short amosite used here have been utilized previously *in vivo* and have been found to have very different abilities to cause disease. By inhalation, the LFA caused severe pulmonary fibrosis whilst the SFA was virtually without

effect.[12] In addition, inhalation exposure to the LFA caused 14 lung tumours to develop in 40 animals, including one pleural mesothelioma and one peritoneal mesothelioma. By contrast, the SFA produced no lung tumours, although one peritoneal mesothelioma developed amonst the exposure group. When the LFA (25 mg) was instilled into the peritoneal cavity, it caused mesotheliomas in 95 per cent of rats, whilst the same mass of SFA caused only 4 per cent tumours.[12] In the mouse peritoneal cavity, the LFA was found to be many times more active in causing inflammation, on a fibre number basis, than the SFA.[13] Many other studies have demonstrated the importance of fibre length in tumorigenicity,[15-17] transforming activity,[18] cell toxicity,[19] lung cell proliferative response,[20] and release of macrophage-derived growth factors.[21]

The different *in vivo* activities shown by the long- and short-fibre asbestos samples were paralleled by a marked difference in the ability of the two different fibre samples to cause macrophages to secrete the cytokine TNF *in vitro*. Thus, incubation of alveolar macrophages with the LFA caused a substantially greater release than did SFA. The LFA effect was greater than that produced by either the pathogenic particulate quartz or the inert particulate TiO_2. This may be explained by stimulation due to incomplete phagocytosis of the long fibres. Additionally, the non-specific receptors involved in the phagocytosis of a particle such as asbestos may be cross-linked[7] and in the case of a very long fibre, this may be sufficient to stimulate the cell. Further experimentation into the nature of the receptors that mediate binding of fibres to macrophages may illuminate this phenomenon.

On opsonization with IgG, there was a marked increase in the ability of the LFA to stimulate the release of TNF. This effect of opsonization with IgG was seen with all of the dusts tested. However, since the starting level was highest with LFA, the effect of opsonization was greatest. Immunoglobulins are present in the lung-lining fluid at around the 50 mg/ml used in the majority of the experiments described above. The levels of immunoglobulin in bronchoalveolar lavage fluid have been reported to be increased in asbestos-exposed lung.[3] We have ourselves reported a marked protein leak into the bronchoalveolar space accompanying inflammation in the lungs of rats inhaling asbestos.[5] Thus, progression of inflammation in the lungs of asbestos-exposed individuals would be expected to lead to opsonization of depositing fibres, leading to a further stimulation of TNF release and an enhancement of the inflammatory process. The same sequence of events is likely to occur in the lungs of quartz-exposed individuals, since we have demonstrated here that the ability of quartz to stimulate macrophage release of TNF is also markedly enhanced by opsonization with IgG.

With LFA and SFA, increasing the opsonizing concentration of IgG failed to even out the difference between the two samples, suggesting that the binding sites for IgG are saturated at 50 mg/ml.

Scheule and Holian,[7] using chrysotile asbestos, demonstrated an approximate doubling in its ability to stimulate an oxidative burst following opsonization with IgG. These workers used guinea-pig alveolar macrophages and also found that crocidolite asbestos was not substantially enhanced in its ability to stimulate an oxidative burst by IgG opsonization. Therefore, although two different types of the amphibole family of asbestos—crocidolite and amosite—have shown different abilities to be affected by opsonization, the type of species used could be an important modifying factor. Measuring oxidative burst chemiluminescence, Nyberg and Klockars[22] demonstrated results similar to those of Scheule and Holian. It may be anticipated that chrysotile asbestos would show even more activity than either crocidolite or amosite in terms of its ability to be opsonized to stimulate TNF release. This would be related to the relatively low binding of IgG to amphiboles compared with its ability to bind to chrysotile.[23]

Like other workers,[7,22] we attribute the effect of opsonized particles in stimulating the increased release of TNF to stimulation of the protein kinase C system of the macrophages via the macrophage Fc receptors. The decrease in TNF release caused by including the protein kinase C inhibitor staurosporine in the incubation medium supports this contention. Increased cross-linking of the receptors caused by contact with the extended surface of the LFA might explain the observed effect of opsonized long fibres.

Other results from our laboratory, to be published elsewhere, show that LFA causes an increased proliferative response and, in the long term, severe fibrosis in the rat lung. TNF has been suggested to be important in the context of experimental pneumoconiosis in mice and rats.[10,11] Because of its pro-inflammatory effects it is likely to be important in the genesis of the inflammation and fibrosis caused by dusts. However, the ability to stimulate macrophages to release other cytokines, such as platelet-derived growth factor,[24]

is likely to be an important fibre length-related property.

Biological phenomen as complex as fibrosis or cancer, with their multiple cellular interactions, will not be explained by the production of single factor. However, a fibre length-related ability to stimulate the protein kinase C system, causing secretion of important mediators such as macrophage TNF, is an important step in explaining the pathogenic role of fibre length. The activity of asbestos substitutes in this respect will be a vital area of study in relation to a unified theory of fibre pathogenesis.

ACKNOWLEDGEMENT

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FIBRE NUMBER-DEPENDENT SECRETION OF TNF BY RAT BRONCHOALVEOLAR MACROPHAGES

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Abstract

Secretion of tumour necrosis factor by rat bronchoalveolar macrophages has been assessed following exposure to a range of mineral fibres of different length and chemical composition. The samples chosen included refractory ceramic fibres, man-made vitreous fibres, silicon carbides and amosite asbestos. Tumour necrosis factor secretion by bronchoalveolar macrophages exposed to fibres at equal mass, in vitro, was markedly increased by one type of silicon carbide fibre and to a lesser extent by four of the man-made vitreous fibres. When levels of TNF were adjusted for fibre number the man-made vitreous fibres then appeared more active than the silicon carbide, indicating that fibre number is of importance in determining stimulation of TNF secretion. However, differences in activity between the fibre types remained even after TNF levels were adjusted for fibre number, suggesting that other factors such as fibre surface activity are likely to be involved in macrophage stimulation. This is supported by evidence from one of the man-made vitreous fibres which contained more fibres per unit mass than any fibre but was virtually inactive in stimulating TNF secretion. We further demonstrated that TNF secretion *in vitro* was related to ability to stimulate an inflammatory response in the lungs of rats following inhalation exposure.

Introduction

Chronic inhalation exposure of rodents to some man-made mineral fibres induces the development of lung tumours and fibrosis (Davis *et al*, 1984). The pathogenesis of the lung response to these fibres is not fully established but it is likely that their toxicity is related to fibre length. This is supported by evidence from studies which strongly implicated fibre length as an important factor in the development of tumours subsequent to asbestos exposure (Davis *et al*, 1986). The early cellular response in the lung to inhaled mineral fibres is considered to be predictive of their pathogenicity in the long term. Studies with long and short fibre asbestos samples have shown that the long fibres which produce more tumours (Davis *et al*, 1986) are also more inflammogenic (Donaldson *et al*, 1989).

We have previously shown that inhaled fibres deposit at the terminal bronchioles and first alveolar duct bifurcations of the lung where they are phagocytosed by macrophages (Brown *et al*, 1991). A consequence of the phagocytosis of fibres by macrophages may be secretion of cytokines that can contribute to both the development of the initial inflammatory response and to subsequent tissue derangements. The cytokine tumour necrosis factor (TNF) has multiple proinflammatory and cell stimulatory activities and is released by macrophages following exposure to long fibre and, to a much lesser extent, by short fibre asbestos (Brown *et al*, 1991). TNF appears to be a central requirement for the development of pathology following exposure to asbestos (Bissonnette and Rola-Pleszczynski, 1989) and other dusts such as silica (Piguet *et al*, 1990; Driscoll *et al*, 1990).

In the present study, we have investigated the potential toxicity of a number of mineral fibres by measuring secretion of TNF by rat bronchoalveolar macrophages exposed *in vitro*, at equal mass of fibre. We chose fibres of different length and

composition in order to assess whether TNF secretion, and thus predicted pathogenicity, was related solely to fibre length or was influenced by other aspects of the fibres. We also report preliminary data comparing TNF secretion by macrophages following *in vitro* exposure to asbestos and vitreous fibre, with development of lung inflammation following inhalation of these fibres.

Materials and methods

In vitro exposure Macrophages were obtained by bronchoalveolar lavage of untreated, control rats of the Wistar strain. The cells were diluted to 1 x 10^6/ml in Ham's F10 medium containing 0.2% bovine serum albumin (<1ng/ml LPS) and adhered at 1ml/well for 1hr in 24 well plates (Sterilin). The plates were then washed with saline, fibres added at 50μg/well in F10/0.2% BSA, in the presence and absence of 100ng/nl LPS, and incubated 24 hours at 37°C. Supernatants were then harvested and stored at −70oC prior to analysis for TNF content by the bioassay of Flick and Gifford (1987), using the L929 cell line. One unit of TNF was defined as that causing 50% lysis of L929 cells.

In vivo exposure rats were exposed in whole body inhalation chambers to asbestos or glass fibres at 1000 fibres/ml (see Donaldson *et al*, in this volume).

Fibres The refractory ceramic fibres (RCF1,2,3 and 4) and man made vitreous fibres (MMVF10,11,21 and 22) were obtained from the Thermal Insulation Manufacturers Association repository (TIMA). A special purpose vitreous fibre (Code100/475) was obtained from Johns Manville and the two silicon carbide fibres (SiC1 and SiC2) were industrial samples. The long fibre asbestos sample is one that we have used in previous studies (Davis *et al*, 1986; Donaldson *et al*, 1989). Fibre size distributions for each sample were obtained by phase contrast optical microscopy (PCOM). The summarised data is presented as numbers of fibres >5μm/μg for all of the fibres used in the TNF assay (Figure 1).

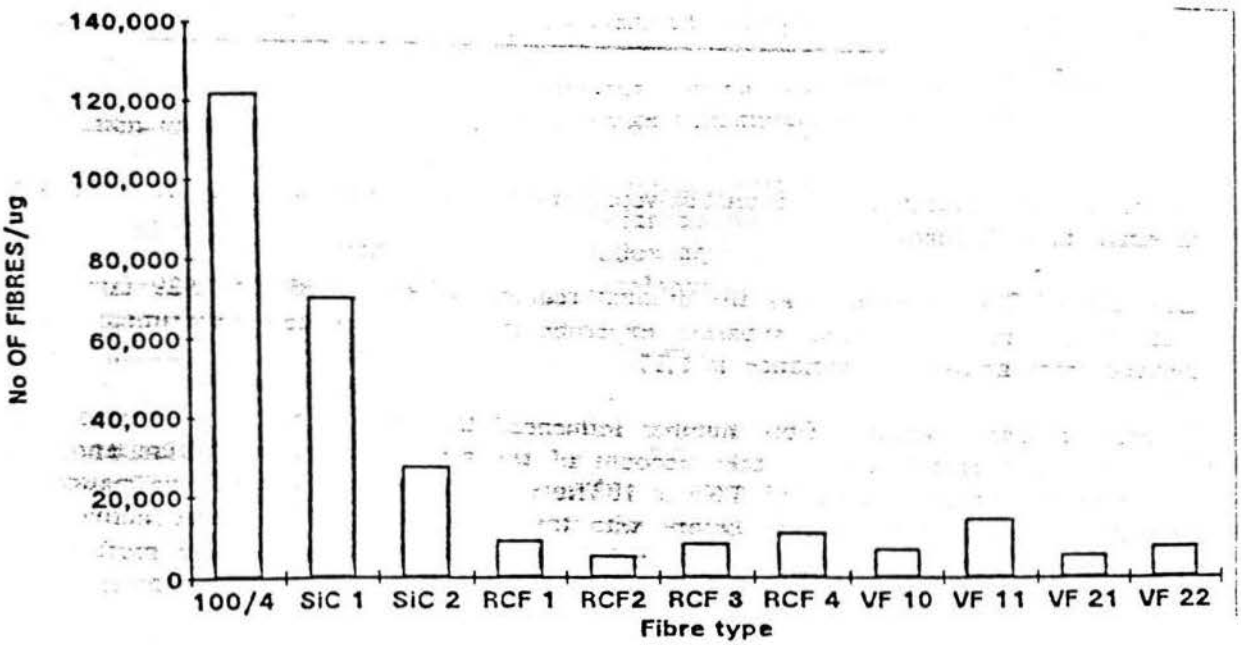


Figure 1 Numbers of fibres >5μm, by PCOM, per μg of the indicated fibres.

Statistical analysis Treatment effects were assessed by analysis of variance of log-transformed data.

Results

Secretion of TNF, shown in Figure 2 as TNF secretion in the presence of 100ng/ml LPS, varied with fibre type. SiC1 was substantially more active than any other fibre while, conversely, SiC2 was of very low activity. Despite the substantial variation in response to the two silicon carbide samples there did appear to be a pattern in the response to some of the other samples. The RCFs in general appeared to be of low activity and the MMVFs more active than the RCFs, code 100/475 or SiC2. However, analysis of variance of the data revealed that the only fibres causing significantly increased release of TNF compared with control cells were MMVF22($p<0.005$) and SiC1($p<0.001$).

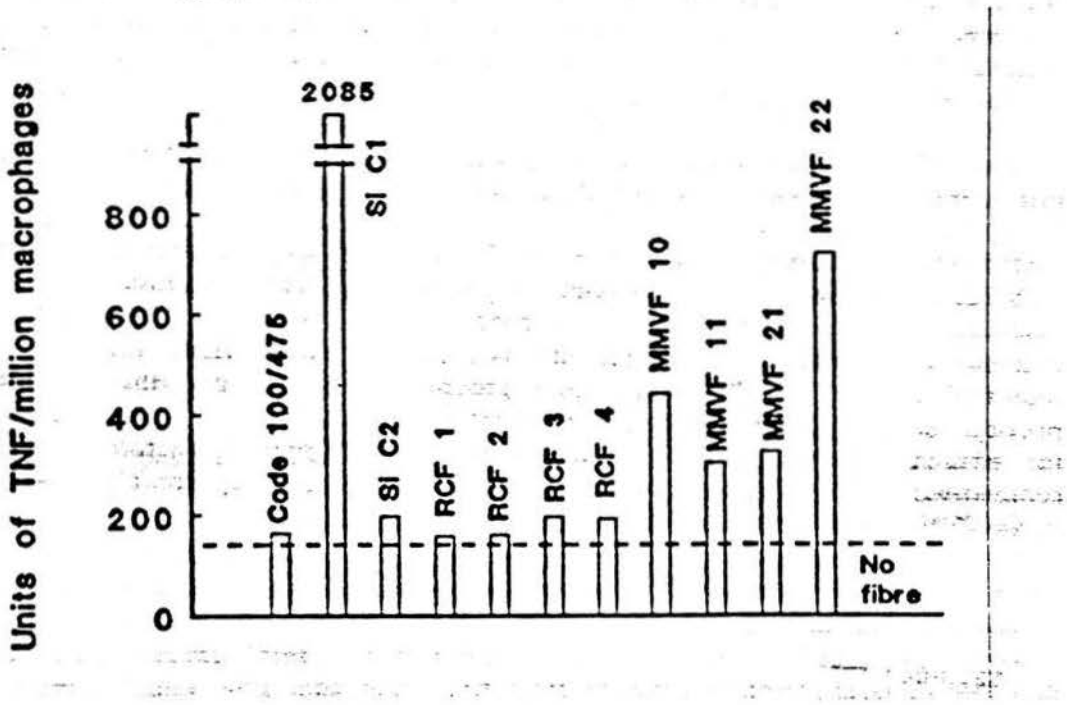


Figure 2 TNF secretion by bronchoalveolar macrophages exposed, *in vitro*, to fibres at equal mass (50μg/ml).

One unit of TNF is defined as the amount required to cause lysis of L929 target cells. Data is the mean of three separate experiments. The overall experimental error, derived from analysis of variance is 1.17.

In order to assess whether fibre number influenced the amount of TNF secreted, the TNF data was recalculated to take account of the number of fibres >5μm and the data then presented as units of TNF x 10^5/fibre (Figure 3). In general the pattern of response remained the same as before with the exception of SiC1 whose activity was reduced to a level similar to that of the MMVFs.

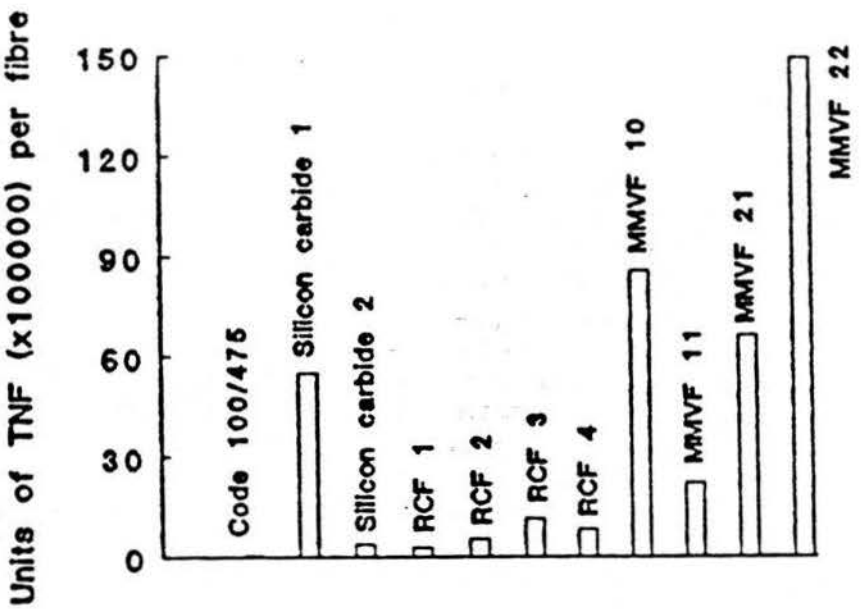


Figure 3 TNF secretion by bronchoalveolar macrophages exposed, *in vitro*, to fibres at equal mass and then adjusted for fibre number. Units of TBF secreted per million macrophages were multiplied by 1×10^5 and then divided by the number of fibres per well. Data are derived from that presented in Figures 1 and 2.

The relationship between ability to stimulate TNF production *in vitro* and inflammogenicity *in vivo* is summarised in Table 1. Full details of the inflammatory response to long amosite and Code 100/475 are presented in Donaldson *et al* (ibid), this volume. The high level of TNF secretion elicited by long amosite corresponded with the induction of a marked inflammatory response following inhalation of the fibres. Code 100/475, which had very low ability to stimulate TNF secretion, induced a very minimal inflammatory response in the lung.

Table 1 Production of TNF by macrophages treated with amosite and Code 100/475, in relation to the ability of the fibres to cause inflammation on inhalation.

	TNF[1] (without LPS)	Bronchoalveolar[2] inflammation following inhalation
Long amosite asbestos	936	+ + +
Code 100/475 vitreous fibre	23	+/−

[1]Data from 3 separate experiments.
[2]Data from 6 rats per group.

Discussion

In this study we have investigated TNF secretion by bronchoalveolar macrophages exposed to various mineral fibres *in vitro*, at equal mass, and have demonstrated differences in the amounts of TNF released. Some of this variation can be accounted for by differences in the number of fibres per unit mass. For example, when TNF secretion by macrophages exposed to SiC1 was assessed without taking account of fibre number, SiC1 appeared to be substantially more active that any other fibre. However, there were many more fibres >5μm per unit mass in the SiC1 sample than in any other, except Code 100/475. When TNF secretion was subsequently calculated as units of TNF per fibre the observed stimulatory activity of SiC1 was markedly reduced. Adjusting for fibre number did little to alter the relative TNF-stimulatory activity of the other fibres and so factors other than numbers of fibres >5μm per unit mass must be considered. Estimation of numbers of fibres >5μm per unit mass is the standard procedure in industrial settings for estimating airborne fibre concentration. However, experimental data indicates that the optimum correlation between fibre length and tumourogenicity is obtained for fibres >8μm (Stanton and Layard, 1981) and so further work is warranted to investigate the relationship between TNF stimulation and number of fibres in other length categories. Surface activity of the fibres is another factor which may influence TNF secretion. This is suggested by the finding that Code 100/475, which contained the greatest number of fibres >5 per unit mass, and hence almost certainly also contained the greatest number of fibres per unit mass in any other length category, did not stimulate TNF secretion.

The relevance of our findings, regarding ability of mineral fibres to stimulate TNF secretion *in vitro* in relation to events occurring in the lung, is shown by the comparison of our *in vivo* and *in vitro* data where we demonstrated a positive relationship between the ability of long amosite asbestos and Code 100/475 glass fibre to induce TNF secretion *in vitro* and to elicit lung inflammation. This data implies that the mineral fibres we have tested here could induce an inflammatory response in the lung and thus have the potential to induce lung injury. However, one caveat remains that this, and other short term assays fail to take account of fibre solubility which is likely to have a major influence on the ability of fibres to exert a continous insult in the lung.

Acknowledgments

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Asbestos fibre length-dependent detachment injury to alveolar epithelial cells *in vitro*: role of a fibronectin-binding receptor

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Summary. A short and a long fibre sample of amosite asbestos were tested for their effects on cells of the human Type 2 alveolar epithelial cell-line A549 *in vitro*. The long amosite sample was found to cause a rapid detachment of the epithelial cells live from their substratum. At the highest dose, on average 28% of the cells present were detached in this way. Studies on the mechanism of the detachment injury showed that it did not involve oxidants since it was not ameliorated by scavengers of active oxygen species. Neither was the effect reduced by treatment of the fibres with the iron chelator Desferal. Treatments reported to increase the interaction between fibres and cells, serum and poly-L-lysine, did not influence the detachment injury, nor did lung lining fluid. Conversely, the fibronectin tripeptide RGD alone could cause detachment which suggested that a fibronectin-binding integrin was involved. This receptor could be reduced in activity by long fibre exposure, leading to detachment. The detaching effect of fibre could be mimicked by the protein kinase C activator PMA, and so the second messenger system of the cell could also be involved. This type of injury could be important in the pathology associated with exposure to long fibres.

Keywords: asbestos, fibre, lungs, epithelium, fibronectin, RGD

Fibre length has been found to be the major descriptor of the ability of industrial fibres of different types to cause lung pathology (Stanton *et al.* 1981; Davis *et al.* 1986). This is not to say that fibre surface chemistry is unimportant since the biological activity of fibres can, without doubt, be modulated by the presence of various substances on their surface both *in vitro* (Scheule & Holian 1989) and *in vivo* (Brown *et al.* 1990). However, for fibre samples that are of similar thinness, such as the short and long amosite samples studied extensively in our laboratory (Davis *et al.* 1986; Donaldson *et al.* 1989), the long fibres have the greatest activity.

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The mechanism whereby long, thin fibres cause lung cancer, fibrosis and mesothelioma is not well understood but much research has been directed towards understanding the role of the alveolar leucocytes in the pathobiological process (reviewed in Rom *et al.* 1991). Less attention has been paid to the epithelial cells that line the airspaces although they are likely to be important players in the pathological process.

Inhaled fibres have been shown to deposit preferentially on the terminal bronchiolar/alveolar duct surface, where they are in close contact with Type 1 and Type 2 alveolar epithelial cells (Warheit *et al.* 1984). The consequences of this contact for the epithelial cell are not known but could be important in the increased epithelial permeability (Gellert *et al.* 1985) and the increased

turnover of epithelial cells (Brody & Overby 1989) detected using a variety of methods in fibre-exposed humans and animals. *In vitro*, epithelial injury (Brown *et al.* 1986) and transformation (Hesterberg & Barrett 1984) have been recorded in epithelial cells exposed to glass and asbestos fibres.

We previously noted that when the two samples of amosite asbestos were added to cells of a human alveolar epithelial cell line (A549) *in vitro*, there was a rapid onset detachment, in the absence of lysis, caused by the long sample and no effect of the short sample. We describe the details of this finding and some studies into the mechanism that underlie epithelial cell adherence and detachment in the presence of fibres.

Materials and methods

Long and short fibre amosite asbestos samples

The size dimensions of these fibres have been detailed elsewhere (Davis *et al.* 1986; Donaldson *et al.* 1989). In essence the short fibre sample has less than 10% of fibres >10 μm whilst the long fibre sample has about 70%. In the >20 μm range the short has virtually none while the long has about 40%. The two samples are of closely similar diameter (Davis *et al.* 1986).

In various experiments the fibres were pre-coated, by incubation for 24 hours at room temperature in the iron chelating agent Desferal (deferoxamine, Sigma, Poole, Dorset, 1 mg/ml) or poly-L-lysine (Sigma, 1 mg/ml). In some experiments, lung lining fluid was obtained by lavaging the bronchoalveolar space of normal Wistar rats with a small volume (3 ml) of saline and discarding the cells to obtain lung lining fluid (LLF). Fibres at a concentration of 1 mg/ml were then incubated in this solution for 1 hour at 37°C on a rotator. In all cases the fibres were then spin-washed and suspended in medium for use in the assay as described below. In all experiments a control aliquot of fibres was incubated in PBS and taken through the same procedure, to control for loss of fibres in the centrifugation and washing steps.

Epithelial cell injury assay

The assay is essentially that described by Donaldson *et al.* (1988) modified so that mineral fibres and not effector cells are added to induce detachment of the target epithelial cells. Cells of the alveolar epithelial cell line A549 (Lieber *et al.* 1976) were plated onto microtitre plates (Greiner, Dursley, Gloucester) at 5×10^4/100 μl of MEM medium + 10% heat-inactivated foetal calf serum (Life Technologies, Paisley, Scotland). The cells were incubated overnight in the presence of 74 kBq of $Na^{51}CrO_4$ (Amersham International, Aylesbury, Buckinghamshire). The monolayers were then washed three times with phosphate-buffered saline (PBS) to remove all except the intra-cellular ^{51}Cr, and fibres were added. The fibres were suspended, with vigorous shaking, in F10 medium (Life Technologies) plus 2% bovine serum albumin (BSA; Sigma) to concentrations ranging from 10–100 μg/ml. Two hundred microlitres of this suspension were dispensed into the wells containing monolayers.

Cells were then incubated with the fibres for 4 hours. At the end of this period the extent of ^{51}Cr released free into the medium (an expression of cell injury) and the amount in detached cells were both determined. Details of the method used to do this are given in Donaldson *et al.* (1988). Briefly, at the end of the incubation period, a centrifugation step is carried out and an aliquot of the supernatant gives an indication of the free ^{51}Cr. The remaining counts include some free and some cell-bound counts both of which can be calculated on the basis of the remaining volume and the free counts calculated from the above.

In some experiments there were modifications to this basic approach. Phorbol myristate acetate (PMA; Sigma) was used in some experiments and was included in the detachment assay at 0.01, 0.1 and 1 μg/ml. PMA was first dissolved in dimethylsulphoxide (DMSO; Sigma) to 1 mg/ml before dissolving in medium. DMSO alone at the final concentration used in the PMA experiments had no effect on detachment. In some experiments the effect of serum on the process of detachment caused by long amosite was tested by increasing the serum concentration up to 25%.

RGD/fibronectin

To assess the role of fibronectin in the attachment/detachment of epithelial cells, the cells were allowed to adhere and label on the normal tissue culture surface modified by pre-coating with 100 μl of 1 μg/ml of fibronectin (Sigma) for 3 days at 37°C to dryness.

These experiments suggested that the epithelial cells bound to the fibronectin. The binding of cells to fibronectin has been ascribed to the presence, on the cells, of a receptor for a 3-amino acid (arginine, aspartic acid, glycine; RGD) sequence on the fibronectin molecule. To determine whether we could cause detachment with the soluble agonist of this receptor, the RGD tripeptide (Sigma) was added to the cells at 50–500 μg/ml for the length of a normal assay in the absence of fibres, and detachment was assessed.

Antioxidants

To study the effect of antioxidants on the detachment caused by long amosite, superoxide dismutase (SOD 50 μg/ml Sigma), catalase (50 μg/ml Sigma) and glutathione (GSH; 200 μM Sigma) were included in assays. Dose responses were carried out for all of these substances and the final doses used represent the highest dose at which there were no toxic effects caused by the antioxidant alone. We have previously shown that detachment of epithelial cells caused by leucocytes could be blocked with the protease inhibitor alpha-1 protease inhibitor (Sigma). This was added to detachment assays in the presence of long amosite, at the concentration found to prevent detachment previously (1 mg/ml).

Statistical analysis

Experiments were repeated at least three times on separate occasions and more where stated. All data, obtained as c.p.m. of ^{51}Cr, were logarithmically transformed to obtain a normal distribution and then analysed by two or three-factor analysis of variance, with 'experiments' always as one factor. This enabled the variance due to between-experiments variability to be taken into account; significant effects of treatment were then identified using variance ratio (F) tests.

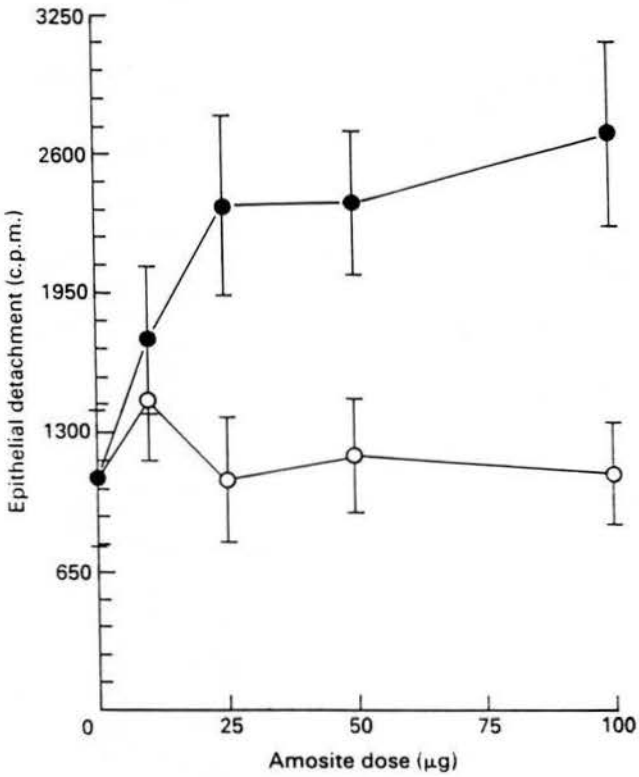


Figure 1. Detachment injury caused by increasing doses of ●, long and ○, short fibre amosite asbestos. Data points represent the mean and the bars denote one standard error in either direction, derived from triplicate measures in seven separate experiments.

Results

Detaching activity of long fibre amosite in the absence of lysis

Figure 1 shows the results obtained from seven separate experiments. The analysis of variance table showed a significant effect of fibre type ($F = 9{\cdot}02$, $P < 0{\cdot}05$) revealing that detachment was different depending on the type of fibre that was used, with the long amosite causing significantly more detachment than short.

To determine if there was an effect of dose a three-factor analysis of variance was used with detachment in c.p.m. as the response variable and fibre type, dose and experiment as the factors. The analysis of variance table revealed a significant interaction between fibre type and fibre dose ($F = 3.72$ $P < 0.05$), showing that the pattern of the dose response was different and depended on the type of fibre. This type of analysis does not provide information on which doses showed significant differences between the two fibre types but the appearance of Figure 1 strongly suggests that the dose–response effect was clearest in the first two dose points, with a plateau thereafter. Some explanation as to the basis of this particular shape of dose–response curve is offered in the discussion.

Figure 2 shows the data obtained for lysis for the same seven experiments as are shown in Figure 1. The analysis of variance table showed, as is suggested by the Figure, no effect of fibre type ($F = 1.04$, $P > 0.25$) or dose ($F = 1.62$, $P > 0.1$). Thus although long amosite caused significantly more detachment than short amosite, it did not cause any excess lysis compared to the short amosite.

Studies on the mechanism of detachment caused by long amosite

The ability of long fibres of amosite to cause detachment was not significantly affected by pretreatment of the long amosite fibres with the iron-chelating agent Desferal ($F = 0.002$, $P > 0.25$) (Figure 3).

Poly-L-lysine has been used to enhance the interaction between amosite fibres and cells. However, treatment of the long and short amosite fibres with poly-L-lysine was

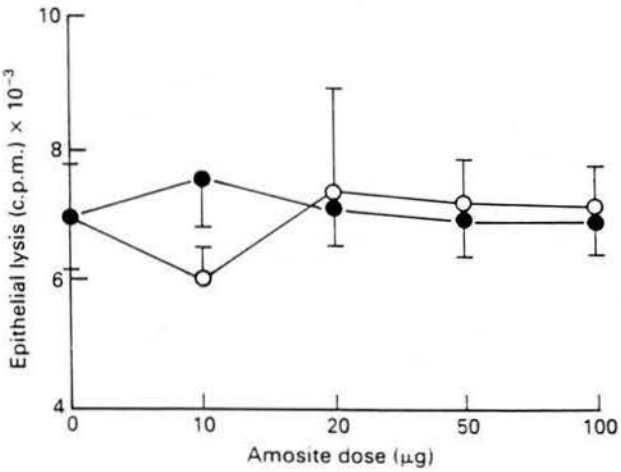


Figure 2. Data on free ^{51}Cr, a measure of lytic cell injury, from the same seven experiments as those in Figure 1; data are derived as described in the legend to Figure 1. ●, Long; ○, short fibre amosite.

not a significant factor ($F = 1.46$, $P > 0.25$) in enhancing the detaching potential of the long amosite or in causing short amosite to become able to cause detachment (Figure 4).

In previous studies the concentration of serum has been found to be a key factor in the ability of amosite to cause injury to epithelial cells. As shown in Figure 5,

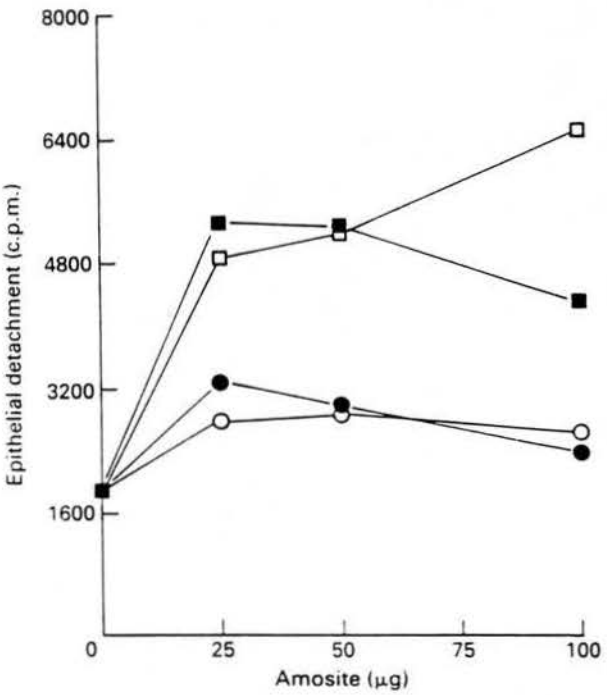


Figure 4. Effect of preincubation of long or short fibre amosite in poly-L-lysine (PLL) on ability to cause detachment of epithelial cells. Data are derived as the mean of triplicate measures in four separate experiments. Standard errors, which were less than 12% of the mean, are omitted for clarity. □, Long; ■, long + PLL; ○, short; ●, short + PLL.

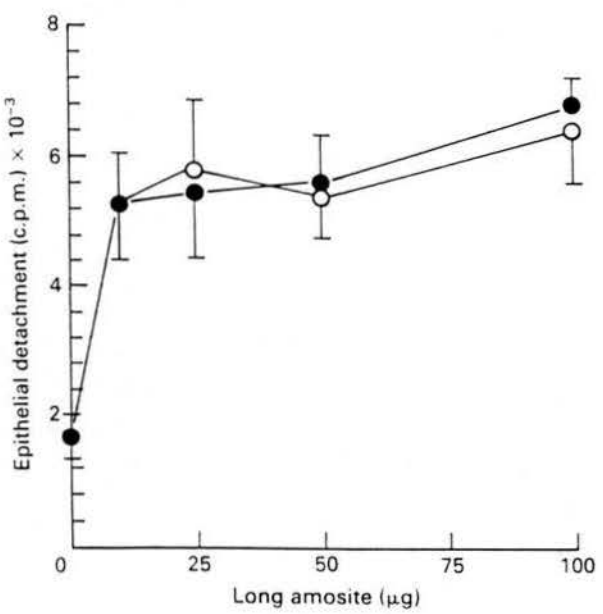


Figure 3. Effect of pretreatment of long fibre amosite with the iron chelator Desferal, on its ability to cause epithelial detachment. Data are the mean and s.e.m. from triplicate measures in three separate experiments. ●, Desferal; ○, no Desferal.

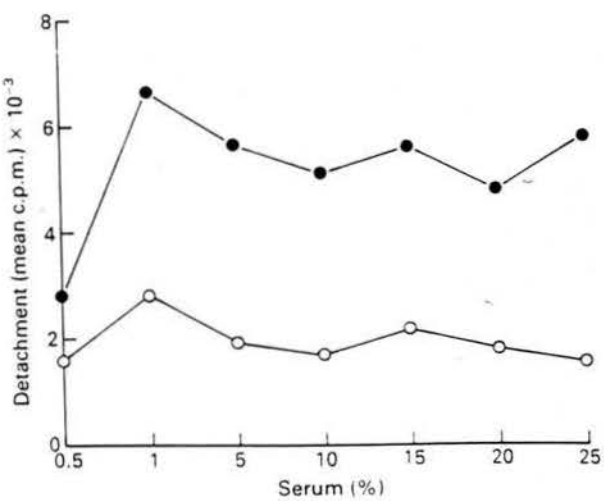


Figure 5. Epithelial cell detachment caused by 100 μg of long fibre amosite (LFA) in varying concentrations of serum. Results are the mean of three triplicate wells in three separate experiments. Standard errors, which were less than 12% of the mean, are omitted for clarity. ●, LFA; ○, no LFA.

however, the detaching effect of long amosite was maintained throughout a wide variation in serum concentration and the effect of serum did not attain statistical significance ($F = 0.60$, $P > 0.25$).

To test the effect of an anti-oxidant on the ability of long amosite to cause detachment we included glutathione, superoxide dismutase and catalase along with long amosite in the detachment assay. The effect of these treatments was that SOD caused reductions in detachment in 2/9 experiments and catalase had this effect in 2/9 experiments, although these reductions were recorded in 4 different experiments. GSH caused reductions in 6/9 experiments and when this was examined by analysis of variance for an effect of GSH treatment, the effect just failed to attain statistical significance ($F = 4.48$, $P < 0.1 > 0.05$). The anti-protease alpha-1-protease inhibitor did not cause reductions in the detachment caused by long amosite in any experiments.

Role of fibronectin and a fibronectin receptor in attachment of epithelial cells in vitro

Because of previous reports of a role for fibronectin in the interaction between amosite and cells *in vitro* (Brown *et al.* 1991), we investigated the effect of the presence of the fibronectin tripeptide RGD in the assay. We found that RGD alone, without long amosite, showed a significant effect ($F = 54.93$, $P < 0.05$; Table 1) in causing the epithelial cells to detach from the surface of the culture plate. We presume that this resulted from competition between soluble RGD and RGD sequences on surface-bound fibronectin, for an epithelial cell membrane RGD receptor. The role of fibronectin in attachment was further supported by the finding that culture of the cells on a fibronectin matrix inhibited both spontaneous and long amosite mediated detachment (Figure 6). The effect of fibronectin was significant ($F = 199.04$, $P < 0.01$) but there was no significant interaction between fibre and fibronectin showing that the pattern of response to the fibres (detachment caused by the long fibres and no effect of the short fibres) was the same whether fibronectin was absent or present.

We also studied the effect of the protein kinase C activator PMA on detachment, demonstrating a significant dose effect ($F = 5.22$, $P < 0.05$, Figure 7). The slight decrease in detachment at 1.0 compared to 0.1 μg of PMA was a consistent finding in all of the five experiments that were used in the analysis.

Table 1. Detachment injury to epithelial cells caused by the fibronectin tripeptide RGD

	Detachment (c.p.m.)
Untreated	1751 (343)
RGD-treated*	5214 (746)

Data derived as the mean (s.e.m.) of three replicates in three separate experiments.

* RGD at 500 μg/ml.

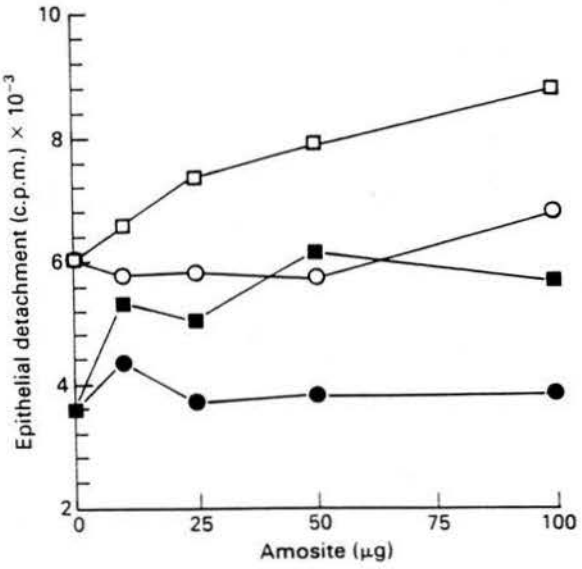


Figure 6. Detachment injury caused by long or short fibre amosite to epithelial cells cultured on plastic or fibronectin (+Fn). Results are the mean of triplicate wells in three separate experiments. Standard errors, which were less than 10% of the mean, are omitted for clarity. □, Long; ■, long + Fn; ○, short; ●, short + Fn.

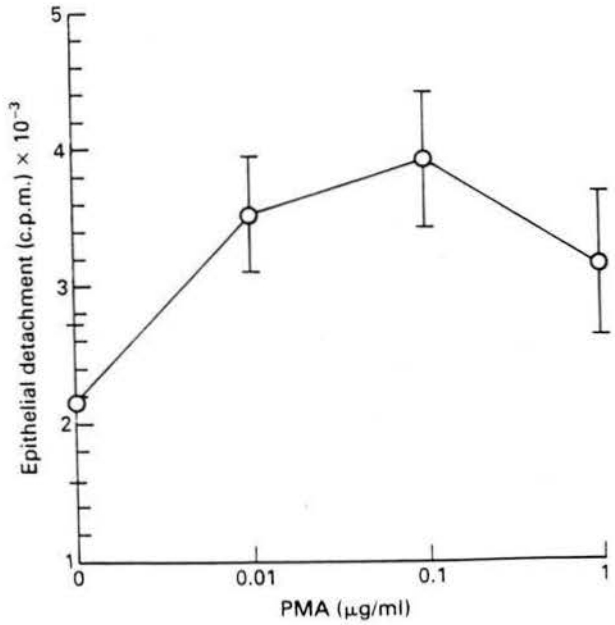


Figure 7. Detachment injury caused to epithelial cells by phorbol myristate acetate (PMA). Results are the mean and s.e.m. of triplicate wells in five separate experiments.

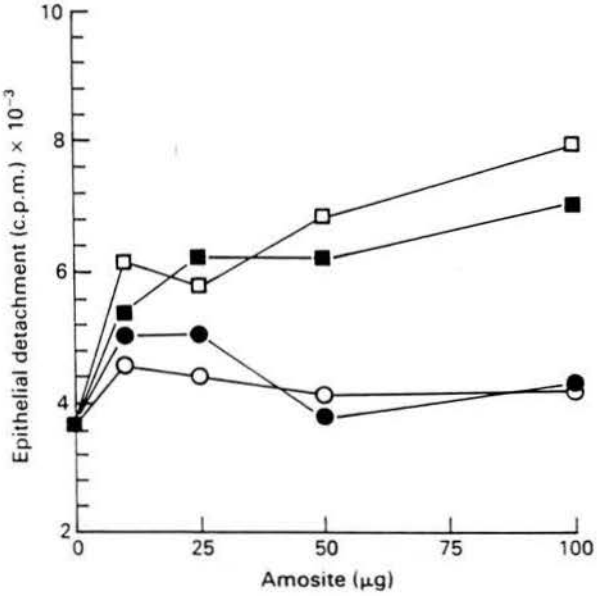


Figure 8. Effect of preincubation in rat lung-lining fluid (LLF) on the ability of long or short fibre amosite to cause detachment injury. Results are the mean of triplicate wells in three separate experiments. Standard errors, which were less than 12% of the mean, are omitted for clarity. □, Long; ■, long + LLF; ○, short; ●, short + LLF.

Depositing fibre is likely to make contact with lung lining fluid and so we tested the effect of pretreatment in LLF on the ability of long fibres to cause detachment. LLF was not a significant effect ($F = 0.11$, $P > 0.25$; Figure 8) demonstrating that the LLF does not have the ability to modify the detaching activity of the long fibres.

Discussion

Particles of many different types, including quartz (Brody *et al.* 1982), chrysotile asbestos (Warheit *et al.* 1984), and mixed dust (Osornio-Vargas *et al.* 1991) deposit in the rat lung parenchyma preferentially at the terminal bronchiolar/alveolar duct region. When they deposit in this position and in higher airways, these particles are in close contact with the epithelial cells for a variable length of time. There has been some consideration of the likely outcomes of the interactions between fibres and epithelial cells. Brody and co-workers (1983) have demonstrated, following a brief inhalation exposure to chrysotile asbestos, that fibres are found inside the epithelial cells in contact with contractile microfilaments. They have suggested that this results in the translocation of the fibres to the interstitium. Adamson and co-workers have shown that instillation of a short fibre sample of crocidolite causes little effect on the bronchiolar/alveolar epithelium (Adamson & Bowden 1987a) whilst long fibres caused epithelial injury (Adamson & Bowden 1987b). Epithelial death and detachment with exposure of the basement membrane were often seen in association with inflammatory cells, making it difficult to separate the directly injurious effects of the fibre from those effects inflicted on the epithelium by the inflammatory cells (Donaldson *et al.* 1988). Moalli *et al.* (1987) have described areas of mesothelial cell detachment, loss of junctions and formation of blebs at 3–6 hours after intraperitoneal instillation only when fibre preparations contained long fibres. Although the authors implicate the inflammatory response itself in the persistence of inflammation and mesothelial injury this is unlikely to be a factor as early as 3–6 hours, and so the fibres themselves are likely to be involved in this early loss of the integrity of the mesothelial barrier.

Increased proliferative activity of the epithelium has been reported as a response to fibres (McGavran & Brody 1989; Adamson *et al.* 1988). This may represent a reparative response to the epithelial injury caused by the fibres although the role of macrophage-derived growth factors must also be considered. In the mouse peritoneal cavity, increased proliferation to replace mesothelial cells injured by long fibres has also been reported (Moalli *et al.* 1987).

In vitro, airway epithelial cells have been shown to take up fibres (Mossman *et al.* 1977) which may be mediated in part by active oxygen species (Hobson *et al.* 1990). The injurious effects of asbestos fibres can be reduced by the addition of scanvengers of active oxidant species (Shatos *et al.* 1987; Mossman & Marsh. 1989; Goodlick & Kane 1986). The toxic effects of fibres on epithelial cells show a spectrum that ranges from genetic effects (Hesterberg & Barrett 1984) to cell fusion (Brown *et al.* 1986) to frank lytic toxicity (Woodworth *et al.* 1982).

In the present paper we describe how long fibres of amosite asbestos can produce a rapid onset, subtle type of injury in cells of a human alveolar epithelial line *in vitro*. The injury is characterized by the detachment of the cells live from their substratum within 4 hours of exposure and by the fact that the detachment is caused by long fibres and not short fibres of the same diameter; we have never detected ^{51}Cr release accompanying the detachment. Although more subtle an injury than lysis, the effects of loss of alveolar epithelial cell integrity could be highly injurious to the lung.

In a companion paper (in preparation) we show that a large range of fibres of different types (glass fibres of various compositions, silicon carbide fibres and ceramic fibres) can all cause this type of injury to varying degrees.

We believe that this detachment injury is fundamentally different from 'classic' asbestos-mediated lytic toxicity because it does not involve release of ^{51}Cr and is not ameliorated by treatment of the amosite fibres with iron chelating agent or by the presence of antioxidants during cell/fibre interaction. In the detachment caused by long fibres, antioxidants had no effect. The thiol GSH had more effect than SOD or catalase, but still did not affect the detachment injury to a statistically significant degree.

Iron on the surface of fibres has been shown to be involved in the toxicity of some forms of asbestos, probably through the generation of active oxygen compounds. We demonstrated here that treatment with the iron chelator Desferal, which attenuates the toxic effects of crocidolite in killing alveolar macrophages (Goodlick & Kane 1990) and epithelial cells (Kamp *et al.* 1990), did not affect the detachment injury caused by the long amosite.

Two treatments reported to enhance the interaction between cells and asbestos, poly-L-lysine (Brown *et al.* 1991) and increased serum content of the incubation medium (Kamp *et al.* 1990), failed to increase the detachment injury. A sample of lung lining fluid obtained from normal lung by lavage failed to influence the detachment caused by long amosite, suggesting that *in vivo* the ability of long fibres to cause this type of injury would not be influenced by the local milieu. However, there was some dilution of the lung lining fluid (by up to 6 times) during harvesting, and this could have influenced the findings.

The biphasic shape of the detachment dose–response curve is worthy of comment, showing a rapid take-off to 10 μg/ml and then a slow climb thereafter. This could be due to the nature of the interaction between long fibres and the cell surface. Even a low dose of amosite fibres, e.g. 10 μg/ml, means that well over 1.5 million fibres are added to the 10^4 cells in each well. Therefore each cell could be exposed to at least a hundred fibres. With fibres of the length of the long fibre sample this means that the cells will be covered with long fibres that could physically prevent the access of other fibres. This would be expected to upset the normal dose–response curve. It could also be that there is a sub-population of cells that are very detachable and it is these that come off first even with a low dose. More work is necessary to clarify these matters.

The fibronectin tripeptide RGD has been implicated previously in the interactions between asbestos and cells (Brown *et al.* 1991). Treatment of the epithelial cells with the RGD alone was sufficient to cause detachment so implicating a receptor for the RGD portion of fibronectin in the attachment of the epithelial cells; this receptor could be a target for modulation during detachment injury. Attachment via this receptor has been demonstrated for bronchial epithelial cells (Rickard *et al.* 1991) and other lung epithelial cells (Albelda 1991). When long and short amosite were added to epithelial cells grown on fibronectin, there was a general reduction in the extent of detachment, both spontaneous and long fibre-mediated. This would be anticipated if the cells were indeed utilizing the fibronectin as an attachment factor via a fibronectin-binding integrin (Rickard *et al.* 1991). Based on the assumption that the receptor could be modulable by the protein kinase C system we used PMA which directly activates protein kinase C. PMA caused a dose-dependent detachment injury at as low as 0.1 μg/ml although for reasons as yet unclear, at higher doses there was less detachment. It seemed possible that there could be quite dramatic effects on cells before they got to the point of detaching so we assessed the size of cells on the assumption that the cells would round up, becoming smaller, before they actually detached. Size data (not shown) revealed that there was a dramatic reduction in size in the PMA-treated cells. No experiments were carried out on the size of epithelial cells after fibre treatment but it can be assumed that there is a size reduction in them also, prior to detachment. Size of epithelial cells may represent a very sensitive indicator of cell injury which could have application in the future.

Whilst detachment injury was shown *in vivo* in the present study it is quite likely that *in vivo* the modulatory effect of the long fibres may be less extreme and may be manifest as a more subtle effect. This is suggested by the decreased detachment caused to cells on fibronectin. If long fibres can cause decreased attachment of epithelial attachment *in vivo* then this could be important in increased interstitial transfer of fibres, increased access of harmful alveolar macrophage products to the interstitium, and altered interactions between interstitial mesenchymal cells and epithelial cells, which could be important in fibrosis (Adamson *et al.* 1990; Brody *et al.* 1981).

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LFA-1 and ICAM-1 in Homotypic Aggregation of Rat Alveolar Macrophages: Organic Dust–mediated Aggregation by a Non–Protein Kinase C–dependent Pathway

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We have examined the role of cell adhesion molecules in the homotypic aggregation of rat alveolar macrophages after exposure to wool and grain dusts. Molecules such as bacterial lipopolysaccharide (LPS) and phorbol myristate acetate (PMA) can upregulate adhesion molecules, resulting in aggregation of lymphocytes. In rats treated intratracheally with an inspirable sample of wool dust collected from the air of British wool textile mills, and sieved grain dust, aggregates of macrophages were present in the bronchoalveolar lavage (BAL). Our hypothesis was that substances present on the dust surface could activate and upregulate adhesion molecules of the CD11/CD18 complex on the BAL cells and account for the aggregates. Macrophages from untreated rats form aggregates *in vitro*, which averaged 19 cells/aggregate; when treated with both wool and grain dusts, this rose to 25 and 24 cells/aggregate, respectively. LPS, PMA, and the proinflammatory cytokine tumor necrosis factor (TNF) also caused increases in aggregate size. Staurosporine, an inhibitor of protein kinase C (PKC), reduced the number of cells per aggregate from 35 cells/aggregate in LPS- and PMA-treated macrophages to 18 cells/aggregate, the same as untreated. In contrast, staurosporine had no effect in reducing the size of aggregates produced by the organic dusts. Removal of divalent cations, which are essential for maintaining integrin stability and PKC activity, resulted in complete abolition of aggregate formation. Treatment with monoclonal antibodies to lymphocyte function-associated antigen-1 (LFA-1) α and β and intercellular adhesion molecule-1 (ICAM-1) resulted in the inhibition of aggregate formation in a dose-dependent manner. Our results strongly suggest that aggregate formation in response to the two organic dusts involves upregulation of adhesion molecules which is not PKC dependent. The consequence of these cell interactions in the lungs of workers exposed to dust in wool textile mills may be important in leading to inflammation.

Cell adhesion is an important event that occurs between cells and other cells, and between cells and the extracellular matrix. For leukocytes, the integrins of the CD11/CD18 complex mediate many of these interactions (1). The three members of the CD11/CD18 family, also referred to as lymphocyte function-associated antigen-1 (LFA-1), complement receptor type 3 (CR3), and p150,95, share structural features of integrins (1–3). They are glycoproteins composed of a distinct alpha subunit (CD11a, CD11b, or CD11c) noncovalently associated with a common beta subunit (CD18) to form a heterodimeric complex that spans the cell membrane. Divalent cations (Ca^{2+} and Mg^{2+}) have roles both in the stabilization of the complex (4, 5) and in binding ligand or counter-receptors (6). One of the key counter-receptors for LFA-1 is intercellular adhesion molecule-1 (ICAM-1 or CD54), which is expressed on a variety of leukocytes, endothelial cells, and epithelial cells in response to molecules such as bacterial lipopolysaccharide (LPS) or inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (7). Many ligands have been identified for CR3, including iC3b, a fragment produced when C3 is cleaved during complement activation, fibrinogen, and also ICAM-1. Also binding iC3b and fibrinogen is p150,95, which shows great structural homology with CR3. However, a ligand present on endothelium distinct from ICAM-1 has also been demonstrated (1).

CD11a/CD18 is present on all leukocytes, whereas CD11b/CD18 and CD11c/CD18 are found mainly on monocytes, macrophages, and polymorphonuclear neutrophils (PMN).

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Abbreviations: bronchoalveolar lavage, BAL; bovine serum albumin, BSA; complement receptor type 3, CR3; intercellular adhesion molecule-1, ICAM-1; lipopolysaccharide binding protein, LBP; lymphocyte function-associated antigen-1, LFA-1; lipopolysaccharide, LPS; normal goat serum, NGS; phosphate-buffered saline, PBS; protein kinase C, PKC; phorbol myristate acetate, PMA; polymorphonuclear neutrophils, PMN; Tris-buffered saline, TBS; tumor necrosis factor, TNF.

Integrin activation is triggered from within the cell, possibly as a result of signaling via other cell surface receptors, providing a stimulus-dependent adhesion-strengthening mechanism. For monocytes/macrophages, a key signaling molecule may be CD14, the receptor for LPS and lipopolysaccharide binding protein (LPS/LBP) (8, 9). LPS from the cell walls of gram-negative bacteria is bound by LBP, which is normally secreted in nanogram quantities by the liver. In infection, secretion of acute-phase proteins is rapidly increased and LBP concentration rises to microgram quantities. Binding of this complex to CD14 can cause homotypic monocyte aggregation, resulting from activation of surface integrin complexes, mainly mediated by LFA-1/ICAM-1 (8, 9). Occupation of the CD14 receptor results in intracellular signaling through a protein kinase C (PKC)-dependent pathway (8), which in turn is responsible for integrin activation.

Previous epidemiologic studies by the Institute of Occupational Medicine have shown that symptoms of bronchitis in workers in wool textile mills in the north of England were related to airborne mass concentration of wool dust to which workers were exposed (10). Dust collected from the air of the wool mills caused lung inflammation and aggregation of bronchoalveolar lavage (BAL) cells in a rat model following intratracheal instillation (11).

We hypothesized that cellular aggregates we have previously observed in the lavage (11) could represent manifestations of antigen processing, possibly indicative of the initiation of an immune response. We therefore set out to characterize the cells in bronchoalveolar cell aggregates from rats treated with wool dusts. We also examined the factors involved in the formation of the aggregates *in vitro*, using wool dust and sieved grain dust. The cytokine TNF, which we have demonstrated to be released from alveolar macrophages cultured with wool and grain dusts *in vitro* (manuscript in preparation), was investigated for the ability to cause aggregation *in vitro*. Monoclonal antibodies to LFA-1 and ICAM-1 were utilized to investigate the role of these molecules in aggregation. The phorbol ester phorbol myristate acetate (PMA) and endotoxin (LPS) were included because these substances can upregulate adhesion molecules on the leukocyte membrane and so contribute to aggregation formation in macrophages (9, 12).

Materials and Methods

Animals

Male SPF HAN rats (Charles River UK Ltd., Margate, Kent, UK) were used throughout.

Collection of Wool Dusts

Dusts were collected from two wool textile mills in the north of England designated S (start) and M (middle), which represented opening/blending and combing processes, respectively. A series of six IOM static inspirable dust samplers (13) were placed at each site in the dustiest zones. Samplers were operated for a full work shift and the dust collected on Gelman GLA filters with a 5-μm pore size (Gelman Hawksley, Northampton, UK). Dust was removed from filters with a soft brush. Dust from each mill was pooled into a tube, weighed, and mechanically rotated for 24 h to ensure mixing, and these samples were stored at −20°C until required.

Collection of Grain Dust

Samples of dust were collected from the ledges of a barn that stored wheat and barley. The dust was sieved through a 200-μm mesh, followed by a 45-μm mesh, by shaking mechanically for 30 min. The dust so obtained was < 45 μm in diameter, and this was used in all subsequent assays.

Intratracheal Instillation of Wool and Grain Dust

Groups of three animals were anesthetized with an intraperitoneal injection of 0.1 ml Valium (Roche, Welwyn Garden City, UK) at a concentration of 5 mg/ml, followed by an intramuscular injection of 0.05 ml Hypnorm (Jansen Pharmaceuticals Ltd., Grove, Oxford, UK) at a concentration of 10 mg/ml fluanisone and 0.315 mg/ml fentanyl citrate. An incision was made to expose the trachea, and 0.5 ml of wool dust suspension contained in phosphate-buffered saline (PBS) (GIBCO, Paisley, UK) at a concentration of 2 mg/ml was instilled via a blunted needle. The incision was closed using surgical clips. The animals were further injected intramuscularly with 0.2 ml of naloxone (Sigma, Poole Dorset, UK) contained in PBS at a concentration of 0.3 mg/ml, to reverse the effects of anesthesia. Animals were conscious within a few minutes and showed no ill effects.

Bronchoalveolar Lavage

At selected time points, animals were killed with a single intraperitoneal injection of nembutal, the thoracic cavity was opened, and the lungs were cannulated and removed. The lungs were then sequentially lavaged with four 8-ml aliquots of saline at 37°C and pooled into a single tube. Cells were spun at 1,000 rpm in a refrigerated centrifuge for 10 min, and resuspended in F-10 medium (GIBCO) containing a 2% bovine serum albumin (BSA) (Sigma) and kept on ice. Total cell numbers were evaluated and cytocentrifuge smears prepared that were stained with May-Grunwald Giemsa. Preparations for immunostaining were fixed in acetone for 10 min, and stored at −20°C until required. Grain-treated BAL was not assessed for aggregate characterization due to a delay in receiving the dust.

Monoclonal Antibody to LFA-1 α and β and ICAM-1

Hybridomas were prepared and kindly supplied by Dr. Takuya Tamatani of the Tokyo Metropolitan Institute of Medical Science, Japan. Hybridomas were raised against rat spleen cell phytohemagglutinin blasts or a rat thymic lymphoma cell line and screened for the ability to block aggregation of concanavalin A blasts. Specificity for ICAM-1 and LFA-1 was confirmed extensively, as described by Tamatani and associates (14). The hybridomas were cultured in RPMI-1640 containing 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (complete medium). Cells were cultured in 75-cm^2 flasks until the color of the medium became yellow (about 7 days), at which point the medium was collected, spun to remove cells, and stored at −70°C until required.

Immunostaining

Characterization of the cell aggregates from BAL of rats exposed to wool dust was carried out using mouse anti-rat

monoclonal antibodies directed against surface markers on macrophages MCA 342 (clone ED2) and T lymphocytes (Pan-T) MCA 52 (clone MRC OX-19) (Serotec, Oxford, UK). Normal goat serum (NGS) was used as a blocking agent to prevent nonspecific staining followed by the appropriate monoclonal antibody. The monoclonal antibodies were further diluted in NGS to give optimal staining. All slides were then washed thoroughly in Tris-buffered saline (TBS) for 10 min with two changes of solution. Subsequent steps involved the use of a commercially available staining kit (ICN Immunochemicals, High Wycombe, Bucks, UK), using alkaline phosphatase for the macrophage/T-lymphocyte characterization or using peroxidase for the anti-integrin binding (Vector Labs, Peterborough, UK), which visualized the bound antibody. When the desired degree of staining was achieved, slides were washed in TBS and counterstained in Harris hematoxylin for 30 s, rinsed in tap water, and mounted.

In addition to the test slides, controls consisting of lymph node and one set omitting the primary antibody were included. Cells were evaluated by counting 10 separate aggregates from each of the test conditions in triplicate. Within each aggregate, numbers of positive, negative, and PMN cells were noted, as was the total number of cells making up each aggregate.

Preparation of Wool and Grain Leachates

Samples of leachate were prepared by mixing ledge wool dust (obtained from wool mills by sweeping ledges and surfaces) and sieved grain dust in PBS at a concentration of 5 mg/ml at room temperature for 24 h. Solutions were then spun at 3,000 rpm for 15 min to remove large fragments and then finally filtered through 0.22-μm filters to sterilize the leachates. Half of each sample was then depleted of endotoxin by passing down polymyxin B columns (Flow, High Wycombe, Bucks, UK) according to the manufacturer's instructions. Endotoxin from *Escherichia coli* serotype 011:B4 (Sigma) was made up to 1 mg/ml in PBS, stored at −70°C, diluted 1:100, and sonicated before use in the aggregation assay.

Measurement of the Endotoxin Content of Wool Leachates

The "Coatest" (ICN) limulus amoebocyte lysis-based spectrophotometric assay was used to assess the endotoxin content of the wool/grain leachates. Values were as follows: (figures as pre- and post-polymyxin treatment, respectively, in ng/ml) Wool S, 21.99; 0.14. Wool M, 19.76; 0.16. Grain, 14.99; 0.14. LPS, 22.45; 0.13.

In Vitro Aggregation of Macrophages by Wool and Grain Dusts, Leachates, and TNF

Rat alveolar macrophages were suspended in each wool leachate, depleted wool leachate, macrophage supernatant, TNF, PMA, LPS, and fibronectin at a concentration of 2.5×10^5 cells in 200 μl of solution. Three different assay systems were compared, all of which yielded similar size and number of aggregates. Cells were incubated at 37°C for 24 h, in teflon-coated wells, 24-well plates, or Tissue-Tek slides (Miles Laboratories, Naperville, IL), and the nonadherent aggregates collected and cytocentrifuge preparations made, which were then stained with Diff-Quik. Adhered aggregates of cells were also assessed and produced results comparable to aggregates in the cell suspensions (medium: 19.7 versus 19.1 cells/aggregate; medium + PMA: 29.3 versus 28.5 cells/aggregate in adhered and suspension, respectively). The cytokine TNF was diluted to 100 U/ml in F-10 + 2% BSA; PMA and LPS were diluted to 10 μg/ml and fibronectin to 1 μg/ml in F-10 + 2% BSA. Cells were also suspended in wool and grain dusts at a concentration of 100 μg/ml.

The number of cells present in each of 10 randomly selected aggregates per cytospin was counted. An aggregate was defined as a cluster of 10 or more contiguous cells that remained after gentle pipetting and cytospin preparation. With reference to Figure 2, there are 11 aggregates that are clearly distinguishable from groups of cells that are merely single cells that are touching. The reproducibility of the counting procedure was verified with different observers counting the same slide, and with one observer reading the same slide twice; results obtained were very closely similar in each case.

Staurosporine (Sigma) was included with PMA, LPS, and whole fractions of wool and grain dusts, as described above, at a concentration of 2 μM. In some experiments, assay medium was depleted of calcium and magnesium ions, using potassium EDTA at a concentration of 2.5 mg/ml.

Effect of Anti-LFA-1 α and β and Anti-ICAM-1 on Aggregate Formation

Alveolar macrophages were cultured in 24-well plates (as above) with either PMA or LPS at the concentrations used previously. Cells/treatments were resuspended either in the supernatants from each of the hybridomas, or complete medium that served as a control. Since the hybridoma supernatant must necessarily contain the metabolites of the hybridoma cells, we wished to control for any effect that these might have on aggregate formation. We therefore prepared a supernatant from rat lymphocytes cultured for the same duration as the hybridoma cells; this is referred to as a "lymphocyte supernatant control." An irrelevant antibody Pan-T (Serotec) MRC OX-19 was included at the same dilutions as the hybridoma supernatants.

Statistics

Experiments were assessed using two- or three-factor ANOVA (General Linear Model; Minitab) where experiment was always a factor. This allowed between-experiments variation to be taken out and the effect of treatment to be assessed by variance ratio (F) test. Data for repeat experiments were logarithmically transformed, when necessary, to comply with a normal distribution. All ANOVAs were carried out in the normally distributed data. The data presented in the figures are untransformed. When there was a positive F test, showing a significant global treatment effect, individual means were compared using *t* tests with a pooled estimate of standard error, as required.

Results

Aggregate Formation

From days 1 to 7, there was a steady increase in the number of aggregates in the BAL, reaching a peak at around 7 days

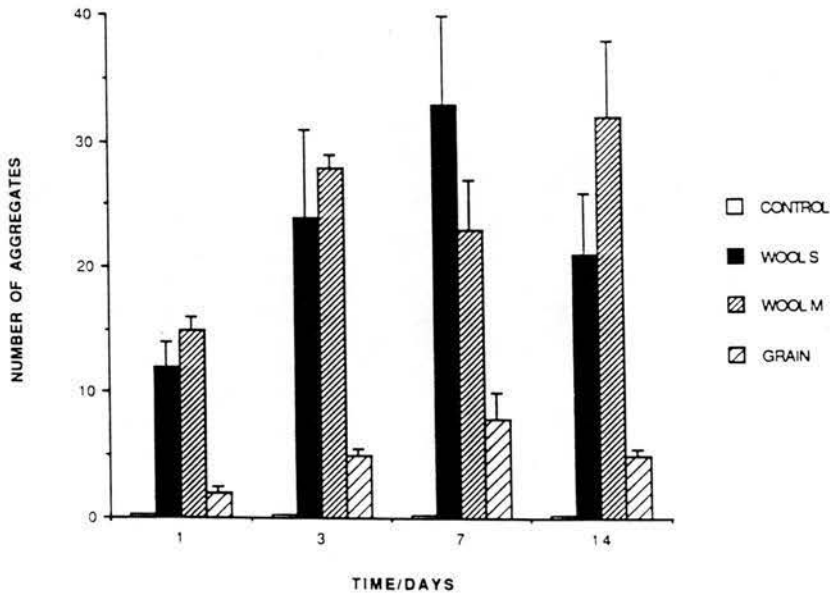


Figure 1. The number of aggregates per cytospin from rats treated intratracheally with 1 mg wool dust S and M 1, 3, 7, and 14 days after exposure. Results are the mean + SEM from triplicate groups of animals at each time point.

(Figure 1). This profile was the same for both samples of wool dust. Grain-treated animals showed a similar, although reduced, number of aggregates in BAL. In saline (unstimulated) animals, there were no aggregates in the BAL at any time point. All BAL samples from grain- or wool-treated animals showed a high percentage of PMN at day 1, which were substantially reduced by day 3 and were negligible by day 7.

Aggregates of cells lavaged from the lungs of rats treated with wool dust are shown in Figure 2.

Characterization of Cells within Aggregates

The numbers of macrophages and lymphocytes per aggregate in wool dust–treated animals were identified using monoclonal antibodies and subsequent immunostaining (Figure 3). In the day 1 exposed animals, aggregates contained PMN that were recognized by the multinucleated morphology in the stained cytospins and were not assessed.

Aggregates were made up almost exclusively of macrophages (Figure 3), which, like the total number of aggregates per cytospin, reached a peak at the day 7 time point. As in the case of the total number of aggregates per cytospin, at day 14, wool dust S–treated animals showed a slight decline in the number of macrophages per aggregate. There were almost no cells staining with the lymphocyte marker, with an average of less than one per aggregate (Figure 3). Aggregates from grain-treated animals were not characterized.

Aggregation of Alveolar Macrophages *In Vitro*

Modulation of aggregate formation. In order to further characterize the aggregation phenomena, we developed an assay for *in vitro* assessment of the effects of various agents.

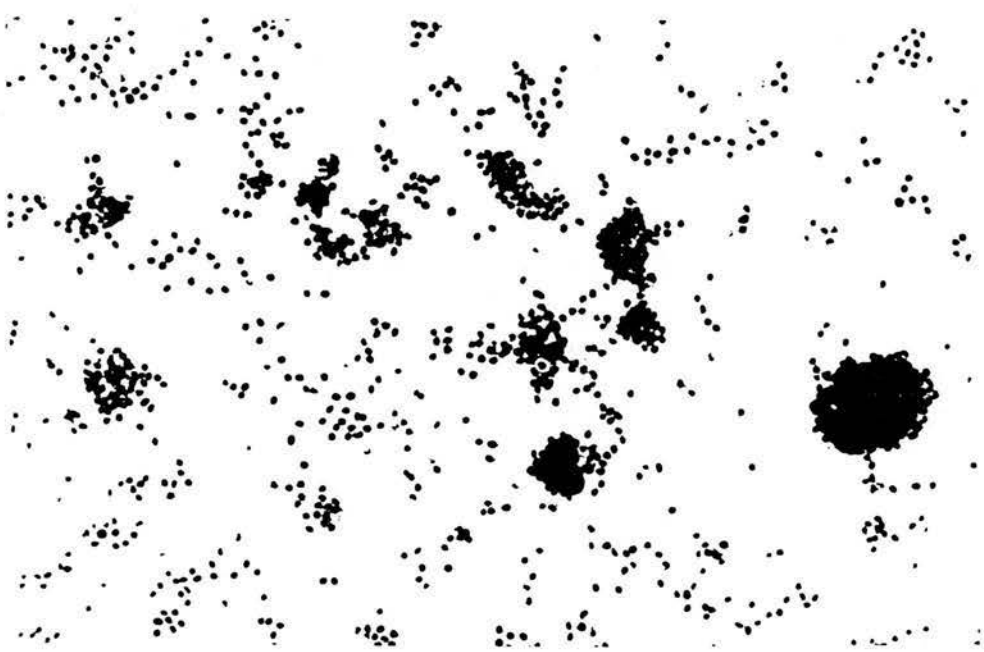


Figure 2. Aggregates of cells lavaged from the lungs of rats treated with wool dust.

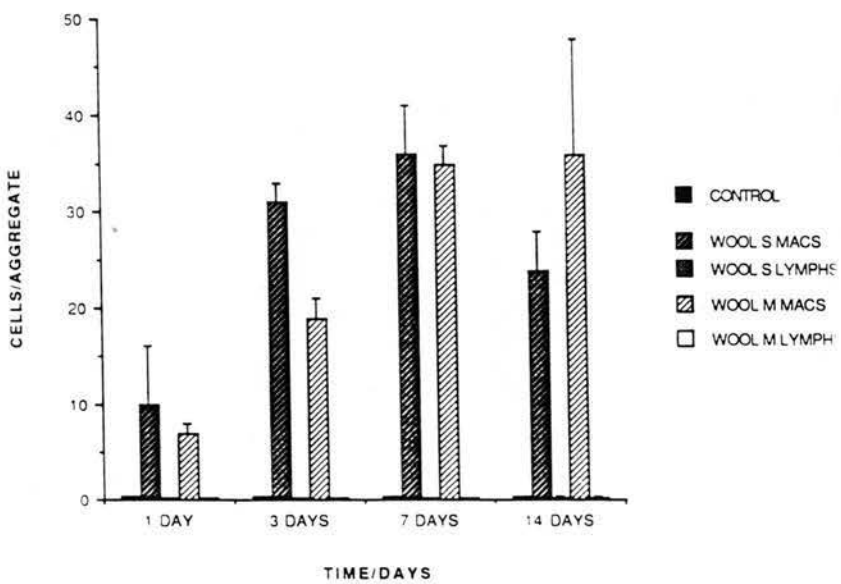


Figure 3. The number of macrophages and lymphocytes per aggregate in BAL from rats treated with wool dusts S and M. The data are represented as the mean + SEM from three separate animals at four time points.

Incubation of alveolar macrophages from untreated animals in teflon wells for 18 h resulted in spontaneous formation of small aggregates of cells.

Wool and grain dusts. Wool and grain dust treatment *in vitro* caused increases in the number of cells per aggregate (medium, 18.78 + 0.67; wool S, 25.28 + 1.03; grain, 23.53 + 1.25) (mean + SEM) (Figure 4). No *in vitro* treatments caused an increase in the total number of aggregates; the major change was a striking increase in the number of cells/aggregate. ANOVA showed a significant effect of treatment ($F = 9.523$; $P < 0.01$). Individual *t* tests showed that all three treatments caused significantly more cells per aggregate than medium alone.

Leachates of wool and grain dusts. Leachates of wool and grain dusts failed to produce significantly different numbers of cells per aggregate compared with the control levels (control, 13.4 + 3.17; wool S, 13.23 + 3.34; wool M, 16.4 + 6.78; grain, 13.8 + 4.41) (mean + SD). ANOVA indicated no significant treatment effect ($F = 1.335$; $P > 0.05$). Be cause dust leachates produced no change in the number c cells per aggregate, leachates of dust depleted of endotoxi were not examined. These results indicate that endotoxi released from the organic dust is not responsible for the ir creased aggregation caused by the organic dust *in vitro*.

Stimulators of macrophage activity. Increased aggreg tion *in vitro* could be observed after treatment of macr phages with endotoxin, PMA, and TNF (Figure 5). significant treatment effect was observed in ANOVA (F

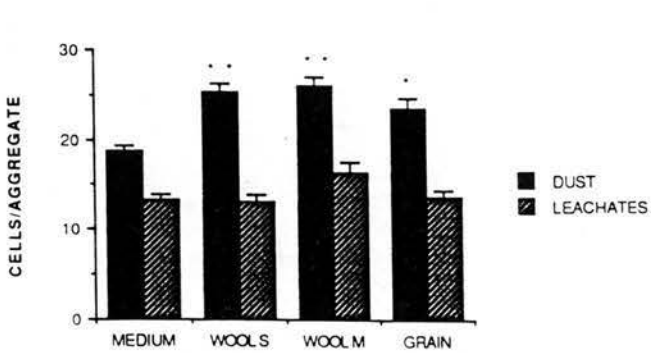


Figure 4. The effect of wool and grain dusts and dust leachates on alveolar macrophage aggregation. Significant increases were observed for each dust (wool S and wool M, $P < 0.01$; grain, $P < 0.05$). Ten random fields per treatment were assessed, and the experiment was performed on three separate occasions. Data are represented as the mean + SEM of the number of cells per aggregate. Difference from control as indicated: ** $P < 0.01$ and * $P < 0.05$. There was no significant effect of treatment in any of the dust leachates compared with medium.

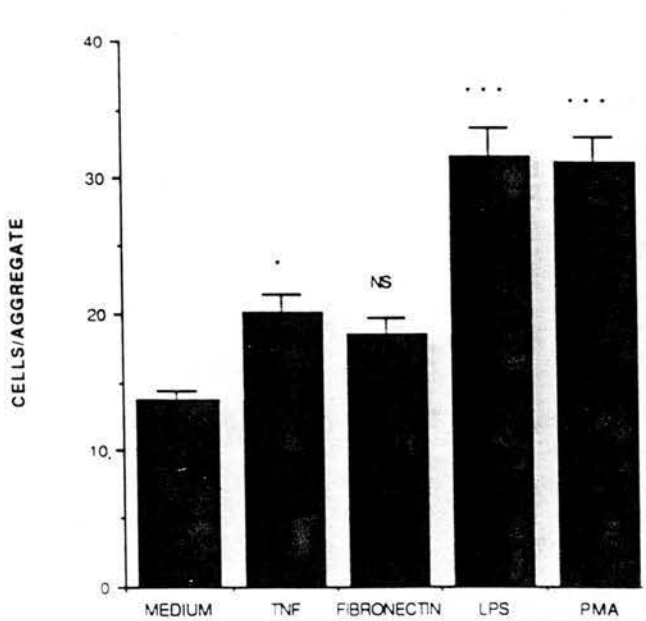


Figure 5. The effect on *in vitro* aggregation of alveolar mac phages of treatment with TNF, LPS, PMA, and fibronectin. D are presented as the mean SEM of the number of cells compris each aggregate. Ten random fields per treatment were assess and the experiment was performed on three separate occasio Asterisks denote significance of difference with medium alo * $P < 0.05$ and *** $P < 0.001$. NS = no significant differen

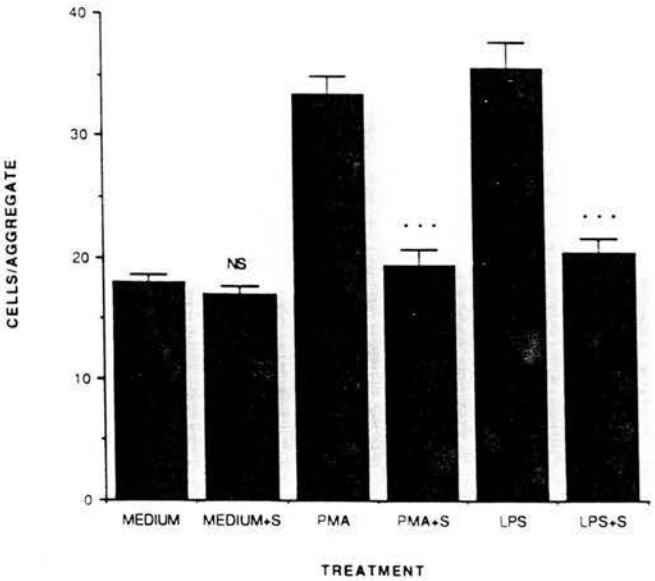


Figure 6. Size of alveolar macrophage aggregates from control, PMA-treated, and LPS-treated cells and the effect of staurosporine. Data are represented as the mean + SEM of 10 randomly selected fields per treatment in three separate experiments. All comparisons indicated by asterisks are between the staurosporine and nonstaurosporine groups for each treatment. NS = no significant difference; *** $P < 0.01$.

8.876; $P < 0.01$). Individual t tests showed significant ($P < 0.001$) differences between medium and both endotoxin and PMA. A lesser but significant effect ($P < 0.05$) was shown by treatment with TNF. The effect of fibronectin was not significant ($P > 0.05$).

Effect of Staurosporine on Macrophage Aggregation

Since PMA exerts its effects by activation of PKC, we examined the effects of staurosporine, a PKC inhibitor, on the aggregation of macrophages. The effect of staurosporine on the spontaneous aggregating ability of macrophages and the increased aggregation caused by LPS and PMA is shown in Figure 6. With treatment (untreated, LPS, or PMA) as classifying variable, there was a significant effect ($F = 7.125$; $P < 0.05$) of PMA and LPS and a significant treatment/staurosporine interaction ($F = 10.909$; $P < 0.05$). Staurosporine thus did not affect spontaneous aggregate formation, but cells that had also been stimulated with LPS and PMA were reduced to control levels. Thus, PKC activation may have a role in enhancement of aggregate formation observed after LPS treatment.

The effect of staurosporine on wool and grain dust-mediated macrophage aggregation is shown in Figure 7. In contrast to the findings with the classic macrophage activators LPS and PMA, staurosporine did not significantly reduce organic dust-mediated aggregate formation ($F = 0.350$; $P > 0.05$) or the interaction between staurosporine and treatment ($F = 1.324$; $P > 0.05$).

Molecular Mechanism of Aggregate Formation

Removal of the divalent cations calcium and magnesium from the culture medium completely abolished the formation of aggregates formed either spontaneously or by treatment with LPS, PMA, or organic dusts, consistent with a role for integrins in the aggregation of macrophages.

Effect of Aggregate Formation of Antibody to LFA-1 and ICAM-1

When cells were treated with LPS and undiluted supernatant containing monoclonal antibodies against intercellular adhesion molecules LFA-1α, LFA-1β, and ICAM-1, there was abolition of aggregate formation as shown in Figure 8. A clear dilution effect was demonstrated with all three supernatants, which even at a dilution of 1:200 was only approaching control levels of 37 cells/aggregate. There was no significant effect of antibody type ($F = 2.96$; $P > 0.05$), but a significant

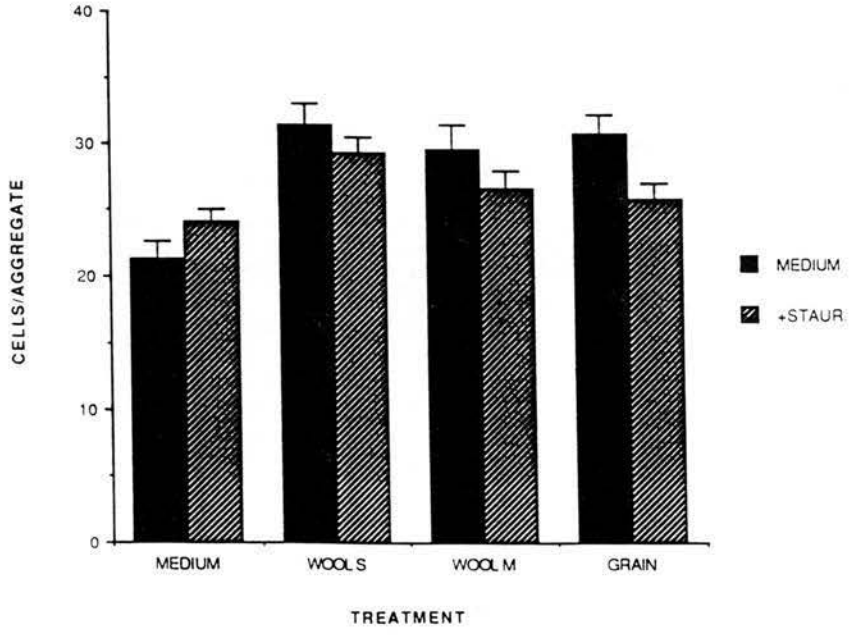


Figure 7. Number of alveolar macrophages in aggregates from wool dust S and M treatments *in vitro* and the effect of staurosporine on aggregate size. Data are represented as the mean + SEM of 10 randomly selected fields per treatment, and the experiment was performed on three separate occasions.

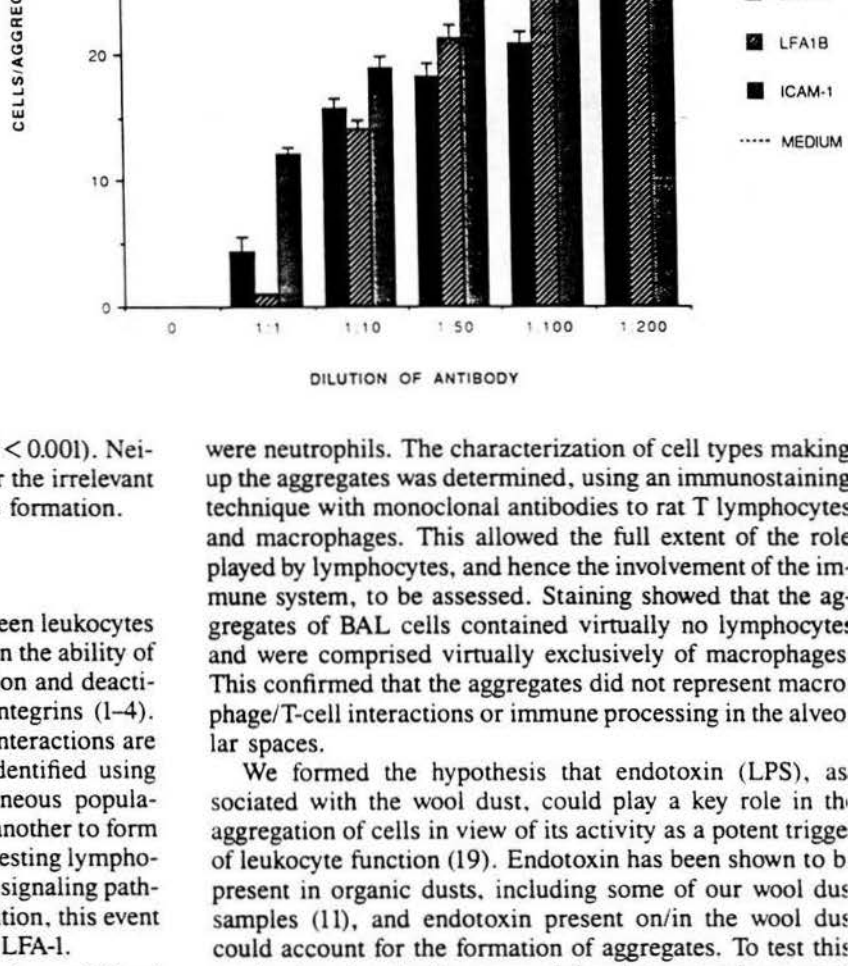


Figure 8. Effect of dilution of hybridoma supernatant on the number of cells per aggregate in macrophages treated with LPS *in vitro*. Data are the mean + SEM of three separate experiments.

effect of dose was demonstrated ($F = 35.21$; $P < 0.001$). Neither the "lymphocyte supernatant control" nor the irrelevant antibody control had any effect on aggregate formation.

Discussion

The interaction between leukocytes, and between leukocytes and the extracellular matrix, depends partly on the ability of a variety of molecules to regulate the activation and deactivation of cell surface molecules known as integrins (1–4). Two molecules mediating cell-cell adhesive interactions are ICAM-1 and the integrin LFA-1 (6), first identified using homotypic adhesion assays, where homogeneous populations of T and B lymphocytes adhered to one another to form aggregates (15). This effect does not occur in resting lymphocytes and requires stimulation of intracellular signaling pathways that result in activation of LFA-1. In addition, this event is blockable with monoclonal antibodies to LFA-1.

Persistent upregulation of adhesion molecules could lead to chronic cell activation, inflammation, and pathology. Organic dusts are known to cause chronic inflammation, with an important role being played by bacterial endotoxin (16). The profile of the bronchoalveolar lavage from organic dust–exposed individuals shows increased numbers of lymphocytes and macrophages (17). If there were a persistent immune response in the lungs of wool dust–exposed rats, this could lead to chronic inflammation mediated either by immune complexes or by T cell–mediated inflammation through the action of lymphokines. The absence of this confirms that, in our model, the macrophage aggregation, and in the longer term the granuloma and giant cell formation (18), is unlikely to be a classic immunologic phenomenon.

We demonstrated an inflammatory response and leukocyte aggregation in our rat model when dust collected from the air of wool mills was instilled into the lungs. We set out to characterize the cell surface adhesion molecules involved in these events. Wool and grain dusts produced aggregates of cells that were all macrophages, except at 1 day after exposure to the dust, when some of the cells in the aggregates were neutrophils. The characterization of cell types making up the aggregates was determined, using an immunostaining technique with monoclonal antibodies to rat T lymphocytes and macrophages. This allowed the full extent of the role played by lymphocytes, and hence the involvement of the immune system, to be assessed. Staining showed that the aggregates of BAL cells contained virtually no lymphocytes and were comprised virtually exclusively of macrophages. This confirmed that the aggregates did not represent macrophage/T-cell interactions or immune processing in the alveolar spaces.

We formed the hypothesis that endotoxin (LPS), associated with the wool dust, could play a key role in the aggregation of cells in view of its activity as a potent trigger of leukocyte function (19). Endotoxin has been shown to be present in organic dusts, including some of our wool dust samples (11), and endotoxin present on/in the wool dust could account for the formation of aggregates. To test this we developed an *in vitro* assay of alveolar macrophage aggregation. *In vitro*, spontaneous aggregation of control alveolar macrophages was observed, but, in the presence of organic dust, TNF, fibronectin, PMA, and LPS, the number of cells comprising each aggregate was increased. With LPS and PMA treatments, there were more than twice the number of cells in each aggregate than in the controls, while organic dust caused increases of up to 30%. In addition, there was a slight increase in the number of cells per aggregate in the TNF- and fibronectin-treated groups. We have shown that large amounts of TNF are produced by alveolar macrophages treated with these organic dusts *in vitro* (manuscript in preparation). Surprisingly, although endotoxin was present in leachates, aggregates of cells were no different from controls in the leachate-treated experiments. A possible explanation is the concentration of endotoxin present in the system. In the leachates, nanogram quantities were present, whereas the concentration of LPS used in the *in vitro* assay was in the microgram range. It is possible that leaching of organic dust into PBS does not result in complete removal of endotoxin from the dust, and this could explain the difference between

the activity of dust particles and leachates of them. No increased aggregation was observed compared with the control when the concentration of reagent LPS was increased up to a concentration of 500 ng/ml. Nanogram quantities are, however, sufficient to produce activation of cells *in vivo*, and this may be a starting point for subsequent aggregation of cells. The CD14 receptor for LPS/LBP is important in aggregation, where binding to CD14 causes homotypic monocyte aggregation (8, 9) mediated through activation of integrins on the cell membrane.

The involvement of LFA-1 and ICAM-1 in the aggregation was demonstrated using supernatants from hybridomas against rat LFA-1α and LFA-1β and ICAM-1 (14). Undiluted supernatant completely abolished aggregate formation. In addition, a clear, dose-dependent effect was shown with the three supernatants. Most and colleagues (18) have described a role for LFA-1 in giant cell formation for which aggregate formation via LFA-1 may be a prerequisite. Interestingly, we have noted giant cells and granuloma in lungs of rats treated with wool or grain dust and left for up to 1 mo (20).

We have also shown that aggregation of macrophages by classic activating agents (LPS and PMA) requires the presence of calcium and/or magnesium ions and that the PKC inhibitor staurosporine can reduce the number of cells per aggregate. In contrast, aggregation induced by wool dusts was not significantly inhibited by treatment with staurosporine. These data suggest that macrophage aggregation in response to wool dusts involves a PKC-independent pathway of activation.

The general hypothesis tested here was whether immune processing in the airways, represented by mononuclear cell aggregation in the rat model, could account for the symptoms of airway irritation or inflammation in the workers in wool mills. The aggregates did not represent a classic immune reaction of macrophage/lymphocyte clusters, which could explain the inflammation since no lymphocytes were present in them. The consequence of upregulation in terms of the cell interactions in the lung is of great potential importance. In the particular case of bronchitis and other upper respiratory symptoms in wool workers, increased interaction between alveolar macrophages and between macrophages and other lung cells could be important in leading to inflammation.

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Long and short amosite asbestos samples: comparison of chromosome-damaging effects to cells in culture with *in vivo* pathogenicity.

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INTRODUCTION

The pathogenic effects of asbestos in humans and laboratory animals are well documented , with lung tumours and mesothelioma amongst the major pathologies being produced (Davis and Donaldson 1993). The ability of asbestos and other fibres to damage chromosomes of cells in culture has also been demonstrated (eg Hesterberg and Barrett 1990) and this has been considered to be a likely factor in its toxicity, particularly its carcinogenic action. However, few laboratories have had the opportunity to study the pathogenic effects of defined fibre samples and, using the same samples, examine their clastogenic effects.

In the 1980s the Institute of Occupational Medicine in Edinburgh carried out a large-scale rat inhalation and instillation study on two amosite asbestos samples that differed only in their length. These studies demonstrated that the short and long samples had dramatically differing pathogenic potentials, with the long sample producing many lung tumours and mesotheliomas whilst the short sample was virtually without activity (Davis et al 1986). Studies on the ability of the same two fibre samples to cause inflammation (Donaldson et al 1988) again demonstrated the long fibre sample to be many times more active than the short sample. We report here on the ability of these two amosite asbestos samples to cause chromosome damage to cells in culture.

We have previously been interested in the anti-oxidant activities of the free radical scavenger reduced glutathione (GSH), in defending the lung against the oxidant damage caused by cigarette smoke (MacNee et al 1991) . It has been suggested that asbestos may cause chromosome damage by oxidant radical-mediated pathways (Kamp et al 1992) . We therefore utilised buthionine sulphoximine (BSO), an inhibitor of the GSH synthetic pathway, to deplete GSH in the target cells and assessed whether this had any effect on the chromosome damage caused by amosite asbestos.

MATERIALS AND METHODS

Amosite asbestos samples The long and short amosite asbestos samples utilised in these experiments have been used extensively and are described in detail elsewhere (Davis et al 1986). In brief the short sample was derived from the long sample by milling and was found to be elementally and crystallographically identical to the parent sample. The diameters of the two samples were almost identical but the long sample was substantially longer as shown in Figure 1.

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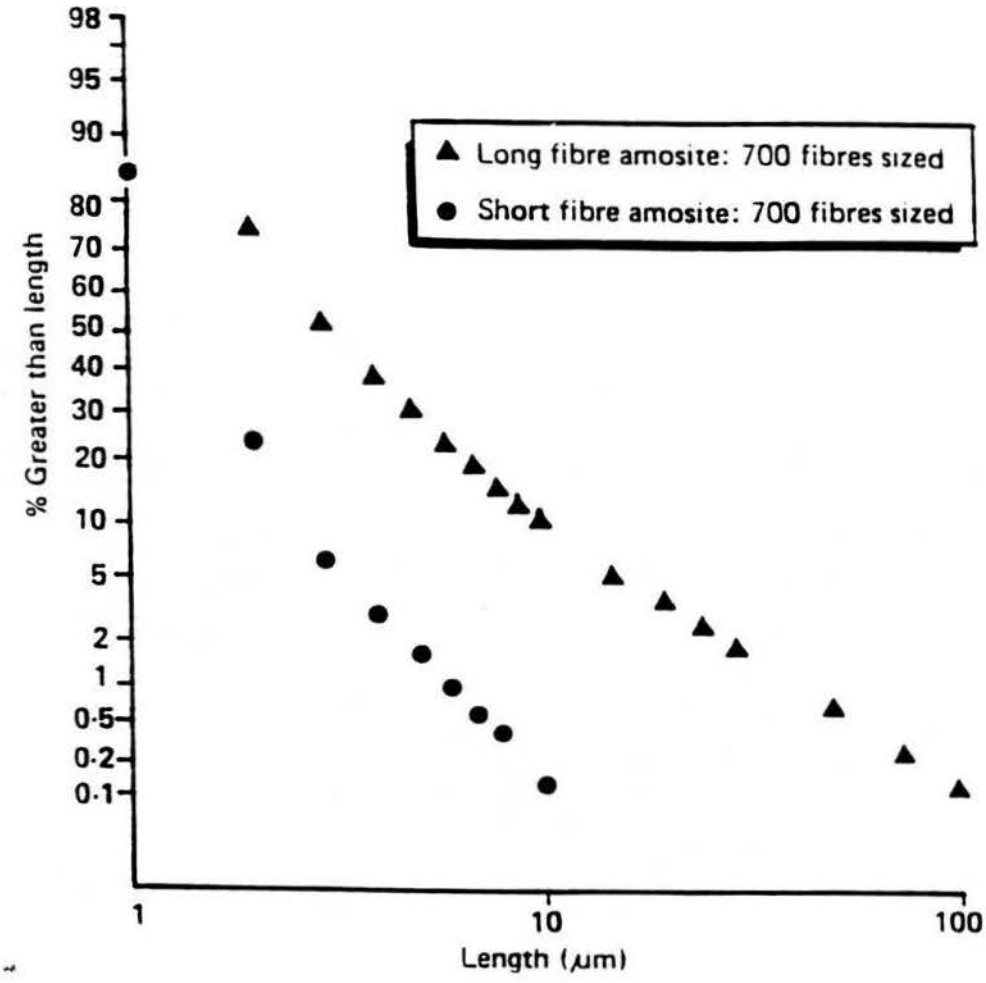


Figure 1 . Length distribution of the long and short fibre amosite samples; 700 fibres counted by SEM.

Experimental Pathology The experimental pathology data summarised below is derived from the published account of experiments where rats were exposed to airborne fibres and the long-term pathological responses were monitored; full details of the exposure systems and analysis can be obtained from Davis et al (1986). Details of the mouse peritoneal inflammation assay can be obtained from Donaldson et al (1989); in brief, mice were injected intra-peritoneally with varying doses of long or short fibre amosite asbestos and the 4 day inflammatory response was assessed by peritoneal lavage and counting of the numbers of inflammatory cells.

Chromosome-damaging effects of fibres Chinese hamster ovary cells (CHO) at 1 x 10^6 cells/ml of F10 medium + 10% foetal calf serum were seeded onto 24-well plates and incubated overnight. The medium was replaced with 1 ml of 10μg/ml of long or short amosite asbestos in medium and incubated for a further 48 hours. Cultures were then treated with 0.1μg/ml colchicine for the final 2 hours of culture and trypsinised off the culture plates. The cells were then spun down and treated with 0.75M KCl for 10 minutes and stained with aceto-orcein. Chromosomal aberrations and hyperdiploidy was assessed in 100 metaphases in 3 separate experiments.

RESULTS

Experimental pathology The results of the experimental pathology experiments are shown in Table 1

Table 1. Results obtained from pathology studies* with the amosite samples

	SHORT AMOSITE	LONG AMOSITE
LUNG TUMOURS (% BY INHALATION)	0	32.5
FIBROSIS (BY INHALATION, ARBRITARY UNITS)	0.5	11.0
MESOTHELIOMA (% BY INSTILLATION)	4	94
INFLAMMATION SCORE (INSTILLATION)	+	++++

*for further details see Davis et al (1986) and Donaldson et al (1989)

Chromosomal and mitotic effects The effects of the amosite samples on chromosomes of cells in culture are shown in Table 2

Table 2. Effects of amosite samples on chromosomes of cells in culture

	CONTROL	SHORT AMOSITE	LONG AMOSITE
CELLS WITH ABERRATIONS (%)	4.5 (0,5)	6.3(0.3)	12.1(0.2)
ABERRATIONS PER CELL	0.06(0.003)	0.07(0.05)	0.19(0.02)
HYPERDIPLOID CELLS (%)	3.3(0.3)	5.7(0.3)	9.5(0.5)
MITOTIC INDEX	7.5(0.2)	6.3(0.2)	4.5(0.05)

All values mean(sd) of 100 cells in 3 separate experiments

EFFECTS OF TREATMENT WITH BSO The effects of treatment of cells with BSO on chromosome aberrations in cells treated with amosite asbestos are shown in Table 3.

Table 3. Effect of BSO on chromosome aberrations

	NO BSO	BSO
CONTROL	4.5(0.5)	9.0(0.5)
SHORT AMOSITE	6.3(0.3)	16.2(1.5)
LONG AMOSITE	12.1(0.2)	18.4(2.3)

All values mean(sd) of 100 cells in 3 separate experiments

DISCUSSION

The increased number of chromosomal aberrations in cells treated with long fibres is consistent with the pathogenic potential of the two samples, with the long sample being substantially more potent than the short in causing lung fibrosis and tumours. Previous workers have shown a relationship between fibre length and pathogenicity of fibres (Hesterberg and Barrett 1990) and Stanton's work with implanted fibres revealed increasing pathogenic potential as the length approached 8µm and over (Stanton et al 1977). Many fibres in the long amosite sample were in this range and longer, whilst there were effectively no 'Stanton' fibres in the short fibre sample. it has been considered that the longer fibres can physically interfere with the migration of chromosomes at anaphase (Hesterberg and Barrett 1990) although this was not observed in the present study. In general, therefore, this study has confirmed the induction of fibre length-related chromosomal abnormalities to cells *in vitro* with two samples of amosite asbestos that have been shown to exhibit the same length -related ability to cause pathology and inflammation in vivo.

GSH is a ubiquitous anti-oxidant found in the lining fluid and cells of the lung and recent studies within our group have suggested that extra-cellular GSH can defend cells in culture against the oxidant injury caused to cells by cigarette smoke (Lannan et al in press). Reactive oxygen species and free radicals have been shown, in many studies, to have a role in asbestos pathogenicity (Kamp et al 1990). The exact role of intra-cellular GSH in maintaining chromosome integrity is unknown but treatment of CHO cells with BSO, an inhibitor of GSH synthesis, increased the amount of chromosomal damage caused to the cells by both short and long amosite. This suggests that free radicals may have role to play in the production of chromosomal abnormalities.

We have found previously that acute exposure of cells in culture to cigarette smoke causes a sharp fall in intracellular GSH (Lannan et al in press). The synergism between asbestos exposure and cigarette smoke in leading to lung cancer is well documented. Effects of cigarette smoke on the GSH levels of the epithelial lining of

the lung could be important in reducing the defences against chromosomal damage and these could be important in enhancing fibre toxicity in smokers.

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

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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 μg/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 μg/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.

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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 symptoms[1]; 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 μg/well (500 μg to 5 μg/ml), which were then tested for their ability to cause epithelial injury.

ALVEOLAR EPITHELIAL CELL LINE

A549 cells derived from a human lung carcinoma[7] 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 μg/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 μg/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 $2·5 \times 10^5$ 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 2×200 μl 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 μg/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 ($1·5 \times 10^6$) 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 $2·5 \times 10^5$ 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 μg/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

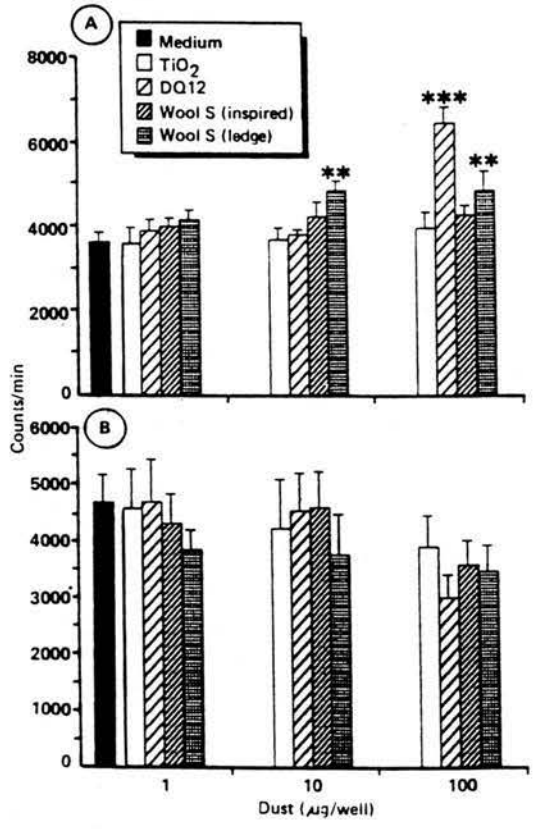


*Figure 1 Effect of wool dust S on A549 epithelial cells with respect to A, lytic injury and B, detachment injury. Injury assessed by measuring ^{51}Cr released from labelled cells and represented by means (SEM) of triplicate experiments (**$p < 0.01$; ***$p < 0.001$ compared with medium control).*

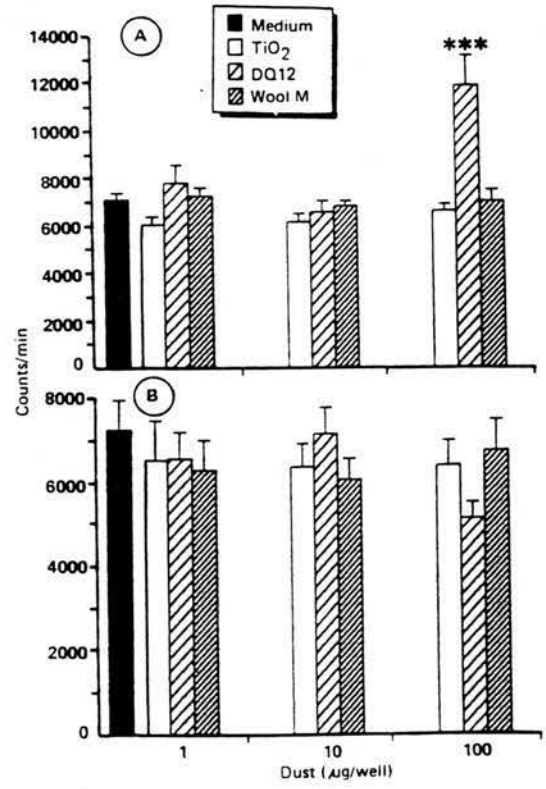


*Figure 2 Effect of wool dust M on A549 epithelial cells with respect to A, lytic injury and B, detachment injury. Injury assessed by measuring ^{51}Cr released from labelled cells and represented by means (SEM) of triplicate experiments (***$p < 0.001$ compared with medium control).*

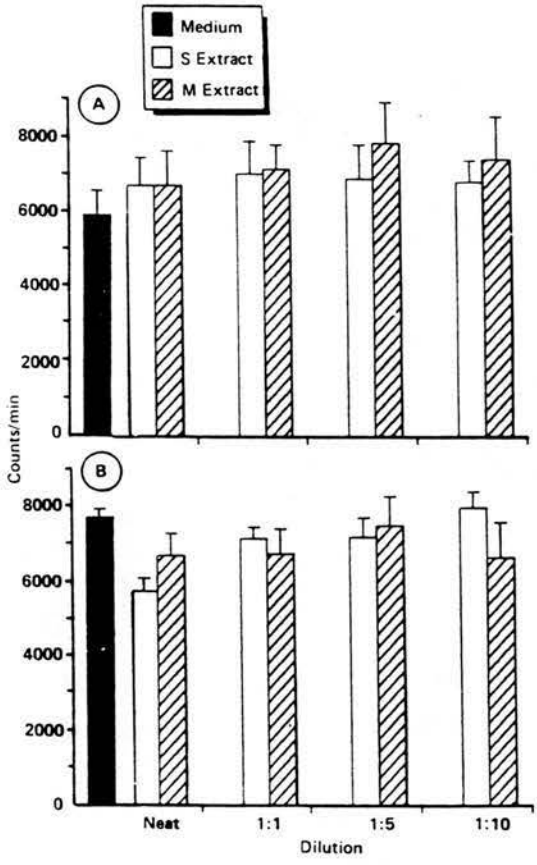


Figure 3 Effect of extracts of wool dusts S and M on A549 epithelial cells with respect to A, lytic injury and B, detachment injury. Extracts were used undiluted and diluted at various ratios with medium. Injury assessed by measuring ^{51}Cr released from labelled cells and represented by means (SEM)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

type C, which showed significant lysis at the highest dose of 10 μg/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 μg/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

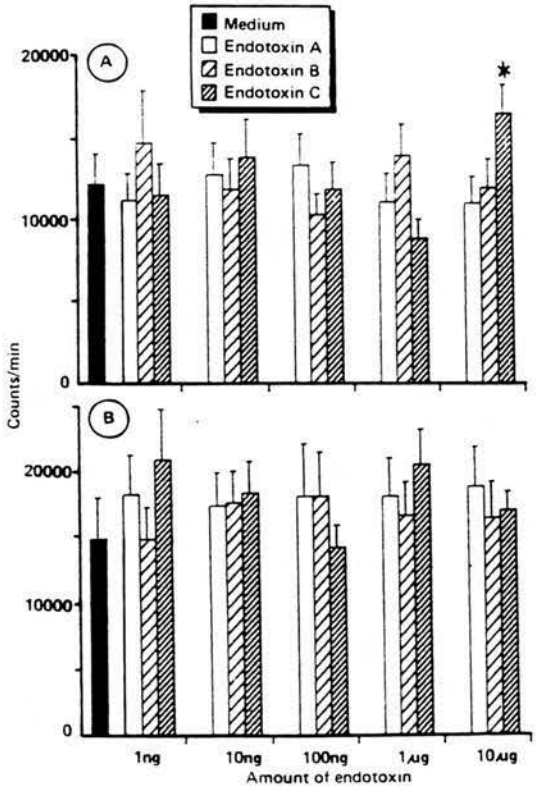


Figure 4 Effect of endotoxins A, B, and C on A549 epithelial cells with respect to A, lytic injury and B, detachment injury. Injury assessed by measuring ^{51}Cr released from labelled cells and represented by means (SEM) of triplicate experiments ($p < 0.05$ compared with medium control).*

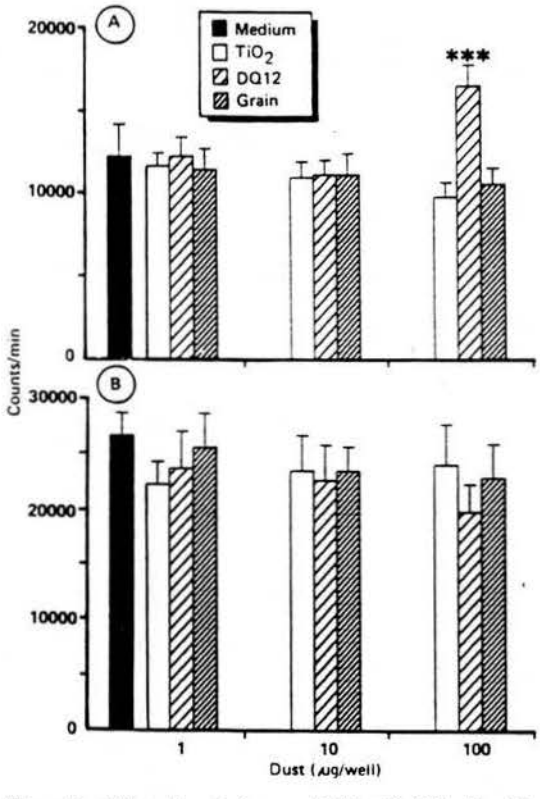


*Figure 5 Effect of grain dust on A549 epithelial cells with respect to A, lytic injury and B, detachment injury. Injury assessed by measuring ^{51}Cr released from labelled cells and represented by means (SEM) of triplicate experiments (***$p < 0.002$ compared with medium control).*

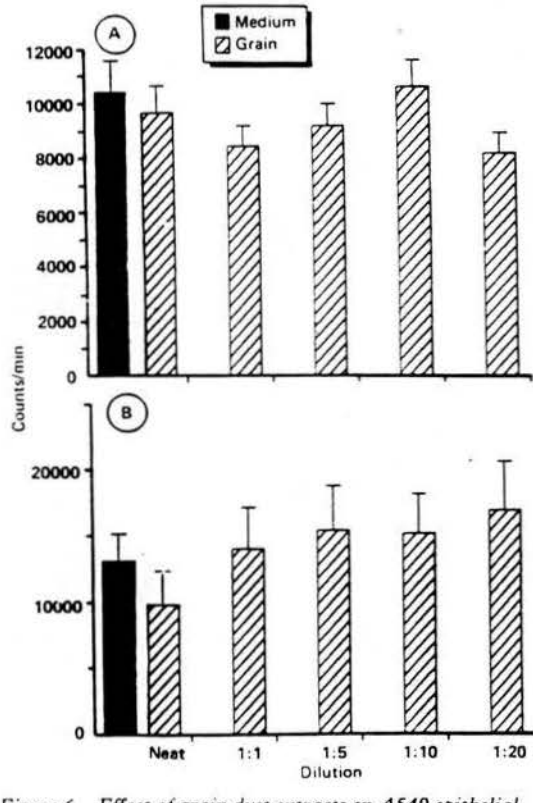


Figure 6 Effect of grain dust extracts on A549 epithelial cells with respect to A, lytic injury and B, detachment injury. Extracts were used undiluted and diluted at various ratios with medium. Injury assessed by measuring ^{51}Cr released from labelled cells and represented by means (SEM) of triplicate experiments (no significant differences were found 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 grain handlers where acute inflammatory reaction can develop, leading to chronic bronchitis with o without airways obstruction, after prolonged ex posure to the dust.[8] The A549 alveolar epithelial ce line has provided a model for the in vitro study o lung injury by various agents and has been used he in an attempt to examine some of the mechanisms b which wool and grain dusts may cause lung inju and account for symptoms present in some membe of the workforce in wool textile mills and in gra workers.

Airborne wool dust has been shown to generate a acute inflammatory response when instilled intr tracheally into the lungs of rats.[9] Neutrophils peak one day after instillation and thereafter decreased background levels by three days. By contra aggregates of mononuclear cells present in the bro choalveolar lavage reached a maximum by sev days, a timescale indicative of an immune respons Also, ledge dust collected from surfaces in the wo

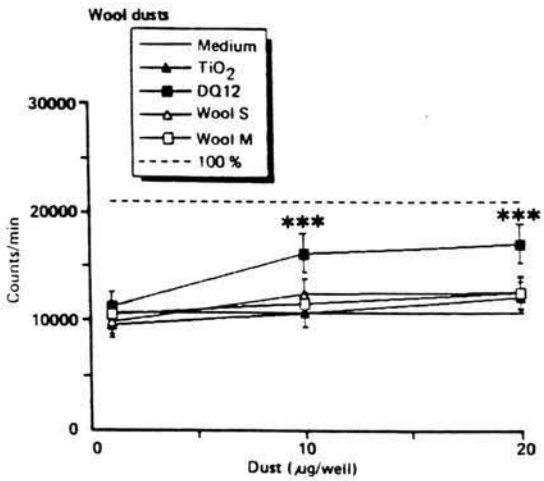


*Figure 7 Cytotoxic effect of wool dusts S and M on ^{51}Cr labelled control alveolar macrophages. Injury assessed by monitoring ^{51}Cr released into the medium. Data represent means (SEM) of triplicate experiments (***$p < 0.001$ compared with medium control).*

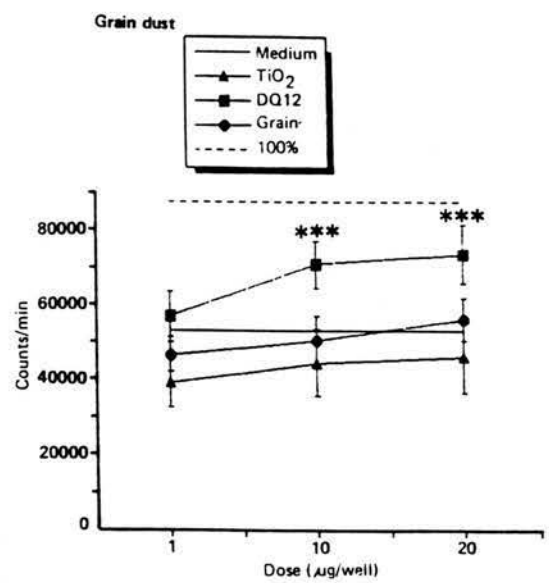


*Figure 8 Cytotoxic effect of grain dust on ^{51}Cr labelled control alveolar macrophages. Injury assessed by monitoring ^{51}Cr released into the medium. Data represent means (SEM) of triplicate experiments (***$p < 0.001$ compared with medium control).*

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.

We acknowledge Professor D M Weir of the University of Edinburgh for his continued interest in this work; and Dr Michael Topping of the Health and Safety Executive for supplying the grain dust. The research was funded by the Health and Safety Executive.

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Differential release of superoxide anions by macrophages treated with long and short fibre amosite asbestos is a consequence of differential affinity for opsonin

I M Hill, P H Beswick, K Donaldson

Abstract

***Objective*—To investigate the ability of short and long fibre samples of amosite asbestos to stimulate superoxide production in isolated rat alveolar macrophages, and to determine how opsonisation with rat immunoglobulin might modify this response.**

***Methods*—Macrophages were isolated from rat lung by bronchoalveolar lavage and challenged with both opsonised and non-opsonised long and short fibres of amosite asbestos. Release of superoxide anions was measured by the spectrophotometric reduction of cytochrome *c*, in the presence and absence of superoxide dismutase.**

***Results*—Both long and short fibre samples of amosite asbestos without opsonisation were ineffective in stimulating isolated rat alveolar macrophages to release superoxide anions in vitro. After opsonisation with immunoglobulin, however, a dramatic enhancement of release of superoxide anion was seen with long fibres, but not short, which confirms the importance of fibre length in mediating biological effects. The increased biological activity of the long fibre sample is explained by increased binding of the opsonin to the fibre surface as, at equal mass, the long fibres bound threefold more immunoglobulin than the short fibres.**

***Conclusion*—Opsonisation is an important factor in modulation of the biological activity of fibres at the cellular level. Differences in binding of opsonin to samples of fibre previously considered to be identical apart from length, suggest that surface reactivity needs to be taken into account when fibres are compared. Binding of biological molecules, in vivo, may thus be an important modifying factor in the pathological processes initiated by fibres.**

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Keywords: opsonin; fibres; surface reactivity

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The critical biochemical processes responsible for derangement of lung tissue caused by asbestos and other respirable fibres are still not fully understood. The concept that reactive oxygen species may underlie the pathogenesis of derangement has become the focus of extensive research in recent years.[1] There are two potential sources of oxidation; the fibres themselves,[2] and the reactive oxygen species produced by inflammatory leucocytes recruited to the sites of fibre deposition. Our study focuses on the reactive oxygen species.

It is known that oxidants derived from phagocytes play a critical part in the host's defence but, accumulating evidence indicates that damage caused by their inappropriate release, can contribute to the development of many types of lung disease.[3–5] Various mechanisms have been proposed by which fibres may induce such production.[6 7]

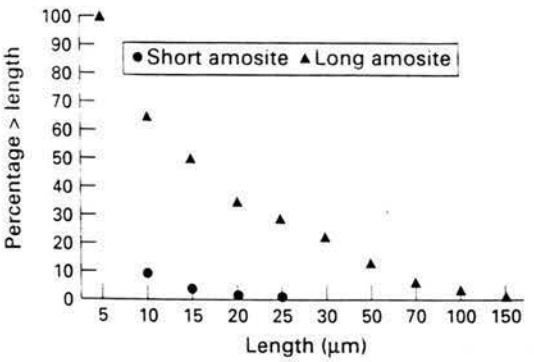
It has been suggested that the release of reactive oxygen species in response to long fibres may be the result of frustrated phagocytosis on the part of the alveolar macrophage[8] or is a direct result of stimulation of NADH or NADPH oxidase when the asbestos fibre interacts with the macrophage cell membrane.[4] Conversely, it may be an as yet undefined combination of the two. Roney and Holian have suggested that phospholipase C is involved in activation of the protein kinase C to stimulate generation of superoxide anions,[7] and Kalla *et al* have also shown that extracellular calcium prolongs this release.[9]

Most of these studies have been carried out with chrysotile and crocidolite asbestos fibres. Both of these types of asbestos have been shown to induce the production of superoxide radicals from alveolar macrophages of the hamster, rat,[4 10] guinea pig,[7 9] and human.[11] Long chrysotile fibres have been found to be more effective than short fibres in eliciting release of superoxide anions from rat alveolar macrophages.[5]

Macrophages interact with their environment through the binding of molecules to receptors on their external surface.[12 13] In general, such interaction with a foreign particle will lead to phagocytosis, but optimum ingestion is mediated by proteins derived from the host, which coat the foreign material and thereby increase its recognition by phagocytes. Various soluble components, present in the bronchoalveolar space, have been shown to be opsonic in this way, including immunoglobulin (IgG). In the presence of IgG, superoxide generation induced by asbestos is significantly enhanced in macrophages of guinea pigs[14] and humans.[11 15]

In phagocytosis mediated by opsonins, the opsonins serve as a ligand, which can attach the xenobiotic to its receptor on the phagocytic membrane. Binding of the ligand to

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receptors on the cell surface is thought to be the recognition step that initiates phagocytosis.[16] It has been proposed that cross linking of cell surface Fc receptors by chrysotile asbestos fibres opsonised by IgG could be the trigger for such superoxide generation,[14] as the superoxide anion is known to be released outside the cell when phagocytosis of long fibres of asbestos is attempted.[17]

Only a few studies have included amosite asbestos in their investigations and Roney and Holian found that, in contrast to chrysotile exposure, guinea pig macrophages did not release superoxide when challenged with amosite.[7] Davis and coworkers showed a difference in pathogenicity between long and short fibres of amosite.[18] The same fibre samples were used by Donaldson *et al* to show an enhancement of inflammation dependent upon fibre length[19] and greater cytokine secretion by alveolar macrophages exposed to long amosite fibres than to short fibres.[20]

It has been suggested that production of oxidants by leucocytes may be important in disease related to fibres, and that long and short fibres are very different in pathogenicity. The purpose of this study was to investigate the ability of both long and short fibres of amosite asbestos to stimulate production of superoxide in rat alveolar macrophages, and to determine how rat IgG might modify these responses.

Materials and methods

Cytochrome *c* type III from horse heart, superoxide dismutase (SOD) from bovine erythrocytes, and rat IgG were from the Sigma Chemicals, Poole, Dorset. Dulbecco's phosphate buffered saline (PBS) was from Gibco, Paisley, Renfrewshire and adjusted to pH 7·4. All other chemicals were from BDH Chemicals, Poole, England.

A stock solution of phorbol myristate acetate (PMA) was prepared by addition of 0·5 ml of dimethyl sulphoxide (DMSO) to 1 mg of PMA and was frozen at −70°C in 10 μl aliquots until required. This was diluted 1:200 with PBS immediately before use. Similarly, 75 000 units of SOD was made up to 1 ml with distilled water, aliquoted, and stored as for the PMA. Rat IgG was prepared at a concentration of 5 mg/ml and frozen at −70°C in 100 μl aliquots. The SOD was added to reaction buffer at a concentration of 1·5 μl of SOD/ml of buffer for use in control tubes. The reaction buffer was freshly prepared with PBS, 1 mg/ml cytochrome *c*, and 2 mg/ml dextrose.

CELLS

Male Wistar rats were killed by a 2 ml intraperitoneal injection of sodium phenobarbitone (60 mg/ml), and the trachea was exposed by blunt dissection. A small incision was made between the cartilagenous rings, and a blunt needle introduced and secured with fishing line. The lungs were then dissected free of the thoracic cavity and lavaged with four 10 ml volumes of normal saline at 37°C. After installation of the lavage fluid, the lungs were massaged gently to increase the yield of alveolar macrophages.

The isolated cells were centrifuged at 350 × *g* for 10 minutes at 4°C and the cell pellets resuspended in 1 ml of sterile PBS. Viability was checked by trypan blue exclusion and cell numbers were counted with an improved Neubauer haemocytometer. Purity was checked by prepared cytocentrifuge slides stained with Diffquick (Merz Dade, Switzerland).

FIBRES

The fibres used in this study were the long and short fibre amosite samples described extensively in previous publications.[18-20] Figure 1 shows the length distribution. The diameter distributions are virtually identical. Due to the ball milling of the long fibre sample to produce the short, there was extensive comminution of fibres, such that only 37% of the short sample by mass was classified as fibre by the World Health Organisation (WHO) classification of >5 μm long, <3 μm diameter, and aspect ratio >3:1.

OPSONISATION OF FIBRES

The asbestos samples were mixed with PBS at a concentration of 1 mg/ml, and 50 μl of rat IgG (5 mg/ml) was added. The suspensions were incubated for 30 minutes at 37°C with constant rotation, and spin-washed three times in PBS, at 900 *g* for 10 minutes. Naked samples (without IgG) were taken through an identical incubation and washing protocol. Pellets were resuspended at stepped suspension concentrations from 125 μg to 20 mg/ml for short fibre amosite, and from 15·6 μg to 5 mg/ml for the long fibre samples. All dust samples were dispersed by passing the suspensions 10 times through a 23 G needle attached to a 20 ml syringe.

ASSAY OF SUPEROXIDE ANIONS

The method used was an adaptation of that previously described.[21] Cell suspensions were diluted to yield 5 × 10^6 cells/ml. The assay was carried out in duplicate with 3 ml tubes and 0·25 × 10^6 cells/tube. Either 15 μl of PMA or 100 μl of the naked or opsonised dust suspension was used as a trigger for superoxide production. Reaction buffer containing cytochrome *c* was added to each tube to give a final assay volume of 1·5 ml. Tubes with no dust or PMA were also set up as a check for

any endogenous superoxide production by untriggered cells.

The tubes were incubated and shaken at 37°C in a water bath for one hour before centrifugation at 900 *g* for five minutes to remove particulates. (Optimum incubation times were assessed by serial incubation studies from zero to two hours; data not shown).

The supernatant extinctions were determined at both 550 nm and 468 nm against a PBS blank, and the difference in these readings converted to concentration of superoxide anions/million cells. An extinction coefficient of 0·021 μM was used for cytochrome *c*.

The complete experimental run was also carried out with tubes that contained the prepared buffered SOD substituted for normal reaction buffer. To correct for any reduction of cytochrome *c* independent of superoxide anions, superoxide production was assessed as only the reduction in cytochrome *c* inhibitable by SOD. This was measured by subtracting the control results from those obtained from the samples. Overall results are expressed as nM of superoxide produced/million cells.

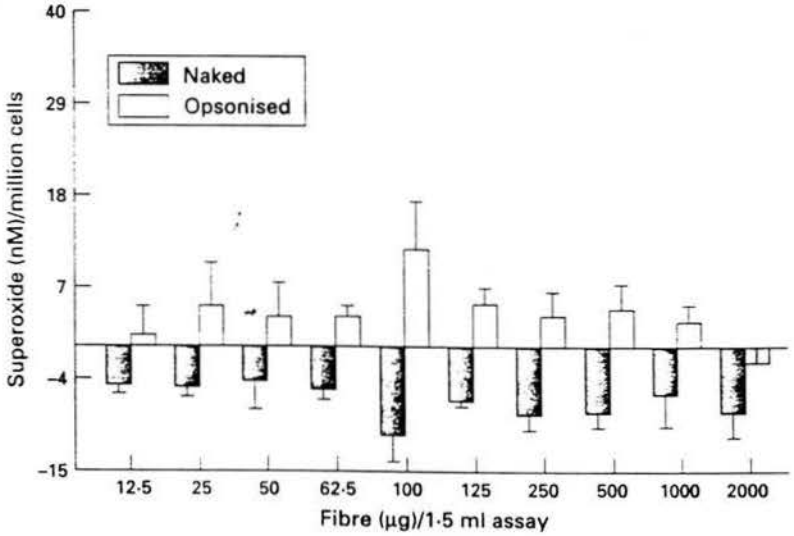


Figure 2 Production of superoxide anions by isolated rat alveolar macrophages treated with naked short fibre amosite asbestos or those opsonised by IgG. Results are shown compared with background. Each bar represents the mean (SEM) of at least three separate experiments with duplicate samples.

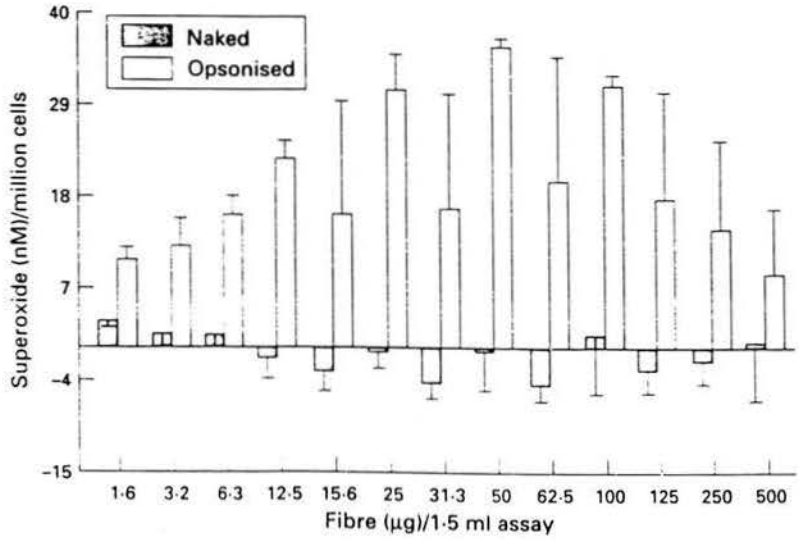


Figure 3 Production of superoxide anions by isolated rat alveolar macrophages treated with naked long fibre amosite asbestos or those opsonised by IgG. Results are shown compared with background. Each bar represents the mean (SEM) of three separate experiments with duplicate samples.

RADIOIODINATION OF PROTEIN

To investigate potential differences in the adsorption of protein by both long and short amosite fibres, the complete opsonisation procedure was repeated with ^{125}I labelled IgG. Rat immunoglobulin was iodinated with a procedure previously described,[22] and the unbound ^{125}I separated from ^{125}I-IgG conjugate by passing through a PD-10 column.

OPSONISATION OF DUSTS WITH ^{125}I-IgG

Dusts were mixed with PBS to a concentration of 1 mg/ml, and 0·5 ml of rat ^{125}I-IgG (5 μg/ml) was added. The mixtures were incubated for 30 minutes at 37°C with constant rotation, and were spin-washed three times in PBS, at 900 *g* for 10 minutes. Opsonised dust pellets were resuspended at a concentration of 5 mg/ml and 100 μl aliquots were read in a gammacounter.

STATISTICAL ANALYSES

All experiments were repeated at least three times under identical conditions. Results of repeat experiments were analysed by analysis of variance, with the general linear model in the Minitab statistical computer program (Minitab, 1989). Effects of treatment are expressed as variance ratio (*F*) statistics, with P values. Data for the binding of labelled IgG to fibres, were log transformed before analysis.

Results

The average production of superoxide by unstimulated macrophages was 17 nM/million cells; this was presumed to be a result of stimulation by contact with the plastic assay tubes. Stimulation by PMA generated an average of 113·7 nM of superoxide/million cells. The results are expressed as the mean of duplicate tubes in at least three separate experiments, with subtraction of the unstimulated control values.

SHORT FIBRES

Figure 2 shows production of superoxide anions by rat alveolar macrophages when exposed to different doses of both naked (unopsonised), and IgG-opsonised, short fibre amosite asbestos. Macrophages challenged with naked fibres produced less superoxide than unstimulated cells (mean (SD) 6·5 (1·99) nM less than background production). Opsonised fibres induced significant stimulation (mean (SD) 4·1 (3·4) nM over background). The ANOVA showed a significant effect of opsonisation: $F = 71{\cdot}83$, $P < 0{\cdot}001$.

LONG FIBRES

With long fibre amosite (fig 3), the magnitude of the macrophage response was slightly suppressed compared with a background of

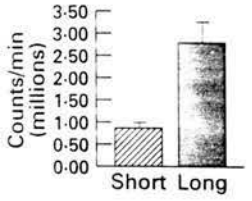


Figure 4 Binding of ^{125}I labelled IgG to samples of short and long fibre amosite asbestos. Bars show mean (SEM) of four separate experiments.

naked long fibres (mean (SD) 0·8 (2·45) nM less than background). Opsonised long fibres, however, produced a substantial stimulation of superoxide release with a mean (SD) of 19·4 (8·46) nM more than background. The ANOVA showed a significant effect of opsonisation: $F = 68{\cdot}27$, $P < 0{\cdot}001$.

When fibre length was considered across all experiments there was an evident and highly significant effect, $F = 14{\cdot}34$, $P < 0{\cdot}001$ that confirmed the increased stimulatory potential of long fibre amosite.

Figure 4 shows the amount of radiolabelled opsonin binding to the short and long fibre samples. It is clear that the short fibres bound much less opsonin than the long fibre sample: $F = 18{\cdot}18$, $P < 0{\cdot}001$.

Discussion

Our study has shown that, in an unopsonised state, neither long nor short fibres of amosite asbestos were effective in stimulating rat alveolar macrophages to release superoxide anions. In fact, unopsonised short amosite seemed to inhibit superoxide release, although this effect was small. There was no obvious toxicity to the alveolar macrophages with any treatment, as assessed by the percentage of total cellular lactate dehydrogenase released into the culture medium during incubation (data not shown). This suggests that short amosite may be capable of inhibiting the membrane NADPH oxidase. This remains speculative, however, and was not pursued.

The dramatic enhancement of release of superoxide anions found when the long fibre amosite was opsonised with IgG, confirmed the greatly increased biological activity after opsonisation,[11 14 15 20] presumably through an increase in the second messenger system of the macrophage induced by Fc receptors.[20]

Of the 13 dust treatments in the dose response from unopsonised long amosite fibres, five showed slight stimulation, two showed virtually no effect, and six showed a slight decrease in superoxide production. We deduce from this that the unopsonised long amosite had no net effect on superoxide release.

Even opsonised short amosite consistently provoked a modest oxidative burst. All data were expressed as release of superoxide anions per unit mass, as very little of the short amosite was present as WHO defined fibres (>5 μm long, <3 μm diameter with an aspect ratio >3:1).[18] Our study confirms the importance of long fibres in the mediation of biological effects, as the long fibres were substantially more able to stimulate superoxide release than the short. It seems likely that the activity that was present in the short fibre sample was mediated by the few fibres that were longer than 5 μm.[18]

One of the most distinctive aspects of the biological effects induced by fibres is the difference that can be found both between dusts and between different samples of the same dust. Some studies indicate that the variations in response reflect the physical characteristics of the minerals involved, such as shape, diameter, fibre length, or surface area,[4 18 23] whereas others have implicated the surface charge or chemical composition.[17 24–28]

The increased biological activity of the long fibre sample in this study could be explained by increased binding of opsonic IgG to the fibre surface as, at equal mass, the long fibre asbestos bound threefold more IgG than the short fibres. Earlier studies with these two samples of amosite suggested that the short fibres (obtained by ball milling the long fibres) were identical elementally, crystallographically, and chemically to the long fibres. No previous effort was made to compare the surface reactivity of the two samples. Our study has shown that the surface of the short fibres is different from that of the long fibres in terms of ability to bind the opsonin IgG. Indeed, the short fibres might have been predicted to bind more protein than the long, because of the increased surface area engendered by the extra number of ends. The process of ball milling could have contaminated the surface of the short fibre sample, or exposed different crystal faces, which may differ from the surfaces of the long sample.

Previous work with this amosite has shown increased pathogenicity,[18] inflammatory potential,[19] and ability to cause release of tumour necrosis factor,[20] in the long fibre sample compared with the short. Our study suggests that an additional factor, surface reactivity, could be important in mediating the increased activity of the long fibre sample, over and above its greater average length. Ball milling is a process that might be expected to cause changes in surface reactivity due to the violent interaction between the fibres and the components of the ball mill during the process of milling. To obtain long and short amosite samples that are similar, a less energetic method would seem to be necessary.

Several groups have attempted to modify the surface of asbestos fibres to alter their bioactivity in vitro and, as fibres deposited in the lung are immediately exposed to pulmonary surfactant, the opsonins present in alveolar lining fluid have attracted much attention.[11 14 15 20 29] A normal component of lung lining fluid, IgG,[30 31] is increased 10-fold in rats exposed to chrysotile asbestos.[32] Our study confirms that fibres that deposit in the lung fluid will increase in ability to generate reactive oxygen species, and to become more pathogenic. Furthermore, deposition in the lungs of people with existing inflammation from any cause, could lead to more biologically active fibres, than deposition in the lungs of normal people with normal concentrations of pulmonary IgG.

Previous studies have shown that proteins (including components of complement,[33] immunoglobulin, and other globular proteins[34]) bind to the surface of asbestos fibres. There is evidence that there could be differences in the site of adsorption that lead to competition between proteins for access to binding sites when fibres are placed in a solution of different proteins.[20] Further work is

required to investigate the lung lining fluid, which is a complex mixture of protein and lipid,[35] to find the relative importance of opsonisation in vivo.

No work was done to characterise the mechanism of increased release of superoxides found in our study with opsonised long fibre asbestos. We presume that the protein kinase C system of the cell is involved, as shown for other similar activities of opsonised fibres, such as release of superoxide anions[14] and release of tumour necrosis factor.[20]

In conclusion, our study has confirmed the importance of opsonisation in modulating the biological activity of fibres at the cellular level, but it has also pointed out important differences in binding of a protein to samples of fibres previously considered to be identical apart from length. The differential binding may explain the biological activity of the fibre. This factor needs to be taken into account when fibres are compared, as binding of biological molecules in vivo may be an important modifying factor in the pathological processes initiated by fibres.

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Superoxide anion release by alveolar macrophages exposed to respirable industrial fibres: modifying effect of fibre opsonisation*

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With 3 figures and 1 table

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Key words: Superoxide anion; Alveolar macrophage; Macrophage, alveolar; Fibres, industrial; Industrial fibres; Opsonisation, fibre; Amosite asbestos; Asbestos, amosite; Radicals, free; Free radicals.

Summary

Phagocyte-derived free radicals are considered to play a role in fibre-related pathology and the components of the lung lining fluid could modify the surface of fibres. Therefore we examined the ability of long amosite asbestos and a range of manì-made fibres to stimulate release of superoxide anion from rat alveolar macrophages when they were in their native form (unopsonised) and opsonised by incubation in rat Immunoglobulin G. We also assessed the specific amount of opsonin adsorbed to each fibre type. In the uncoated form all of the fibres produced modest amounts of superoxide release from macrophages. When they were opsonised however there was an effect on stimulation of release of superoxide that was fibre-specific. Both MMVF21 and RCF 1 were dramatically enhanced in their ability to stimulate release and this was related to a high affinity of their surface for IgG. Code 100/475 and SiC were not substantially affected by opsonisation and this was reflected in their low affinity for IgG. Long amosite had low affinity for IgG but showed dramatic enhancement of capacity to stimulate superoxide release. These fibre-specific differences in the effect of a coating of material that is found in the lung lining points out the problems of interpretation of *in vitro* data and more work on this important area is warranted.

Introduction

Reactive oxygen intermediates (ROI) are considered to be important in the pathogenesis of fibre-related disease (DONALDSON et al. 1994). These could be derived from the fibre surface or from inflammatory leukocytes that are recruited to the sites of fibre deposition and tissue injury. In regard to the latter source we previously examined the ability of long amosite asbestos to stimulate release of superoxide from rat alveolar macrophages and found an important enhancing effect of rat immunoglobulin, an opsonic component of the normal lung lining fluid (HILL et al. 1995). We have therefore gone on to examine a range of respirable fibres for ability to stimulate the release of superoxide from macrophages, for native fibres and fibres incubated in the opsonin.

Material and methods

Fibres: The fibre used were the amosite asbestos fibres used previously (DAVIS et al. 1986) refractory ceramic fibre 1 (RCF1), and man-made vitreous fibres 21 (MMVF 21), were obtained from the TIMA Fibre Repository; Code 100/475 glass microfibre and Silicon carbide were as used in the Colt Programme of the Institute of Occupational Medicine. All of the fibres were 100 % respirable. Fibres were counted by Phase Contrast Light Microscopy by WHO rules. A summary of the length distributions of the fibres are shown in the table:

	% of fibres greather than length		
Fibre	10 µm	15 µm	20 µm
Code 100/475	50.0	33.3	19.3
MMVF 21	85.2	79.5	67.2
Long amosite	64.8	50.4	35.2
Silicon carbide	60.9	43.4	27.6
RCF 1	77.4	65.4	45.3

Fibres were incubated in rat immunoglobulin and spun washed along with control fibres that were incubated in saline but similarly spun-washed to correct for any losses. For

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quantification of binding of IgG, rat IgG was incubated with ^{125}I-IgG and the fibres treated as above, before measuring the specific radioactivity of 3 million fibres.

Superoxide anion: Superoxide anion release from control rat alveolar macrophages was measured as described previously (HILL et al. 1995).

Results

Although dose responses were carried out, in the interests of brevity figure 1 shows only the data for the amount of superoxide anion released by rat alveolar macrophages in response to a single dose of 3 million unopsonised or opsonised fibres. There are clear differences in the effect that opsonisation has, with amosite, MMVF21 and RCF1 all causing substantial enhancement of release.

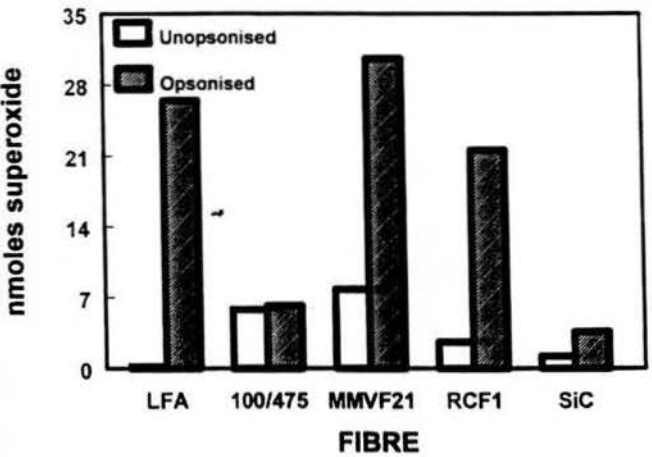


Fig. 1. Release of superoxide anion from rat alveolar macrophages treated with 3 million unopsonised or opsonised fibres of the 5 different fibre types. LFA = long amosite, 100/475 = Code 100/475 glass microfibre, SiC = Silicon carbide. See text for further details of the fibres. Data is the mean of at least 3 experiments.

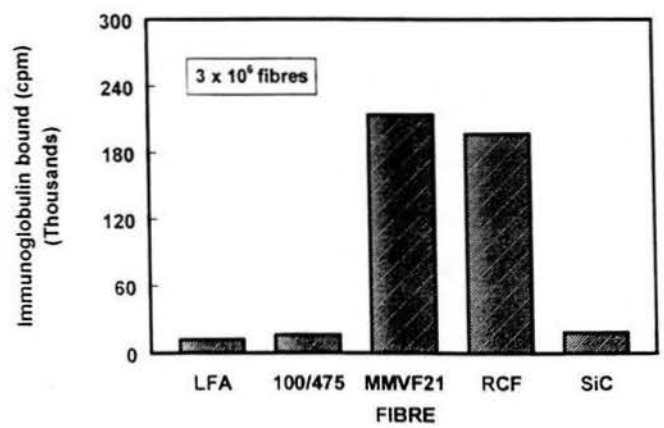


Fig. 2. Amount of radioactive IgG bound to 3 million of the various fibre types. Data is the mean of at least 3 experiments.

Figure 2 shows the amount of ^{125}I-IgG that bound to 3 million fibres of each type of fibre. MMVF21 and RCF showed the greatest affinity for IgG.

Figure 3 shows, for 3 million fibres, the amount of superoxide released in relation to the amount of IgG bound to the surface. There is a good relationship between the two except in the case of long amosite which shows much more activity that would be predicted from the amount of opsonin bound.

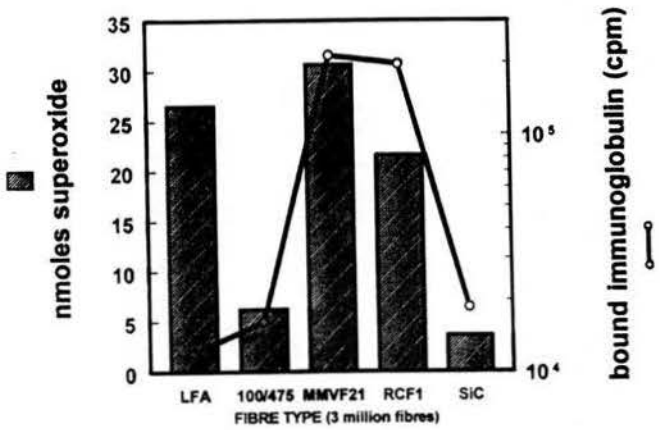


Fig. 3. For 3 million fibres, the figure shows the relationship between the amount of IgG bound (line) and the ability to stimulate release of superoxide anion from macrophages (bars).

Discussion

In the unopsonised form, all of the fibres showed modest ability to stimulate release of superoxide from macrophages. On opsonisation, however, long amosite, MMVF21 and RCF1 all showed dramatic enhancement of this property. Having found differences between different fibres in terms of enhancement of ability to stimulate superoxide release following opsonisation with IgG, we hypothesised that the ability to stimulate superoxide anion release following opsonisation would be a function of the amount of opsonin bound. This proved to be the case for 4 out of the 5 fibres but long amosite appeared to be quite different from the other fibres in terms of a simple explanation for the effects of opsonisation.

Thus the 5 fibres could be divided into 3 categories:

1) MMVF21 and RCF 1 – fibres whose biological activity could be substantially enhanced by opsonisation most likely related to the high affinity of the fibre surfaces for opsonin.

2) Code 199/475 and SiC – fibres whose biological activity was barely changed by opsonisation, no doubt a consequence of the low affinity of the fibre surface for opsonin;

3) Long fibre amosite – a fibre that was dramatically increased in activity by opsonisation but had very low affinity for opsonin.

We believe this to be the first example of fibre-speci-

fic differences in modulability of the biological activity of a range of fibres by a simple modification of the fibre surface by a normal component of the lung lining fluid.

There is no simple correlation between the ability of the panel of fibres used here to be enhanced in their biological activity by opsonisation, and the *in vivo* pathogenicity of these different fibres in different animal studies, nor would we expect there to be. The complex nature of the lung lining fluid milieu means that there should be numerous competitive interactions for binding at the fibre surface and this warrants further investigation. It does however point out the problems of interpreting *in vitro* data when there is no effort to mimic residence in the lung.

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CYTOGENETIC AND PATHOGENIC EFFECTS OF LONG AND SHORT AMOSITE ASBESTOS

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SUMMARY

This study utilized two samples of amosite asbestos which differ in their length, but not in their diameter and which have been shown previously to have very different abilities to cause pathology in rats exposed by instillation or inhalation. The activity of these amosite samples in causing chromosomal aberrations in Chinese hamster ovary cells in culture was examined, along with the effect of the glutathione (GSH) synthesis-inhibiting agent buthionine sulphoximine. The incidence of chromosomal aberrations in cells treated with the short fibre sample was similar to control levels; the long amosite sample caused significantly more chromosomal aberrations than the short fibre sample. When cells were treated with buthionine sulphoximine to decrease the levels of intracellular glutathione, the incidence of chromosomal aberrations was increased in the control cells, but also on treatment with both short and long amosite, the long sample again being considerably more active than the short. The pathogenicity of the long amosite may result from the ability of the fibres to cause chromosome damage, while the enhancement of this damage caused by decreasing intracellular glutathione suggests that the asbestos fibres may impose an oxidant stress on the cells which contributes to these aberrations.

KEY WORDS—asbestos; fibres; chromosome; pathology; glutathione

INTRODUCTION

The pathogenic effects of asbestos in humans and laboratory animals are well documented, with fibrosis, lung tumours, and mesothelioma amongst the major pathologies being produced.[1] The ability of asbestos and other fibres to damage chromosomes of cells in culture has been demonstrated[2] and these cytogenetic effects have been considered to be a likely factor in its toxicity, particularly its carcinogenic action. However, few laboratories have had the opportunity to study the pathogenic effects of defined fibre samples and, using the same samples, to examine their ability to cause chromosome damage.

In the 1980s, the Institute of Occupational Medicine in Edinburgh carried out a large-scale rat inhalation and instillation study on two amosite asbestos samples that differed only in their length. These studies demonstrated that the short and long samples had dramatically differing pathogenic potential, the long sample producing fibrosis, lung tumours, and mesotheliomas, whilst the short sample was virtually without activity.[3] Studies on the ability of the same two fibre samples to cause inflammation[4] again showed the long fibre sample to be many times more active than the short. We report here on the ability of these two amosite asbestos samples to cause chromosome damage to cells in culture.

We have previously been interested in the anti-oxidant activities of the free radical scavenger reduced glutathione (GSH) in defending the lung against the oxidant damage caused by cigarette smoke.[5,6] It has been suggested that asbestos may cause chromosome damage by oxidant

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radical-mediated pathways.[7] Buthionine sulphoximine (BSO), an inhibitor of the GSH synthetic pathway,[8] was therefore used to deplete GSH in the target cells and the effects of this treatment on the chromosome damage caused by amosite asbestos were assessed.

MATERIALS AND METHODS

Amosite asbestos samples

The long and short amosite asbestos samples utilized in these experiments have been used extensively and are described in detail elsewhere.[3] In brief, the short sample was derived from the long sample by milling and was found to be elementally and crystallographically identical to the parent sample. The majority of the short sample, by mass, did not classify as fibres by the WHO criteria; i.e., a particle longer than 5 μm, less than 3 μm wide, and with an aspect ratio greater than 3:1. In the short sample, of the material that was fibrous, less than 10 per cent was longer than 10 μm and none was longer than 20 μm. In the case of the long sample, which was virtually all fibrous by WHO criteria, 70 per cent of fibres were longer than 10 μm and 40 per cent were longer than 20 μm.

Chromosome-damaging effects of fibres

Chinese hamster ovary cells (CHO; ECACC Porton Down, Wiltshire) at 1×10^6 cells/ml of F10 medium + 10 per cent fetal calf serum (Sigma Chemicals, Poole, Dorset) were seeded onto 24-well plates and incubated overnight. The medium was replaced with 1 ml of 10 μg/ml of long or short amosite asbestos in medium and incubated for a further 48 h. Cultures were then treated with 0·1 μg/ml colchicine (Sigma) for the final 2 h of culture and trypsinized off the culture plates. The cells were then spun down and treated with 0·75 M KCl for 10 min and stained with acetoorcein. Chromosomal aberrations and hyperdiploidy were assessed in 200 metaphases in three separate experiments.

Buthionine sulphoximine

Buthionine sulphoximine (BSO) is a specific inhibitor of gamma-glutamyl cysteinyl synthetase, the enzyme which catalyses the rate-limiting step in the synthesis of glutathione (GSH).[8] The cells were cultured with added BSO (Sigma; 100 μg/ml) during the incubation with fibres and in the matched controls. We have found this level of BSO to decrease the intracellular concentration of GSH to less than 20 per cent of normal.[5]

Table I—Effects of amosite samples on chromosomes of cells in culture

	Control	Short amosite	Long amosite
Cells with aberrations (%)	4·5 (0·5)	6·3 (0·3)	12·1 (0·2) *
Hyperploid cells (%)	3·3 (0·3)	5·7 (0·3)	9·5 (0·5) ***
Mitotic index	7·5 (0·2)	6·3 (0·2)	4·5 (0·05) **

All values are the mean (SD) of 100 cells in three separate experiments. For short amosite versus long amosite, *denotes $P<0·05$; **denotes $P<0·01$; and ***denotes $P<0·001$.

RESULTS

Chromosomal and mitotic effects of long and short fibre amosite asbestos

The effects of the amosite samples on chromosomes of CHO cells in culture are shown in Table I. There was no significant difference between the background levels of chromosomal aberrations and the short level produced by short amosite, but the long amosite sample produced significantly more chromosomal aberrations and hyperploid cells (see tables for details of differences). In addition, the mitotic index was significantly less in the long amosite-treated cells. Amongst the aberrations seen were breaks, acentric fragments, dicentric chromosomes, translocations, ring chromosomes, and chromatid exchanges.

Effect of treatment with BSO

The effects of treatment of cells with BSO on chromosome aberrations in control cells and cells treated with amosite asbestos are shown in Table II. In the case of control, short amosite, and long amosite treatment, there were significantly more chromosomal aberrations in BSO treated cells than in untreated cells.

Table II—Effect of BSO on chromosome aberrations

	No BSO	BSO
Control	4·5 (0·5)	9·0 (0·5) **
Short amosite	6·3 (0·3)	16·2 (1·5) ***
Long amosite	12·1 (0·2)	18·4 (2·3) **

All values are the mean (SD) of 100 cells in three separate experiments. For comparison of BSO with no BSO. **denotes $P<0·01$ and ***denotes $P<0·001$.

DISCUSSION

This study compares two samples of amosite asbestos which differ in their length, with regard to their ability to cause chromosomal aberrations in CHO cells. Experiments using these two samples of asbestos have previously provided compelling evidence for the importance of fibre length in mediating the pathogenicity of asbestos fibres, showing that the long fibres were highly pathogenic whilst the short fibres were not pathogenic.[3,4] These data are summarized in Table III, where the increased pathogenicity of long amosite is evident from the increased incidence of tumours, fibrosis, and inflammation caused by this sample, compared with the short sample.

The present study set out to determine whether cytogenetic effects correlated with inflammogenic, fibrogenic, and tumourigenic activities. The number of chromosomal aberrations seen in cultured cells paralleled the pathogenic potential of the two samples, the long sample being substantially more potent than the short.

Table III—Results obtained from pathology studies* with the amosite samples

	Short amosite	Long amosite
Lung tumours (% by inhalation)	0	32·5
Fibrosis (by inhalation, arbitrary units)	0·5	11·0
Mesothelioma (% by instillation)	4	94
Inflammation score (instillation)	+	++++

*For further details see refs 3 and 4.

Many workers have described similar cytogenetic changes to the ones described here, in cultured cells treated with asbestos. The level of chromosome abnormalities seen in the control cells is similar to that found in other studies.[9] Although these changes may be premalignant, the fact that the cells are in continuous culture prevents any testing of the role of these changes in malignancy. Jaurand *et al.*[10] used chrysotile asbestos and rat pleural mesothelial cells and described up to 21 per cent of metaphase nuclei with abnormal chromosomes: similar results were described using chrysotile asbestos and Syrian hamster ovary cells.[9] Both crocidolite and chrysotile asbestos caused anaphase aberrations in human mesothelial cells in the study of Pelin *et al.*[11] The chromosomal aberrations seen with long amosite may be related to mutation of the p53 gene, which leads to an unstable karyotype and chromosomal aberration and has been demonstrated to be present after experimental asbestos treatment.[12]

Previous studies have shown a relationship between fibre length and pathogenicity.[13] For example, Stanton *et al.*[15] working with implanted fibres revealed an increasing ability to cause mesothelioma as the length approached 8 μm and over.[14] Davis *et al.*,[3] however, reported dramatic differences in the pathogenicity of long and short amosite samples when given by the physiological inhalation route as well as by intraperitoneal instillation in rats. Many fibres in the long amosite sample were in the 'Stanton range' (longer than 8 μm), whilst there were effectively no 'Stanton' fibres in the short fibre sample. In the elegant studies of Hesterberg and Barrett,[2] fibre length was found to be an important factor in the clastogenic activity of a range of fibre types. These workers observed that the longer fibres physically interfered with the migration of chromosomes at anaphase, although this was not observed in the present study. In the present study, the failure of the short fibre sample to have any effect on chromosomal aberration incidence compared with controls supports the idea that the longer a fibre is, the more likely it is to interfere with anaphase and to interact with chromosomes, leading to effects on ploidy and chromosomal integrity.

It is notable that the short fibre sample should, in fact, have a greater surface area than the long sample, in view of the number of ends that are present and the general comminution of fibres

produced by the milling process. Despite this, the long sample shows greater activity, which suggests either that surface area is not the main descriptor of pathogenicity or that there is a difference in the surface reactivity of the short and the long samples. Recent evidence from our own laboratory suggests that the latter may be the case and that there is differential reactivity of the short and long fibre samples, the short being less reactive;[15,16] this may be a result of the surface being altered during the ball-milling process.

Reactive oxygen species and free radicals have been shown, in many studies, to have a role in asbestos pathogenicity (reviewed in ref. 7). GSH is a ubiquitous anti-oxidant found in the lining fluid and cells of the lung and intracellular GSH is important in maintaining redox homeostasis.[5] Recent studies within our group have suggested that extracellular GSH can defend cells in culture against the oxidant/free radical injury caused to cells by cigarette smoke.[6] Treatment of the CHO cells with BSO, an inhibitor of GSH synthesis, increased the amount of background chromosomal damage and also increased the chromosomal damage caused to the cells by both short and long amosite. This suggests that free radicals have a role play in the production of background levels of chromosomal abnormality and that normal mitosis is redox-sensitive.

In addition, both long and short amosite appear to place an oxidative stress on the cells, since BSO treatment increased the levels of aberrations with both, to the same degree. This could be related to the iron mobilized from the amosite surface contributing to free radical generation,[7] although this might be anticipated to be the same for both the long and the short samples. However, we have recently found differences between the short and the long amosite samples in terms of surface affinity for protein,[15] which could be a result of the milling process used to derive the short from the long. The two samples, therefore, could differ in other surface reactivities, such as iron mobilization, which could underlie the effect seen here. It is clear, however, that some factor associated with long fibres poses a stress that leads to chromosomal aberrations.

The role of intracellular GSH in maintaining chromosome integrity is unknown, but GSH is a powerful mediator of redox balance within the cell; altered redox status, caused by oxidative stress, has an important impact on a range of proteins such as those of the cytoskeleton,[6] which could be important in leading to cytogenetic defects.

We have found previously that acute exposure of cells in culture to cigarette smoke causes a sharp fall in intracellular GSH.[7] The synergism between asbestos exposure and cigarette smoke in leading to lung cancer is well documented.[1] We postulate that in asbestos workers who smoke, cigarette smoke could lower the GSH levels in the epithelial lining of the lung and this could favour cytogenetic injury by asbestos fibres, leading to lung cancer.

In addition to the chromosomal effects of the long amosite sample described here, its pathogenicity *in vitro* may also be related to the previously described greater ability of the long sample to cause cytokine[17] or oxidant[12] release from macrophages or to cause detachment injury to epithelial cells,[18] compared with the short fibre sample.

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Effect of Long and Short Fibre Amosite Asbestos on *In Vitro* TNF Production by Rat Alveolar Macrophages: The Modifying Effect of Lipopolysaccharide

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Abstract: The influence of long and short fibre amosite on the generation of tumor necrosis factor (TNF) by rat alveolar macrophages was investigated *in vitro*. TNF rich supernatants were prepared from macrophages cultured in F10 medium +2% Bovine Serum Albumin (BSA). Spontaneously released TNF from unstimulated macrophages and TNF rich supernatants from macrophages exposed to Lipopolysaccharide (LPS) and fibres were stored at -70°C and then tested for their cytotoxicity towards L929 cells. Maximum spontaneously released TNF was obtained from 24 hour macrophage cultures. Short amosite fibres had no significant effect in stimulating alveolar macrophages to release TNF while the 50 μg dose of long fibres resulted in significantly increased release of TNF. Co-treatment of alveolar macrophages with LPS and fibres further enhanced the TNF production and maximum production was obtained with LPS +50 μg dose of long fibre resulted in significantly increased release of TNF. Co-treatment of alveolar macrophages with LPS and fibres further enhanced the TNF production and maximum production was obtained with LPS +50 μg of long fibre amosite. The present study indicates that fibre dimension is a major factor in *in vitro* dust activity and TNF has a possible active role to play in dust induced inflammation *in vivo*.

Key words: Amosite fibres — Tumor necrosis factor — Macrophages — LPS — L929 cell lines

Introduction

Exposure to asbestos fibres results in chronic inflammatory reactions and interstitial fibrosis leading to asbestosis[1—4]. However not all asbestos samples have the

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The study was carried out at Institute of Occupational Medicine, Cottage-12, City Hospital Edinburgh, EH10, 5SB, U.K.

same potential to cause damage to lungs. Studies carried out using asbestos of different fibre lengths have revealed that dimension is a major factor in disease development with long fibres generally being more pathogenic than short[2-5]. Studies have shown that long thin fibres are highly carcinogenic as well as more active in short term *in vitro* assays[5-7].

The exact mechanism by which long fibres induce lung disease is not well understood. However a number of reports suggest an important role for alveolar macrophages which deal with the deposited particles. In fibre exposed lungs macrophages accumulate at sites of fibre deposition and can be lavaged from the bronchoalveolar space in increased numbers[8]. Macrophages are an integral part of various aspects of immune response and play a pivotal role in cell to cell communication[9]. This is attained partly through the release of a range of mediators, including tumor necrosis factor (TNF). This factor is a well characterised pro-inflammatory protein produced by activated macrophages and other cells. It is also believed to play a role in fibrogenic activity of mineral dusts *in vivo*[10-15].

We have previously demonstrated that a long amosite asbestos sample has much more fibrogenic[2] and inflammatory[5] activity than a short fibre amosite sample. In view of the possible fibrogenic role of TNF it was considered worthwhile to look into the generation of TNF by alveolar macrophage in response to the long and short asbestos samples.

Materials and Methods

Animals

Inbred female Wistar HAN rats approximately 12 weeks of age, from the Institute of Occupational Medicine, animal unit were used throughout.

Dusts

Long and short fibre amosite was prepared from the commercially available amosite from South Africa. Nearly all the short fibres were below 10 μm while long fibres consisted of 40% greater than 10 μm and 15–20% greater than 20 μm[2, 16].

Bronchoalveolar Lavage (BAL)

Normal (untreated) rats were killed by intraperitoneal injection of nembutal. The animal was opened and trachea tied tightly with suture thread after inserting a canula into it. Lungs were dissected free from thoracic cavity along with the trachea. 8 ml of saline, warmed at 37°C, was slowly injected into the lungs using a 10 ml syringe. Lungs were massaged lightly for approx. 30–40 sec and the BAL pushed back into the syringe using minimum pressure. Lungs were lavaged thrice and pooled lung washings collected in pre-cooled tubes. The cell suspension was spun at 1,200 rpm for 10 minutes at 4°C.

Collection of Supernatants Containing Spontaneously Released TNF from alveolar macrophages

Cells were counted in a haemocytometer and suspended in culture medium consisting of F10 medium (Gibco, Paisley, U.K.) plus 2% bovine serum albumin (BSA, Sigma, Dorset, Poole, U.K.), 5% heat inactivated foetal calf serum (FCS, Gibco, Paisley). The cells were adjusted to a concentration of 1 x 10^6/ml. One ml of cell suspension (1 x 10^6 cells) was added into each of the 24 wells of tissue culture plates and incubated in a humidified 5% CO_2 incubator at 37°C. In the first set of wells (culture I), the cells were allowed to attach for 1 hr and supernatants removed. One ml of fresh medium was added to the same wells and the supernatents removed at intervals of 2, 4, 8, 24 and 48 h, adding fresh medium each time, thus collecting spontaneously released TNF rich supernatants. The supernatants from culture I thus respresent the release of TNF between 0 and 1 h, between 1 and 2 h, between 2 and 4 h, between 4 and 8 h, between 8 and 24 h, and between 24 and 48 h. Another set of wells containing the cell suspension (culture II) was not disturbed and supernatants were removed at 24 h. Fresh medium was added and supernatants removed after incubation for another 24 h. TNF rich supernatants were thus collected at 24 and 48 h only from the start of culture II. These supernatants comprise the TNF release between 0 and 24 h and between 24 and 48h. All the supernatants were spun at 3,000 rpm for 10 minutes and cell free aliquots were stored at -70°C till analysis for TNF.

Time course curve for spontaneous TNF release was plotted and the time of maximum spontaneous release determined. This time point was therefore chosen for all future experiments.

Collection of Supernatants Containing TNF Released in Response to Stimulants

One ml of macrophage cell suspensions (1 x 10^6) was added into each of the 24 well tissue culture plate and incubated as above for 24 h. Non adherent cells were removed and 1 ml of fresh medium having short and long amosite fibres at a concentration of 10, 25 and 50 μg/ml added. In another set of wells with attached cells, 1 ml of fresh medium supplemented with 100 ng/ml Lipopolysaccharide (LPS, Sigma L3880) plus long and short fibre amosite as above were added. The 24 well plates were incubated for 24 h. The cell free supernatants were stored as above, till analysis for TNF production in response to stimulants like LPS and long and short fibre amosite asbestos or without them.

Viability of Cells in Culture

The cell viability was assessed by trypan blue dye exclusion method both before and after treatment with stimulants by running a parallel culture.

L929 Cytotoxic Assay

The presence of TNF in macrophage supernatants was assessed by measuring

their ability to cause lysis of TNF sensitive L929 cell lines (mouse fibroblasts) obtained from the Department of Rheumatology, Northern General Hospital Edinburgh. The cytotoxicity of TNF was estimated following the method of Ruff and Gifford[17]. Briefly L929 cell lines were cultured in minimum essential medium (MEM, Gibco, Paisley) containing 5% heat inactivated FCS and antibiotics. The assay was carried out using the L929 cell suspension at a concentration of 0.3 x 10^6/ml in assay medium consisting of MEM and 5% heat inactivated FCS but without antibiotics.

The cell suspension was dispensed at a rate of 100 µl/well, into 96 well flat bottomed microtiter plates and incubated for 20 h in humidified 5% CO_2 incubator at 37°C. The unattached cells were removed, the cultures washed with assay medium and 100 µl of fresh medium containing 1 µg/ml actinomycin D (Sigma L3880) added. In experimental wells 50 µl of test substance (TNF rich supernatants) and 50 µl of 2 µg/ml actinomycin D were added. Serial dilution was done using a multichannel pipette. The microtiter plates were incubated for 20 h in 5% CO_2 at 37°C. The supernatant medium was discarded and staining of L929 cells carried out by adding 100 µl of 0.5% crystal violet in 20% methanol, filtered through a 0.22 µm filter. After 2 minutes, the cells were washed vigorously with tap water, dried and the optical density read in micro-ELISA plate reader (Dynatech Laboratories Inc., USA) at 540 nm, blanking in the cell free wells.

To verify that cytotoxicity was due to TNF, the effect of different dilutions (1:6400–1:4000) of anti TNF serum (a gift from Dr. S. Kunkel, University of Michigan) on alveolar macrophage supernatants was assessed. Control wells contained preimmune serum.

A standard TNF curve was constructed using human recombinant TNF alpha (rTNFa) (a gift from Dr. J. Symond, Department of Rheumatology, Northern General Hospital Edinburgh).

Units of activity were determined by probit analysis and comparison to TNF standard curve. The OD read is the % killing of the target L929 cells by rTNF. One unit of TNF activity = the amount of TNF required to lyse 50% of the target cells.

Results

Validity of TNF Assay and Abolition of TNF Activity by Anti TNF Serum

The addition of TNF to L929 cells caused a clear dose related loss of viability (Fig. 1). To relate the specificity of the cytotoxic activity released by alveolar macrophages to the presence of the TNF, specific anti TNF serum or pre-immune serum was added to alveolar macrophage supernatants. The alveolar macrophage supernatants with control serum showed appreciable amount of TNF activity (Fig. 2). The treatment of supernatants with anti-TNF serum completely neutralised the TNF activity, thus confirming the specificity of the assay for rat alveolar macrophage TNF.

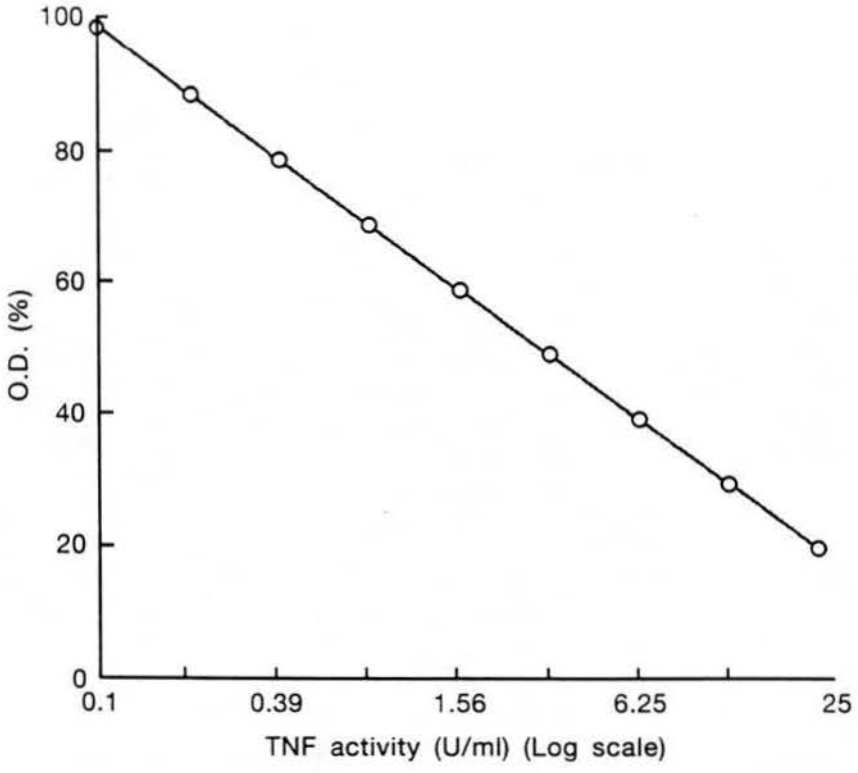


Fig. 1. Standard curve demonstrating the activity of human rTNFa in the L929 assay. The OD read is the % killing of the target L929 cells by rTNF. One unit of TNF activity = the amount of TNF required to lyse 50% of the target cells.

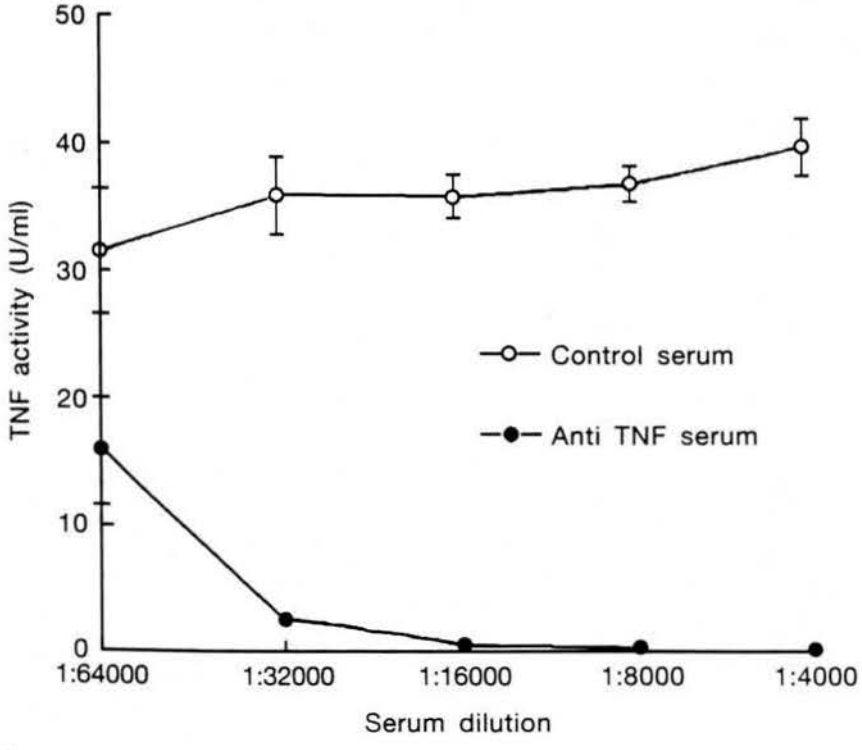


Fig. 2. Neutralisation of rat alveolar macrophage derived TNF by anti-TNF serum. No effect of pre-immune control serum.

Spontaneous Release of TNF by Alveolar Macrophages

The time course for production of TNF by alveolar macrophages showed that the maximum amount of TNF was released spontaneously between 2 and 8 h. Between 8 and 24 h and at 48 h there was no detectable release of TNF into the supernatants (Fig. 3). Supernatants obtained from macrophages cultured continuously for 24 h, without their medium being changed, showed a very large release of TNF (Fig. 3). This was more than twice the amount that would be anticipated by adding all the production at different intervals described in the method section. During 24 h to 48 h, there was no increase in detectable TNF in the supernatant.

Effect of in vitro exposure of long and short amosite fibres on TNF secretion by alveolar macrophages

TNF assay on supernatants from alveolar macrophages cultured for 24 h and then treated with 10, 25, and 50 μg/ml of long and short amosite fibres for a further 24 h revealed that short fibres had no significant effect in stimulating alveolar macrophages to release TNF. In contrast 50 μg of long fibres resulted in significant release of TNF (Fig. 4).

Co-treatment of long amosite fibres and LPS (100 ng/ml) further enhanced the secretion of TNF by alveolar macrophages (Fig. 5). Maximum TNF production was obtained from supernatants of alveolar macrophages stimulated with LPS and

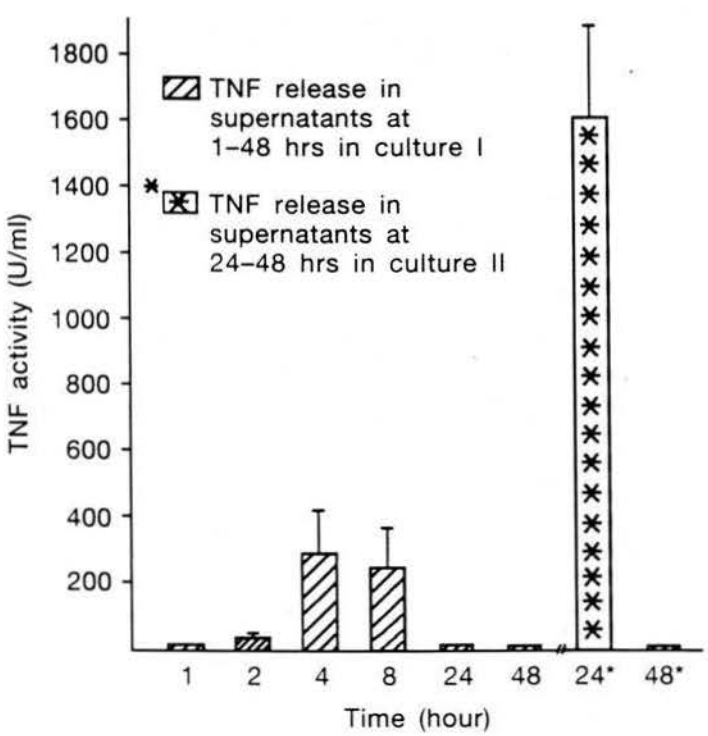


Fig. 3. Spontaneous TNF production by normal rat alveolar macrophages *in vitro*. The data represent the mean of triplicate readings from 4 separate experiments.

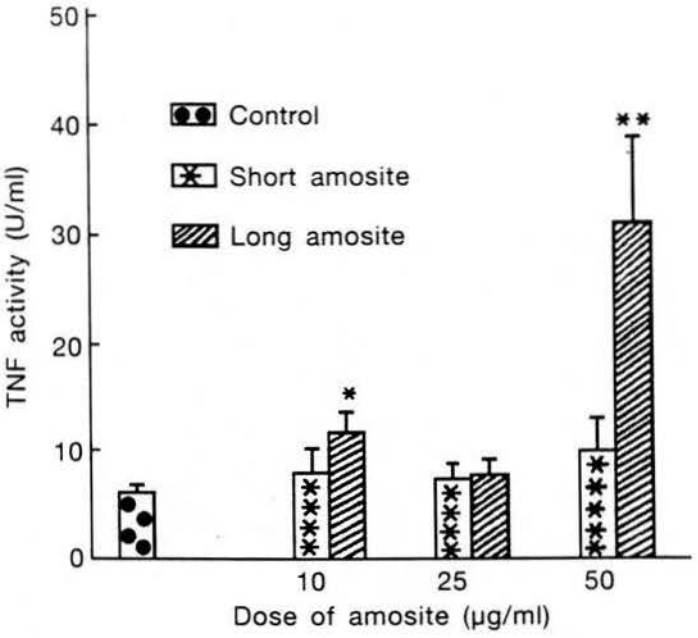


Fig. 4. Effect of different doses of long and short amosite fibres on TNF activity of rat alveolar macrophages *in vitro*, 1 x 10^6 cells cultured for 24 h at 37°C and then treated with dusts for 24 h.
The data represent the mean of triplicate readings from 4 separate experiments.
* $P \leq 0.05$, ** $P \leq 0.01$

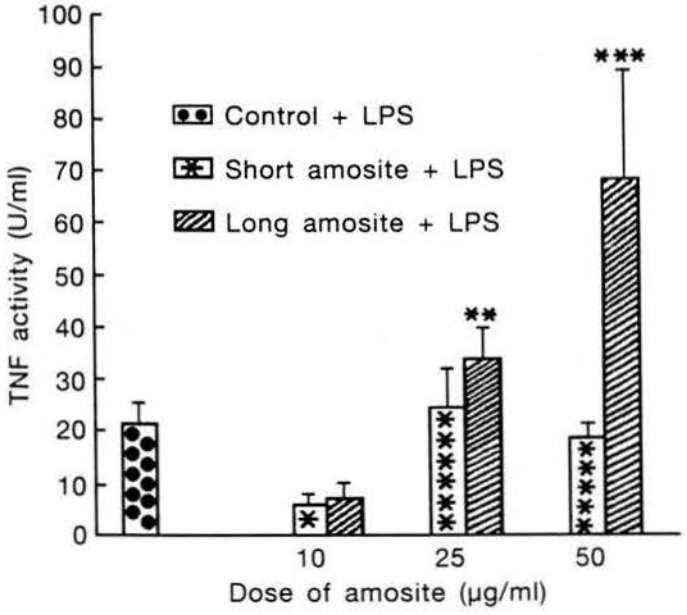


Fig. 5. Effect of LPS+ different doses of long and short amosite fibres on TNF activity of rat alveolar macrophages *in vitro*. 1 x 10^6 cells in 1 ml of F10 medium plus 2% bovine serum albumin supplemented with 100 ng/ml LPS cultured for 24 h and dusts (as in Fig. 4) for 24 h.
The data represent the mean of triplicate readings from 4 separate experiments.
** $P \leq 0.01$, *** $P \leq 0.001$.

50 μg of long fibres. Short amosite fibres had no significant effect on the release of TNF by LPS stimulated alveolar macrophages.

Effect of Amosite Asbestos on Viability of Macrophages

Bronchoalveolar lavage cells from untreated control rats comprised 90–95% macrophages while 88% were viable after 24 h of culture (Table 1). The treatment of macrophage cultures run in triplicate with long and short fibres for 24 h and treatment in combination with LPS resulted in only modest reductions in cell viability.

Table 1. % Viability of Alveolar Macrophages Cultured for 24 hours then treated with dust.

	% VIABLE CELLS	
Dust	No LPS	LPS
None	86 (±2.65)	88 (±1.0)
Short amosite	84 (±2.0)	84 (±2.65)
Long amosite	79 (±2.93)*	81 (±2.0)

% Viability of cultured cells was 88% at the start of the experiment.
The data represent the mean of triplicate readings.
* $p \leq 0.05$

Discussion

Direct interaction between dust particles and the alveolar macrophages is known to result in the release of TNF *in vitro* and the release of TNF due to cell-fibre interaction is believed to initiate inflammatory response following dust deposition in lungs[18]. In an earlier report we described fibre-length related ability of asbestos dust to simulate release of cytokine by alveolar macrophages[16] and that release is enhanced when fibres are opsonised with immunoglobulin-G (IgG).

The present study confirms that normal alveolar macrophages are a rich source of TNF and produced the cytokine spontaneously in culture, without addition of any stimulant. The specificity of the TNF released by rat alveolar macrophages was ascertained by using anti-TNF serum. It completely neutralized the TNF activity as demonstrated by lack of killing of L929 cells[19, 20]. The release of detectable TNF in supernatants without addition of stimuli can be attributed to cell handling and culturing procedures[21, 22]. In the present study appreciable TNF was spontaneously released between 4 and 8 h of culture but was not maximal as the cultures were disturbed by replacing the media at intervals of 2, 4, 8 and 24 h. This repeated handling and removal of TNF and other macrophage secretions from

the cell layer may interfere with autocrine stimulation loops, resulting in sub-optimal TNF release by alveolar macrophages. The likely importance of autocrine stimulation was further confirmed when maximum TNF production was obtained from macrophages cultured undisturbed for 24 hours. At 48 h no detectable TNF production was achieved. The production of TNF was therefore highest at 24 hours and was more than the total TNF obtained at 1, 2, 4 and 8 hours added together.

The treatment of alveolar macrophages with the long amosite fibres resulted in enhanced TNF release in the present study as previously reported[16]. The TNF production was greater from long amosite-treated macrophages whilst short amosite fibre-treated macrophages released equivalent amounts of TNF to untreated controls. The marked difference in stimulation by long and short fibre amosite *in vitro* can be correlated to a marked difference in their ability to cause disease *in vivo*[16]. The earlier reports attributed this difference to the incomplete phagocytosis of long fibres by macrophages. The non-specific receptors at the cell surface involved in phagocytosis are crosslinked and the extended surface of long fibres provides greater surface for crosslinking. This increased crosslinking might cause cell stimulation leading to increased TNF secretion by long fibre stimulated macrophages[23]. Long amosite fibres therefore cause more activation and show increased ability to produce injury and inflammation. The short fibres, on the contrary, may produce low levels of inflammation and less TNF is produced by short fibre-exposed macrophages[2, 5, 24]. Another explanation could be that the accumulation of macrophages at the site of inflammation initiated by asbestos, may increase the functional capability of macrophages contributing to increased elaboration of TNF[25] and our data suggestive of autocrine stimulatory factors in the supernatants support this contention.

The possibility of different adsorption of TNF onto long and short fibre amosite surface does not seem to be responsible for the observed different effects of long and short amosite. Our earlier experiments have revealed that increasing the opsonising concentration of IgG failed to even out the difference between the long and short amosite in the ability to stimulate cytokine secretion by alveolar macrophages[16].

The highest dose of 50 μg/ml of long amosite fibres resulted in remarkable enhancement of TNF activity in the present study, confirming that asbestos fibres increase TNF production in a concentration dependent fashion[3]. Co-treatment with long amosite and LPS further enhanced the TNF production by alveolar macrophages and maximum production was obtained with 50 μg/ml of long fibres and LPS. It has been postulated that priming of alveolar macrophages with LPS alters them physiologically and induces them to produce more TNF than untreated alveolar macrophages[19, 20].

The results obtained in the present paper have possible significance in individuals exposed to fibres and who have concurrent infection since the modifying effects of a bacterial product such as lipopolysaccharide could alter the cytokine response

to the dust. Evidence that this indeed occurs with some dust-induced lung disease can be found in the work of Chiappino and Vigliani[26] who reported that the low level infection associated with conventional housing substantially enhanced the response to silica compared to speficic-pathogen-free-housed rats. Previously Powell and Gough[27] had reported a more florid fibrotic response to quartz in rabbits with concomitant lung inflammation due to horse serum hypersensitivity. A potential mechanism for these effects is the production of autostimulatory cytokine by the macrophages since Driscoll *et al.*[28] demonstrated that gamma interferon could dramatically enhance the TNF secretory response of macrophages to mineral dust. Since macrophages can release interferon[29] there may be an autocrine-mediated effect on the macrophages via LPS-mediated interferon production. Taken together with undoubted importance of TNF in experimental silicosis[13] the present findings may be important in explaining individual difference in response to fibre. Thus individuals who had infection/immune responses in their lungs, even at a low level, could experience heightened responses to asbestos fibres compared to normal, individuals.

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Section 3

Section 3 Studies on inflammatory leukocytes elicited by particles

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Characteristics of Peritoneal Macrophages Induced by Asbestos Injection

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The effect of single intraperitoneal injections of UICC crocidolite, UICC chrysotile, and a latex control particulate on the induced murine peritoneal macrophage population was measured. Spreading, Fc receptor avidity, and phagocytosis were measured 3, 18, and 70 days after injection. When activation of the induced macrophage populations was found at all three times with asbestos, but not with latex, experiments were undertaken to determine whether the asbestos-activated macrophages had attained the full tumoricidally activated state. An *in vitro* assay measuring macrophage cytotoxicity to tumor cells revealed that the tumoricidal potential of asbestos-activated macrophages was low at 3 and 5 days and negligible by 35 days after injection. An *in vivo* assay measuring the effect of asbestos on tumor growth generally supported the contention that asbestos-activated macrophages were not tumoricidal, although one dose of UICC chrysotile did produce a small, significant reduction in growth of a concomitant tumor. It was concluded that a single intraperitoneal asbestos injection in mice induces activated macrophages which do not become fully tumoricidal.

INTRODUCTION

The pathology associated with asbestos exposure in man and animals is well documented and includes lung fibrosis, mesothelioma, and carcinoma of the lung (Harington *et al.*, 1975; Becklake, 1976; Selikoff and Lee, 1978). Additionally, disturbances of the immune system have been detected in asbestos workers (Kagan *et al.*, 1977; Haslam *et al.*, 1978; Lange, 1980), although the place of these disturbances in the etiology of asbestos-related disease is speculative.

The alveolar macrophage, due to its intimate interaction with foreign particulates during their clearance from the lung, has been intensively studied as to its possible role in these diseases (Miller, 1978). The toxic effect of asbestos, particularly chrysotile, on normal macrophages in culture has been compared to the action of silica and a model of asbestos-induced lung fibrosis has been evolved whereby macrophages, damaged and dying following asbestos phagocytosis, release hydrolytic enzymes and cell contents leading to stimulation of fibrogenesis (Allison, 1971). This model was subsequently modified when it was shown that normal macrophages in culture could be stimulated by low levels of asbestos to selectively release hydrolytic enzymes without cell death (Davies *et al.*, 1974). Evidence that this stimulatory, rather than toxic, effect of asbestos was operative *in vivo* was obtained by Miller and Kagan (1976), who found activation of the alveolar macrophages of rats inhaling crocidolite asbestos. Bateman *et al.* (1980),

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using an *in vivo* diffusion chamber containg macrophages and chrysotile asbestos at a dose found by Davies *et al.* (1974) to be stimulatory but not toxic, found evidence that a diffusible fibrogenic factor was released from the asbestos-treated macrophages.

Miller (1978) emphasized the likely importance of the secretory products of asbestos-activated macrophages in chronic asbestos inflammation, while the multiple roles of the macrophage in both humoral and cellular immunity (see reviews in Nelson, 1976; and Unanue and Rosenthal, 1980) suggest that immunological factors may also be important. The macrophage is also important in tumor development (see papers in James *et al.*, 1977) and so the role of the asbestos-activated macrophage in asbestos tumorigenesis requires investigation.

Although peritoneal macrophages differ in some properties from alveolar macrophages (Walker, 1976), they have been used as convenient alternatives in studies of the pathogenesis of asbestos-induced fibrosis (Miller, 1978). Injection of dust into the peritoneal cavity has also been used frequently as an alternative to inhalation since fibrosis and mesothelioma can also be produced in the peritoneal cavity using suitable dusts (Selikoff and Lee, 1978).

The experiments reported here were undertaken as part of a study into the effects of asbestos on cell membranes. The effect of a single intraperitoneal injection of asbestos on spreading activity, Fc receptor avidity, and phagocytic activity was measured since these properties of the macrophage membrane are known to be altered on activation (Cohn, 1978; Rhodes, 1975). The asbestos-induced peritoneal macrophages were found to be activated by the criteria of enhanced spreading and Fc receptor avidity. In view of the suggestion (Cohn, 1978; Hibbs *et al.*, 1980) that activation is a sequential development of properties leading to the fully microbicidal tumoricidal macrophage and since in the present study activation was present as measured by the criteria of spreading and Fc receptor avidity, the tumoricidal potential of the asbestos-activated macrophages was measured using an *in vitro* assay. An *in vivo* assay measuring the effect of intraperitoneal asbestos on the growth of a concomitant subcutaneous (s/c) tumor was also used.

MATERIALS AND METHODS

General. Male CBA/Ca mice 10–14 weeks of age at the time of injection were used throughout. Groups of three mice were injected intraperitoneally with (a) 2.5 mg of crocidolite asbestos (UICC sample) in 1 ml of Dulbecco A; (b) 2.5 mg of chrysotile asbestos (UICC sample) in 1 ml of Dulbecco A; (c) 1 ml of 1% latex (Bacto-latex, 0.81-μm diam, Difco); and (d) 1 ml of Dulbecco A. Groups of animals were killed 3, 18, and 70 days after treatment. Two separate experiments were carried out and the results were pooled.

Preparation of peritoneal exudate macrophages. Mice were killed by ether overdose at the selected time points and the peritoneal cavity was washed out with 6 ml of RPMI 16/40 medium containing 10 U/ml Heparin. The peritoneal exudate cells (PEC), which were always >95% viable by trypan blue exclusion, were washed and adjusted to 1×10^6/ml in RPMI containing 10% fetal calf serum (FCS). One hundred microliters of this suspension were dropped onto 6 × 22-mm coverslips which were incubated on racks for 1 hr at 37°C to allow for attachment of

macrophages. Before they were used in any assay these coverslip cultures were washed vigorously in a large volume of phosphate-buffered saline (PBS) to remove nonadherent cells. Cultures for the spreading assay were stained immediately after the 1-hr incubation and the other assays were carried out with 4 hr.

Spreading assay. After exactly 1 hr of incubation washed coverslip cultures were placed in May Grünwald solution and a May Grünwald/Giemsa stain was carried out. The number of spread macrophages in a total of 200 cells was expressed as a percentage.

Fc receptor assay. Sheep red blood cells (SRBCs) (Oxoid) sensitized with a 1:10 or 1:1000 solution of anti-SRBC serum, or 1:10 normal rabbit serum, were prepared at 1% in RPMI. One hundred microliters of sensitized SRBCs were added to coverslip cultures and left for 30 min at room temperature. Coverslips were washed carefully and inverted on a drop of toluidine blue on a glass slide. More than two red cells attached to a macrophage constituted a rosette and the number of rosette-forming cells in 200 cells was expressed as a percentage. Nonspecific rosettes demonstrated with normal rabbit serum-coated SRBC were present as a background of 7.6 ± 2.2% (SD).

Phagocytosis assay. One hundred microliters of 1% latex were added to coverslip cultures and incubated at 37°C for 1.5 hr. Coverslips were then vigorously washed in PBS to remove free latex and the coverslip was inverted on a drop of toluidine blue on a glass slide. The number of cells which had phagocytosed latex in a total of 200 cells was expressed as a percentage.

In vitro assay of macrophage cytotoxicity to tumor cells. The method used was essentially the radioactive release assay described by Mantovani *et al.* (1980) with the modification of Poste (1979), where the radioactivity associated with the target cells at the end of the assay period was measured. Cultured CCH_1 fibrosarcoma target cells (James *et al.*, 1979) were labelled by culturing in RPMI with 10% FCS, antibiotics, and 30 μCi [^{3}H]thymidine. Cells maintained in this way had a specific activity of 10,000–30,000 cpm/10^6 cells and spontaneous loss of activity over the 2 days of the assay was around 10%/day. To obtain the macrophage effector cells, PEC were collected 3, 5, and 35[1] after saline or asbestos injection and 10^6, 5×10^5, 10^5, and 5×10^4 cells were seeded into triplicate wells of Falcon 24-well plates. Wells were washed after 1 hr to remove nonadherent cells. 10^5 washed and labelled target cells were then added to each well yielding ratios of initial PEC to target cells of 10:1, 5:1, 1:1, and 0.5:1. After 2 days of culture 0.2% ethylene diamine tetraacetic acid (EDTA) was added to detach the tumor cells and their removal was further aided by pipetting. The EDTA-released tumor cells were harvested onto glass fiber paper in a cell harvester (Skatron) and the amount of [^{3}H]thymidine associated with the tumor cells was assessed by scintillation

[1] Previous data have revealed that induction of the tumoricidal response in macrophages following stimulation is an early and rapid event, e.g. 3 days for ip *C. parvum* (Flexman and Shellam, 1980 *Brit. J. Cancer* **42**, 41–51) and 4 days for subcutaneous glass coverslip (Poste, 1979). We therefore expected any tumoricidal macrophage induction by asbestos possibly by Day 3 and certainly by Day 5. Induction of the tumoricidal state suddenly, a long time after initial stimulus, has no precedent known to us: choice of the 70-day time point instead of the 35-day one would therefore have provided no additional information.

counting. As a positive control 5-day *Corynebacterium parvum* (1.4 mg heat killed, Wellcome Laboratories)-induced macrophages were included in all experiments. Since asbestos was always injected in saline suspension and saline alone was found to induce a small amount of tumor cell cytotoxic activity in the peritoneal macrophage population, specific toxicity to tumor cells was calculated as follows for each ratio:

$$\% \text{ specific toxicity to tumor cells} = 100 - \left[\frac{\text{tumor cell bound counts following culture with asbestos-induced macrophages}}{\text{tumor cell bound counts following culture with saline-induced macrophages}} \times 100 \right].$$

Three separate experiments were carried out for each time point.

The effect of intraperitoneal asbestos injection on the growth of a concomitant subcutaneous tumor. As an adjunct to the *in vitro* assay of macrophage cytotoxicity to tumor cells an assay designed to detect any effects of asbestos on tumor growth *in vivo* was utilized. Various amounts of asbestos, saline, or *C. parvum* were injected intraperitoneally either 2 days prior to, or 3 days after, subcutaneous inoculation with 10^6 or 5×10^2 CCH_1 fibrosarcoma cells. Groups of ten mice were used for each condition and tumor growth was measured as the mean of the two major diameters of the tumor.

Analysis of results. Results were analyzed for significance using the *t* test.

RESULTS

The mean number of cells in the control peritoneal exudate was approximately 4 $\times$ 10^6 throughout and the latex-induced exudates never differed from this level (Fig. 1). The asbestos-induced exudates, however, contained approximately five times the control number of cells at Day 3, had fallen to control values at Day 18, and had risen again to approximately three times control levels by Day 70. Be-

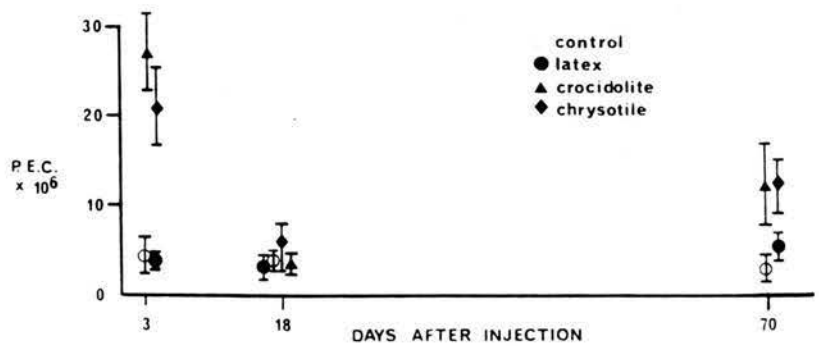


FIG. 1. Mean number of peritoneal exudate cells harvested from mice at various times after intraperitoneal injection of 1 ml saline (control), 1 ml 1% latex, 1 ml/2.5 mg UICC crocidolite or 1 ml/2.5 mg UICC chrysotile. Bars denote ± one standard deviation.

tween 50 and 70% of the cells in all exudates were macrophages by adherence difference; the remainder of the cells were polymorphs, which were increased at 3 days, and lymphocytes, with a few mast cells also present.

Both chrysotile and crocidolite injection resulted in the presence of activated macrophage populations in the peritoneal cavity at all three time points as judged by increased spreading (Fig. 2) and increased ability to form Fc rosettes with lightly sensitized SRBCs (Fig. 3). Spreading activity of asbestos-induced macrophages was maximal at 3 days, declining thereafter, but at all three time points the percentage spread was still significantly greater ($P = < 0.02$, see Fig. 2 for details) than that of saline-induced macrophages. Fc receptor avidity of asbestos-induced macrophages, demonstrated by increased ability to bind and form rosettes with lightly (1:1000 dilution of anti-SRBC) sensitized SRBC, remained similar at all three time points, being significantly greater ($P = < 0.05$, see Fig. 3 for details) than that of latex- or saline-induced macrophages. The latex-induced macrophages were never different from the saline-induced macrophages in any parameter except for the 3-day Fc receptor avidity which was significantly greater ($P = < 0.05$). The mean proportion of macrophages able to phagocytose latex was decreased on Day 3, but only significantly reduced ($P = < 0.05$) for crocidolite (Fig. 4). At succeeding time points, however, all macrophage populations had the same proportion, approximately 95%, of cells able to phagocytose latex. At Day 3, and to a lesser extent at Day 18, phagocytosed asbestos fibers were clearly visible inside crocidolite-induced macrophages and also inside chrysotile-induced macrophages, although chrysotile is less easily seen by light microscopy. The presence of such an ingested fiber load at Day 3 may have rendered some macrophages refractory to further phagocytosis during the latex phagocytosis assay.

The *in vitro* assay for macrophage cytotoxicity to tumor cells revealed that, compared to the *C. parvum*-elicited positive control macrophages, asbestos was relatively ineffective in inducing macrophage tumor cell cytotoxic activity (Fig. 5).

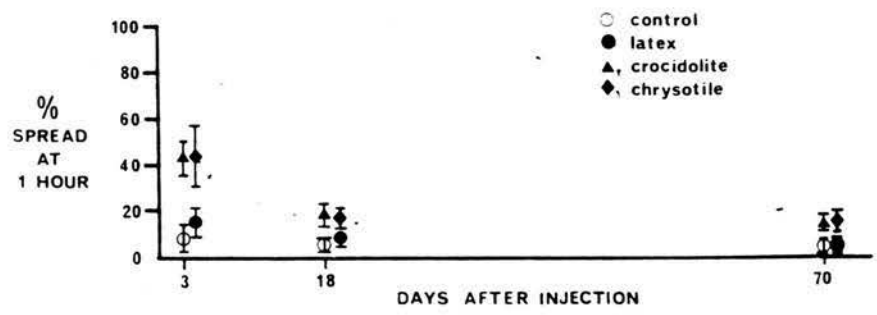


FIG. 2. Mean percentage of macrophages spread after plating onto glass coverslips and incubating for 1 hr at 37°C. Macrophages from PEC induced as in Fig. 1. Bars denote ± one standard deviation. At all three time points there was a statistically significant increase in the spreading of crocidolite- and chrysotile-induced macrophages compared to saline-induced macrophages. Levels of significance: 3-day crocidolite and chrysotile, $P < 0.01$; 18-day crocidolite and chrysotile, $P < 0.01$; 70-day crocidolite, $P < 0.002$; 70-day chrysotile, $P < 0.02$.

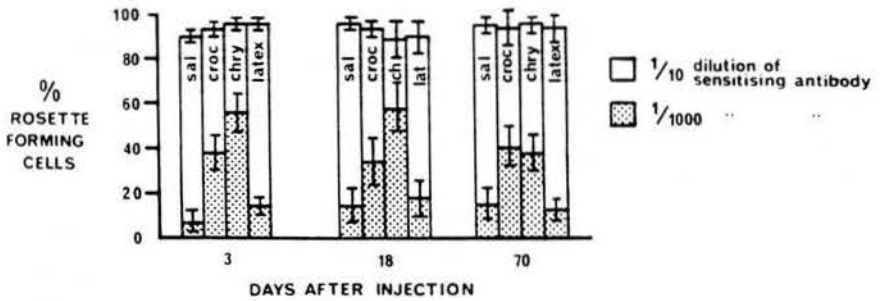


FIG. 3. Mean percentage of macrophages forming Fc rosettes with SRBCs coated with 1:10 and 1:1000 dilutions of anti-SRBC serum. Macrophages from PEC induced as in Fig. 1. Bars denote ± one standard deviation. At all three time points crocidolite- and chrysotile-induced macrophages had a statistically significant increase in the percentage able to form rosettes with lightly sensitized SRBC compared to saline-induced macrophages. Levels of significance: 3-day crocidolite and chrysotile, $P < 0.01$; 18-day crocidolite, $P < 0.05$; 18-day chrysotile, $P < 0.01$; 70-day crocidolite and chrysotile, $P < 0.02$. At 3 days latex also produced a significant increase ($P < 0.05$) in this parameter over saline-induced macrophages.

At 3 days, with chrysotile and crocidolite, macrophages had mean toxicities between 10 and 15%, while 5-day chrysotile-induced macrophages had attained a mean of 20% toxicity; 5-day crocidolite macrophages had only 5% mean toxicity. By 35 days the two asbestos types had very low cytotoxicities to the target cells. The highest toxicities obtained were always at the greatest effector: target ratio of 10:1. In this system 5-day *C. parvum*-induced macrophages produced a mean from all experiments of 27.4% toxicity at 5:1 and 54.0% toxicity at 10:1.

The low level of tumor cell cytotoxic activity induced by intraperitoneal asbestos was reflected in the results of the *in vivo* assay measuring the effect of intraperitoneal asbestos on the growth of a subcutaneous tumor (Table 1). Treatment with chrysotile 3 days after subcutaneous inoculation with 10^6 tumor cells was the only regimen which produced small but significant reductions in tumor size compared with saline-injected controls; this result was obtained in two separate experiments. The same regimen but with 2.5 mg or 20 mg chrysotile given

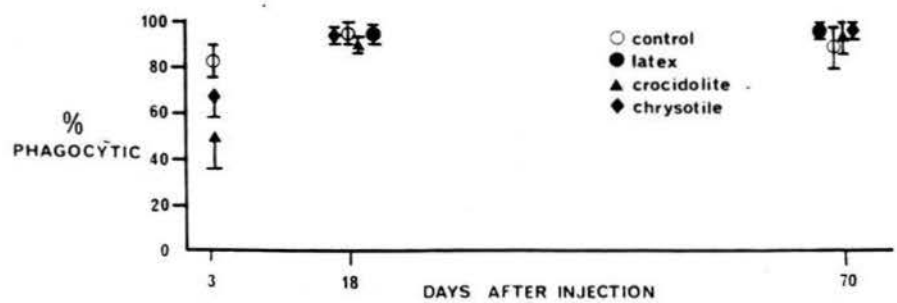


FIG. 4. Mean percentage of macrophages which had ingested latex particles following incubation for 1.5 hr in 1% latex. Macrophages from PEC induced as in Fig. 1. Bars denote ± one standard deviation. At 3 days crocidolite-induced macrophages had a significantly smaller ($P < 0.05$) population able to phagocytose latex than did saline-induced macrophages.

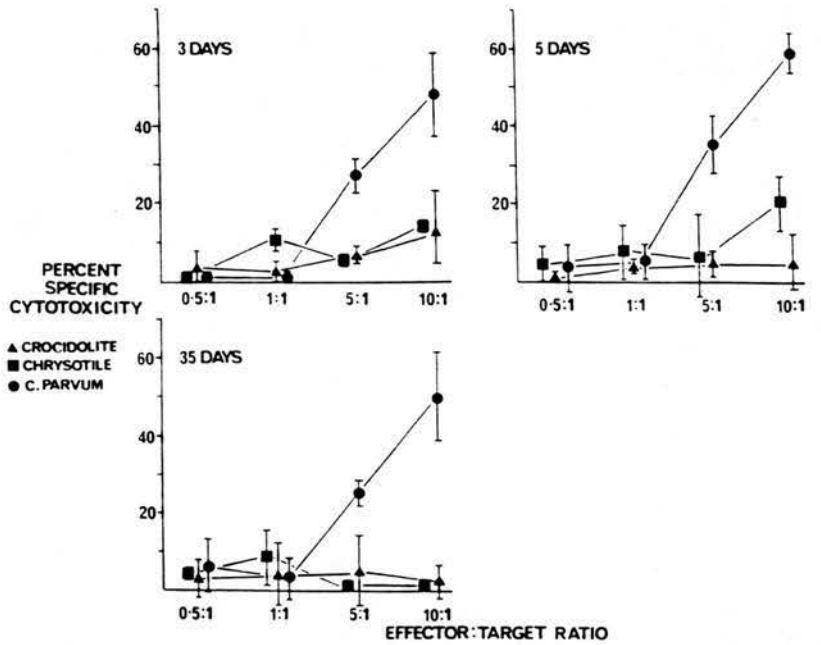


FIG. 5. Mean percentage specific cytotoxicity to tumor cells of macrophages induced by intraperitoneal injection of 2.5 mg UICC crocidolite, 2.5 mg UICC chrysotile, or 1.4 mg *C. parvum*. See text for calculation of specific cytotoxicity. Bars denote ± one standard deviation.

intraperitoneally did not produce significant reductions in tumor size. The use of 5 mg crocidolite in the same regimen also failed to produce significantly smaller tumors. Decreasing the dose of inoculating tumor cells to 5×10^2 in the hope of emphasizing the 5-mg chrysotile effect resulted in abolition of the effect. Treating with 5 mg chrysotile 2 days before inoculation with 5×10^2 tumor cells, a regimen which greatly increased the efficacy of *C. parvum* in reducing tumor growth (Table 1), produced no significant reduction in tumor growth over that of saline-injected controls.

DISCUSSION

This study has shown that a single intraperitoneal injection of both chrysotile and crocidolite asbestos induces increased numbers of cells in the peritoneal exudate at 3 and 70 days, while the number of cells at 18 days was close to that of saline-injected controls. Latex injection never produced a cellular exudate greater in magnitude than that produced by injection of saline. The biphasic variation in numbers of peritoneal exudate cells with time following asbestos injection suggests that the cytotactic stimulus for recruitment of cells into the peritoneal cavity does not simply decay with time after the acute large-scale recruitment at Day 3 but is renewed between Days 18 and 70.

The macrophages present in the asbestos-induced exudate were activated at all three time points as measured by the criteria of increased Fc receptor avidity (Rhodes, 1975) and increased macrophage spreading (Cohn, 1978). Increased

TABLE 1

EFFECT OF VARIOUS DOSES OF INTRAPERITONEAL ASBESTOS, GIVEN EITHER BEFORE OR AFTER 10^6 OR 5×10^2 SUBCUTANEOUSLY ADMINISTERED CCH_1 FIBROSARCOMA CELLS, ON THE MEAN DIAMETER OF THE TUMOR

Number of tumor cells injected s/c on Day 0	Asbestos type and amount	Day of ip asbestos injection	Tumor size [mm ($\bar{x} \pm SD$)][a]	
			Asbestos group	Control group
10^6	2.5 mg chrysotile	+3	16.8 ± 1.2	17.6 ± 1.2
10^6	5 mg chrysotile	+3	13.8 ± 0.8	15.6 ± 1.2[b]
10^6	20 mg chrysotile	+3	14.5 ± 3.4	15.6 ± 3.3
5×10^2	5 mg chrysotile	+3	12.3 ± 2.1	13.1 ± 2.9
5×10^2	5 mg chrysotile	−2	13.2 ± 3.9	13.0 ± 3.8
10^6	5 mg crocidolite	+3	12.3 ± 0.7	13.6 ± 2.0
10^6	1.4 mg *C. parvum*	+3	10.1 ± 1.8	13.6 ± 2.0[b]
5×10^2	1.4 mg *C. parvum*	−2	2.8 ± 3.7	13.0 ± 3.8[c]

[a] For mice injected with 10^6 CCH_1 cells the size given is for Day 17; for mice injected with 5×10^2 CCH_1 cells the size given is for Day 31.

[b] Significant difference $P < 0.002$.

[c] Significant difference $P < 0.001$.

phagocytosis is also a marker of macrophage activation (Cohn, 1978), although decreases in phagocytosis with activation have also been reported (David and Remold, 1973). In the present study the 3-day crocidolite and chrysotile macrophage populations had a smaller percentage of macrophages which could phagocytose latex than did the 3-day saline-induced macrophages; only crocidolite macrophages, however, were significantly less. The 3-day saline-induced macrophages also had a mean phagocytic population which was smaller, but not significantly, than at subsequent time points. It must be emphasized that the assay used here measured the proportion of cells able to phagocytose latex and not the amount of latex phagocytosed by the total population. The macrophages induced by intraperitoneal injection of latex only once differed significantly from control values in any measurement, thus confirming that latex is not a macrophage-activating agent (Schnyder and Baggiolini, 1978).

The *in vitro* assay for macrophage cytotoxicity to tumor cells revealed a low level of tumor cell cytotoxicity in the 3- and 5-day asbestos macrophages and the virtual absence of such activity by 35 days. This low level of tumoricidal activity was present despite evidence of macrophage activation by spreading and Fc receptor avidity, suggesting that, in the murine peritoneal model, asbestos induces only partial activation in the major proportion of the activated population and that only a small proportion have obtained the fully activated state.

The *in vivo* assay measuring the effect of intraperitoneal asbestos on tumor growth produced conflicting, mostly negative, results. Chrysotile produced significant reductions in tumor size at 5 mg but not at 20 mg, while crocidolite at 5 mg did not produce significant reductions in tumor size. Adoption of a regimen which profoundly increased the ability of *C. parvum* to produce reductions in tumor size caused 5 mg chrysotile to lose its effect in reducing subcutaneous tumor growth.

These results are difficult to interpret, but one interpretation could be that the mechanism whereby 5 mg chrysotile ip reduces tumor size is different from the mechanism whereby *C. parvum* ip brings about the same effect.

A model of macrophage activation to the tumoricidal state has evolved, suggesting that there is a complex multistep pathway to the fully tumoricidal state involving serial intermediate stages obtained by differentiation signals (Russell *et al.*, 1977; North, 1978; Cohn, 1978; Hibbs *et al.*, 1980). Such studies have suggested that macrophages activated to a nontumoricidal state by sterile inflammatory irritants, BCG, or toxoplasma infection are in a condition where they can be raised to the fully tumoricidal state by endotoxin treatment or, as is more likely *in vivo*, by the lymphokine MAF (macrophage-activating factor) produced as a result of a cell-mediated immune response (Russell *et al.*, 1977; Hibbs *et al.*, 1980). A corollary of this model is that only newly recruited macrophages are capable of being activated to the tumoricidal state and indeed Poste (1979) has shown experimentally that only newly recruited inflammatory macrophages can have their tumoricidal function induced, or natural tumoricidal function augmented, by lymphokine treatment.

The results of the present study could be interpreted, therefore, as evidence that a single injection of asbestos provides the inflammatory stimulus, and a large influx of newly recruited macrophages into the peritoneal cavity was certainly observed at Day 3, but that levels of MAF were insufficient to induce the fully activated tumoricidal state at any of the time points. In this respect it is of interest that Miller *et al.* (1979) observed alveolar macrophage activation and manifestations of cellular immunity in the lungs of rats inhaling asbestos, although they did not measure lymphokine levels. It is possible, therefore, that the single injection, peritoneal model used in the present study is an unsuitable one for studying asbestos activation of macrophages due either to the use of the peritoneal site or to the single dose compared to the multiple dosing produced by inhalation. If, however, the model used here does result in a cellular immune response and MAF generation then the asbestos-induced macrophages could be rather insensitive to its effect in generating tumoricidally activated macrophages. Such a refractory state, if it existed, could be a contributory factor in asbestos carcinogenesis.

The results presented here for the tumor cell cytotoxicity of asbestos-activated macrophages are rather less than that for inflammatory macrophages generated using glass, another insoluble irritant which is also carcinogenic (Bischoff and Bryson, 1964). In this study Poste (1979) collected macrophages on subcutaneously implanted glass coverslips and demonstrated greater than 40% spontaneous toxicity to tumor cells for a limited period between 4 and 7 days.

In the present study no attempt was made to measure Ia antigen expression in the membrane of asbestos-elicited macrophages. Ia expression is an important parameter in the antigen-presenting function of macrophages and it has been shown to be inducible in peritoneal macrophages by ip injection of activating agents (Beller and Unanue, 1981). Complete characterization of the asbestos-activated macrophage and its role will require study of the kinetics of Ia expression and the antigen-presenting ability of these macrophages.

The present study has shown that a single intraperitoneal injection of asbestos

results in the induction of activated peritoneal macrophages in the short and long term, but in neither case is the fully activated, tumoricidal state attained. These results lend support to previous findings that asbestos can activate alveolar macrophages following inhalation (Miller and Kagan, 1976) and can activate peritoneal macrophages *in vitro* as shown by increased release of lysosomal enzymes in the absence of cell death (Davies *et al.*, 1974). They also confirm the findings of Hamilton (1980), who showed that intraperitoneal asbestos produced macrophage activation as judged by increased plasminogen activator secretion.

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Interactions of Asbestos-Activated Macrophages with an Experimental Fibrosarcoma

by K. Donaldson,* J. M. G. Davis,* A. Ewing† and K. James†

Supernatants from *in vivo* asbestos-activated macrophages failed to show any cytostatic activity against a syngeneic fibrosarcoma cell line *in vitro*. UICC chrysotile-induced peritoneal exudate cells also failed to demonstrate any growth inhibitory effect on the same cells in Winn assays of tumor growth. Mixing UICC crocidolite with inoculated tumor cells resulted in a dose-dependent inhibition of tumor growth; this could, however, be explained by a direct cytostatic effect on the tumor cells of high doses of crocidolite, which was observed *in vitro*.

Introduction

Recently there has been an accumulation of experimental evidence suggesting that asbestos can have a stimulatory or activating effect on macrophages *in vivo* and *in vitro*. Davies et al. (*1*) reported that peritoneal macrophages treated with asbestos *in vitro* released lysosomal hydrolases without evidence of cell death, and Hamilton and co-workers (*2, 3*) demonstrated that asbestos *in vivo* and *in vitro* induces selective release of the neutral protease plasminogen activator from peritoneal macrophages. Humes et al. (*4*) and Sirois (*5*) have reported the induction of prostaglandin release by peritoneal and alveolar macrophages treated with asbestos *in vitro*. Selective release of lysosomal hydrolases and neutral proteases from macrophages can be induced by a range of macrophage activating agents, both immunological and nonimmunological (*6*).

Miller and Kagan (*7*) have reported that the alveolar macrophages of rats inhaling crocidolite show evidence of activation by morphology, Fc receptor avidity and the ability to stimulate T-lymphocytes to take up thymidine. We have studied the macrophages induced in the peritoneal cavity by intraperitoneal asbestos injection and have found macrophages to be activated by several criteria (*8, 9*) but full activation to the tumor cell cytotoxic state wa not found (*8*). Small significant reductions in the siz of experimental subcutaneous fibrosarcomas were however, found in mice which had received UIC chrysotile intraperitoneally compared with mic which had received saline intraperitoneally.

In this paper we present further studies into th interactions of asbestos, macrophages and an e perimental fibrosarcoma.

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Materials and Methods

Macrophage Plasma Membrane 5′-Nucleotidase and Lysosomal Acid Phosphatase

These assays were carried out according to t method of Raz et al. (*10*) using glycerophospha (Sigma) and 5′-adenosine monophosphate (Sigma) substrates, and assaying for phosphate release the method of Ames and Dubin (*11*). Protein was sayed by the method of Lowry (*12*).

Experimental Fibrosarcoma

The experimental tumor line used was the CC fibrosarcoma derived from a subcutaneous meth cholanthrene-induced fibrosarcoma in CBA/Ca mi It has been maintained in long-term culture and p saged through mice at intervals (*13*).

Macrophage Supernatants, Leachate and Asbestos

Peritoneal exudate cells (PEC) were harvested 3 days after intraperitoneal injection of UICC crocidolite, chrysotile, latex spheres (0.81 μm diameter, Difco) or saline. The macrophages were maintained in culture over 24 hr in complete RPMI (RPMI 1640, Gibco) containing 10% fetal calf serum (Gibco) and antibiotics (cRPMI). The supernatant was centrifuged, aliquoted and stored frozen at −20°C until use. Leachate was prepared by incubating crocidolite or chrysotile (5 mg/mL) in cRPMI for 3 days at 37°C then spinning out the fiber by centrifuging at 3500*g* for 1 hr. Asbestos was serum-coated to reduce direct cytotoxic effects by preincubating for 1 hr at 37°C in cRPMI.

CCH_1 Tumor Cell Proliferation Assay

The same assay system was used to measure the effect of macrophage supernatants, asbestos or asbestos leachate on CCH_1 cell proliferation *in vitro*. 5×10^4 CCH_1 cells were inoculated into microtiter plates (Sterilin) in 100 μL of cRPMI. Various volumes of supernatant, leachate or asbestos (preincubated in cRPMI for 1 hr at 37°C) were then added, and the final volume was adjusted to 200 μL with cRPMI. After 24 hr, 0.25 μCi of ^{3}H-thymidine were added to each well and after a further 24 hr the cells were harvested in a cell harvester (Skatron); cell-bound ^{3}H-thymidine counts were measured by liquid scintillometry.

Winn Assay

The assay used was essentially the same as that described by Gabizon and Trainin (*14*). PECs obtained 3 days after IP injection of saline or chrysotile (5 mg) were harvested, counted and mixed with CCH_1 tumor cells in the following ratios: (1) 10:1, i.e., 10^5 CCH_1 cells plus 10^6 PEC in 0.1 mL Dulbecco's phosphate buffered saline (Dul. A) and (2) 100:1, i.e., 5×10^2 CCH_1 cells plus 5×10^4 PEC in 0.1 mL Dul A. Groups of 10 mice received 0.1 mL subcutaneously in the right hind limb. Tumor growth was monitored by taking the mean of the two major diameters of the tumor.

Table 1. Plasma membrane 5′-nucleotidase and lysosomal acid phosphatase activity in 3-day saline- and 3-day chrysotile-induced macrophages.

Macrophage source	5′-Nucleotidase, μmole phosphate/ mg protein/hr	Acid phosphatase μmole phosphate/ mg protein/hr
Saline-induced (3-day)	3.7	0.4
Chrysotile-induced (3-day)	0.5	4.7

Chrysotile was arbitrarily chosen for this assay since previous studies (*8*) had shown crocidolite and chrysotile to be similar in their ability to activate macrophages.

Inclusion of Crocidolite in Tumor Cell Inoculum

UICC crocidolite was incubated in normal mouse serum for 1 hr at 37°C and then mixed with CCH_1 cells so that 0.1 mL of inoculum contained 10 μL of normal mouse serum; 0, 5, 50, or 500 μg of crocidolite; and 5×10^2 CCH_1 cells. Groups of 10 mice received 0.1 mL subcutaneously in the right hind limb and tumor growth was monitored.

Statistical Analysis

All differences were examined for statistical significance using Student's *t*-test.

Results

Table 1 shows that chrysotile-induced macrophages have decreased plasma membrane 5′-nucleotidase and increased acid phosphatase content, compared to saline-induced macrophages.

The effect of 24-hr supernatants of macrophages induced by various agents on tumor cell proliferation is shown in Figure 1. Statistical analysis re-

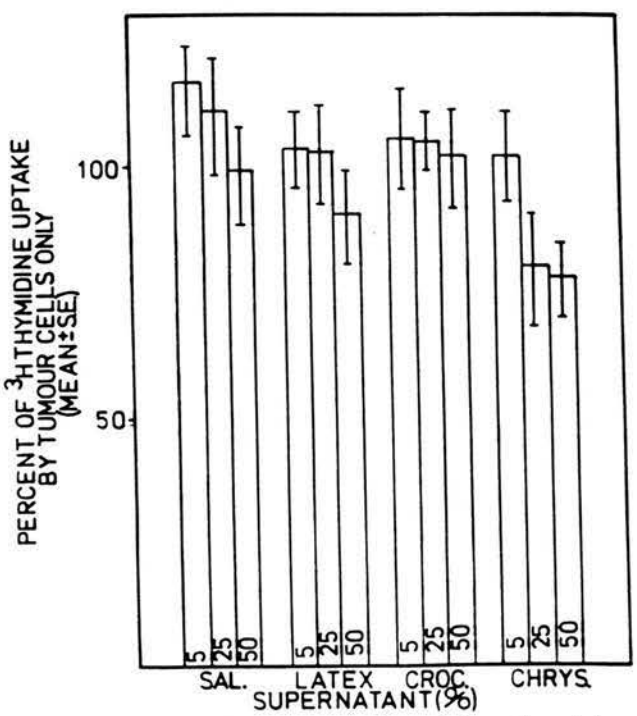


FIGURE 1. Effect of supernatants from 3-day saline-, latex-, crocidolite- or chrysotile-induced peritoneal exudate macrophages on proliferation of tumor cells *in vitro* as measured by ^{3}H-thymidine uptake.

vealed no significant effects of macrophage supernatants from any source, on CCH_1 cell proliferation, compared to CCH_1 cells alone. There was considerable variation in the response of CCH_1 cells to these supernatants between experiments, and this is reflected in the large amount of variation evident in Figure 1, which represents the mean and standard errors of five separate experiments.

Although there were differences in the mean tumor diameter produced by tumor cells alone, tumor cells mixed with saline-induced PEC and tumor cells mixed with chrysotile-induced PEC, at both 10:1 and 100:1 (Figs. 2 and 3), these differences were not statistically significant.

Figure 4 shows a dose-dependent inhibition of tumor growth produced by mixing crocidolite that had been preincubated with normal mouse serum with the CCH_1 tumor cell inoculum.

The effects of crocidolite and chrysotile, at doses which extend over four orders of magnitude, on proliferation of CCH_1 tumor cells *in vitro* are shown in Figure 5; remarkably little effect is seen except at the very highest doses of asbestos, where crocidolite caused marked inhibition of ^{3}H-thymidine uptake by tumor cells. Figure 5 also shows that crocidolite leachate, at the highest concentration, produced inhibition of ^{3}H-thymidine uptake.

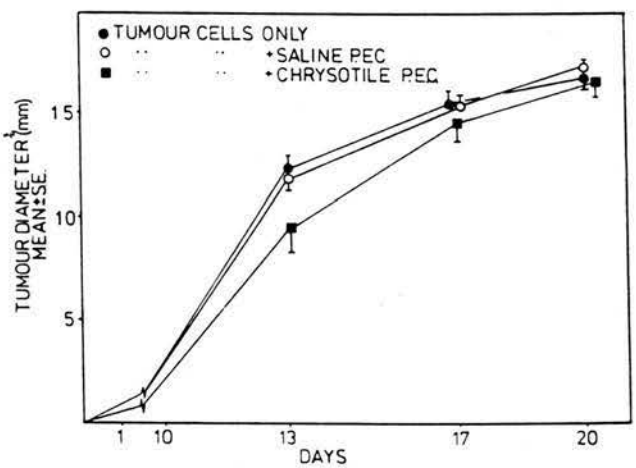


FIGURE 2. Effect of 3-day saline- and 3-day chrysotile-induced peritoneal exudate cells on subcutaneous tumor growth in a Winn assay; peritoneal exudate cell: tumor cell ratio of 10:1 at subcutaneous inoculation. Although no positively cytostatic Winn assay control was included in these experiments, intraperitoneal *C. parvum* is known to inhibit this tumor model by inducing tumor cell cytotoxic macrophages (*8*).

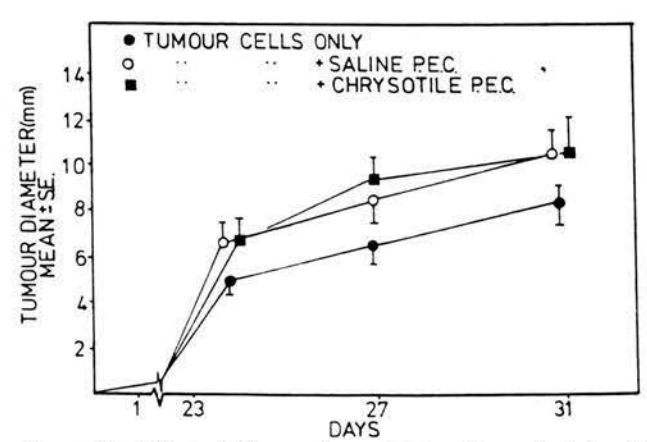


FIGURE 3. Effect of 3-day saline- and 3-day chrysotile-induced peritoneal exudate cells on tumor growth in a Winn assay; peritoneal exudate cell:tumor cell ratio of 100:1 at subcutaneous injection.

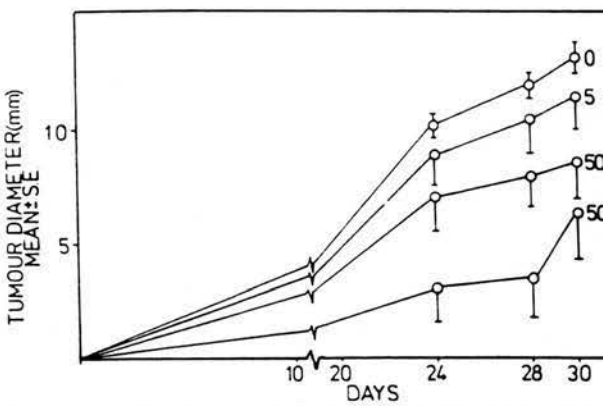


FIGURE 4. Effect of tumor growth of 0, 5, 50 or 500 μg of c cidolite percent along with tumor cells at subcutaneous ino lation.

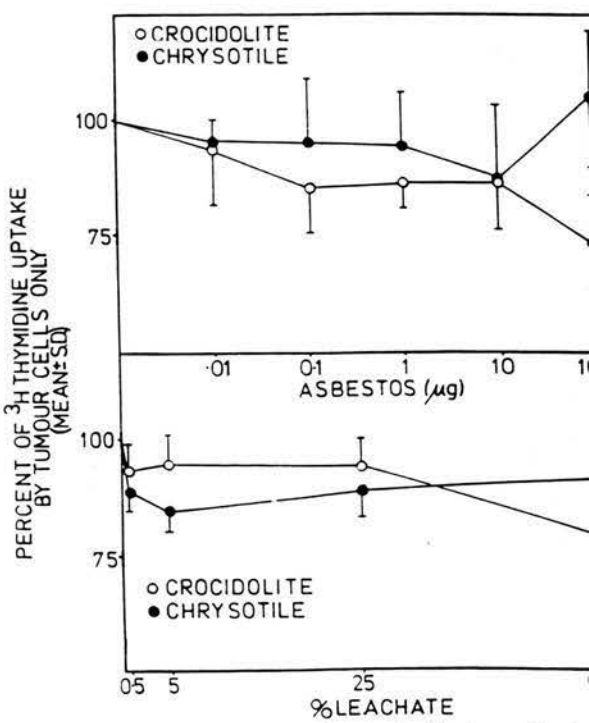


FIGURE 5. Effect of various doses of asbestos and asbe leachate on tumor cell proliferation *in vitro*.

Discussion

In this paper we describe experiments which were aimed at further elucidating the degree of activation of macrophages induced in the murine peritoneal cavity by injection of asbestos.

Macrophage activation apparently involves the sequential adoption of properties which can culminate in the fully tumoricidal state (*15*). In keeping with other reports, we have found that asbestos, can lead to activation of macrophages *in vivo* but, using an *in vitro* assay, we failed to detect any evidence of tumor cell cytotoxicity by asbestos-activated macrophages although *Corynebacterium parvum*-activated macrophages were tumoricidal in this system (*8*). However, in the same study, there was the contradictory finding *in vivo* that the administration of asbestos by intraperitoneal injection to mice caused a small but significant reduction in the growth of an experimental subcutaneous tumor. The present study follows on from this work and examines whether the tumor-retarding effect that was noted *in vivo* could be associated with macrophage activation.

Macrophage cytostatic activity, as well as cytotoxic activity, can play a role in retarding tumor growth, and this can be mediated by soluble factors (*16*). The supernatants from asbestos-activated macrophages were therefore tested in an assay of CCH_1 tumor cell proliferation *in vitro*. No significant inhibitory activity against tumor cell proliferation was detected in crocidolite, chrysotile, latex or saline-induced macrophage supernatants using Student's *t*-test.

In a further attempt to detect cytostatic activity in asbestos-activated macrophages, and in an effort to circumvent the problem produced by difference between the site of the tumor (leg) and the site of primary macrophage activation (the peritoneal cavity), two approaches were tried. In the first approach, Winn assays were used to bring 3-day asbestos- or saline-induced PEC into close contact with the target tumor cells by mixing them together at various effector : target ratios; this mixture was then inoculated and tumor growth compared to that of tumors produced with the appropriate number of tumor cells alone and with similar effector : target ratios of saline-induced PEC and tumor cells. No significant differences in tumor size were obtained with chrysotile-activated macrophages at ratios of either 10:1 or 100:1 compared to controls, and therefore no evidence of tumor cell cytostatic activity in asbestos-activated macrophages was detected.

In a second approach various doses of crocidolite asbestos that had been preincubated in serum-containing medium for 1 hr were mixed with inoculating tumor cells and tumor growth monitored. The dose-dependent decrease in tumor size which was obtained could have been due to local macrophage activation to the cytostatic state or could have been due to a direct toxic effect of crocidolite on the tumor cells. It was specifically to avoid such a direct toxic effect that crocidolite was chosen rather than chrysotile, since the amphiboles have a less active surface than chrysotile, and this is also why the crocidolite was precoated by incubation in serum-containing medium. However, in order to test for such a direct toxic effect of crocidolite on tumor cells, crocidolite was used in the *in vitro* CCH_1 cell proliferation assay. It was clear that both chrysotile and crocidolite and a fiber-free leachate of both asbestos types produced very little effect at all doses, except for inhibition at the highest doses with crocidolite. Since the doses of crocidolite present in inocula compared closely with, or exceeded, the doses used in the *in vitro* assay, it is evident that the reduction of tumor size produced could be accounted for by direct toxic effects of crocidolite on CCH_1 tumor cells. The induction of local tumor cell cytostatic macrophages does not therefore have to be evoked as a mechanism.

It seems likely that the small but significant reduction in the size of experimental tumors in mice injected with chrysotile which we reported previously (*8*) was not macrophage-mediated and that the overall adjuvant effect of asbestos could result in a nonspecific mobilization of some of the other well-documented antitumor immune responses.

The tumoricidal potential of macrophages has been used in these studies in the context of assessing the degree of macrophage activation and the results suggest that asbestos-activated macrophages have not attained the fully activated tumoricidal state. Tumor cell cytotoxic macrophages have been considered to be part of the surveillance system which acts to eliminate transformed cells before tumors can develop, and so the potential of asbestos-activated macrophages to attain full tumoricidal status has relevance for asbestos carcinogenesis. Previous studies have produced evidence that asbestos can act as a tumor promoter in classical two-stage carcinogenesis after initiation by polycyclic hydrocarbons (*17, 18*). A recent report has shown that a tumor promoter can block lymphokine-mediated activation of macrophages to the tumoricidal state (*19*). If this is shown to be a general property of tumor promoters, then it could be of particular relevance to asbestos carcinogenesis.

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Cytotoxic Effect of Asbestos on Macrophages in Different Activation States

by Annette Wright,* K. Donaldson* and J. M. G. Davis*

The *in vitro* effects due to phagocytosis of asbestos by mouse peritoneal macrophages in various stages of activation have been compared. The amphiboles proved relatively inert; chrysotile, however, expressed a greater degree of cytotoxicity toward those populations of macrophages induced *in vivo* with asbestos, than toward any of the other populations of cells. These results are compared with data concerning the enzyme release from the different populations of macrophages following phagocytosis of asbestos. The results indicate that those macrophages that have been exposed to a prior stimulation of either amphibole or serpentine asbestos *in vivo* are particularly sensitive to exposure to a second dose of a toxic fiber.

Introduction

The realization that the alveolar macrophage is the first phagocytic cell in the lung to ingest inhaled asbestos fibers prompted considerable research into the direct effects of fibers on such cells *in vitro*. Early studies (*1*, *2*) have shown chrysotile to be more toxic than crocidolite or amosite, a finding confirmed by many research groups. Macrophage-activating agents, such as zymosan, are known to initiate selective release of lysosomal enzymes (*3*). A study by Davies et al. (*4*) demonstrated that mouse peritoneal macrophages, upon phagocytosis of chrysotile *in vitro*, showed a selective release of lysosomal enzymes in the absence of cell death. However, Jaurand et al. (*5*) demonstrated an additional release of lactate dehydrogenase, thus suggesting some loss of viability, for alveolar macrophages exposed to chrysotile *in vitro*. Studies by Hamilton and colleagues (*6*, *7*) showed that macrophages exposed to chrysotile *in vitro* and *in vivo* release greater quantities of the neutral protease plasminogen activator. In addition, Miller (*8*) and Donaldson et al. (*9*) have shown that asbestos-induced macrophages obtained from either lung or peritoneal cavity possess an altered surface morphology and increased number of membrane receptors consistent with cell activation.

McGee and Myrvik (*10*) demonstrated that activated macrophages are more readily injured upon phagocytosis of toxic agents than resident cells. Wright and Davis (*11*) showed that chrysotile-activated macrophages are more sensitive to the cytotoxic action of chrysotile *in vitro* than unstimulated cells; in addition, Morgan and Allison (*12*) also suggested that macrophages elicited by different biochemical means may show a different response to a further stimulus. The aim of this study was to investigate further the response of populations of macrophages in different stages of activation to the action of asbestos *in vitro*, and enzyme release from these cells was also compared.

Materials and Methods

Stimulation and Harvesting of Peritoneal Exudate Cells (PEC)

Male CBA mice, 12 weeks old, were either untreated or injected intraperitoneally with one of the following stimulating agents: 1 mL saline (Dulbecco's A); 1 mL 10% protease peptone (Difco); 1.4 mg heat killed *Corynebacterium parvum* (Wellcome), a well known macrophage activating agent (*13*); 1 mL of 0.1% latex beads (0.81 μ) (Difco); 2.5 mg of UICC crocidolite, UICC amosite or UICC chrysotile suspended in 1 mL saline.

Three days following injection, the mice were killed by ether overdose. The PEC were harvested by peritoneal lavage and washed.

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Spreading Assay to Assess Degree of Macrophage Activation

PECs (1×10^5) were cultured on 6×22 mm glass coverslips in Ham's F10 medium (14) + 20% fetal calf serum (FCS) at 37°C. After precisely 1 hr, the coverslips were washed vigorously to remove nonadherent cells. The remaining adherent macrophages were stained by May-Grunwald and Giemsa stains. The relative degree of activation of the population was expressed in terms of the percentage of cells completely spread. This means of assessment has been shown to correlate with other methods of activation measurement such as Fc receptor (*9*).

Culture and Treatment of PEC Populations

After harvesting, 1×10^6 PECs were cultured in 35 mm dishes in F10 + 20% FCS. After 1 hr, the cells were washed with saline to remove nonadherents. The resulting macrophage populations were cultured in F10 + 20% FCS either untreated or treated with 0.1% latex beads (0.81 μ), or 100 μg UICC crocidolite, UICC amosite or UICC chrysotile per plate.

Assessment of Phagocytic Ability of Macrophages

The phagocytic ability of the macrophage populations was assessed microscopically after 24-hr culture with latex heads. A cell was termed phagocytic if it contained more than three latex beads.

Assessment of Viability of Marcrophages

Viability of the cells was assessed, using Trypan Blue exclusion, 24 hr following treatment with latex, crocidolite, amosite or chrysotile.

Enzyme Assays

Lactate dehydrogenase (LDH) (*15*) and *N*-acetyl-β-*D*-glucosaminidase (glucosaminidase) (*16*) levels were assessed in both cells and culture medium after 24-hr culture with crocidolite or chrysotile.

In Vitro Activation of Macrophages by Lymphokine

Lymphokine, a known macrophage activating agent, was produced according to the method of Lazdins et al. (*17*) by exposing mouse splenocytes to 10 μg/mL of Concanavalin A (Con A) *in vitro* for 24 hr. Saline-induced macrophages were exposed to either the resulting lymphokine or a Con A supplemented control medium for 24 hr. The activated Con A control and untreated macrophages were then exposed to UICC chrysotile for a further 24 hr and their viabilities assessed.

Statistical Analyses

The data from the spreading assay, macrophage viability estimates and enzymes assays were examined by statistical analyses of variance, the within-experimental replication being used to provide estimates of random variation.

Results

All of the populations of PECs were found to contain 99% viable cells upon isolation from the groups of treated mice. The relative degree of activation of the adherent macrophages, according to their ability to spread on glass, is shown on Figure 1. A high degree of activation was found in those populations induced by asbestos and *C. parvum*, according to this method of activation assessment; they did not differ significantly in their ability to spread on glass. All of the remaining populations showed a much lower ability to spread on glass, the protease peptone population showing an increase over the unstimulated population ($p < 0.05$).

After 24-hr culture *in vitro* with latex heads, the macrophage groups were all found to contain 95% phagocytic and 95% viable cells following ingestion of latex heads. The effect of 24-hr incubation with chrysotile was very different from that of the other two types of asbestos (Figs. 2 and 3). Crocidolite and amosite proved noncytotoxic, and there were no significant differences observed between the macrophage populations ($p > 0.9$ overall). Chrysotile, however, while exhibiting a low degree of cytotoxicity towards the unstimulated and saline-induced populations, showed a slightly increased level of cytotoxicity towards the *C. parvum*-induced cells. All three types of asbestos-induced populations proved particularly susceptible to the cytotoxic action of chrysotile; viabilities of around 30% were obtained and no significant differences were found among these three populations. The populations of macrophages stimulated *in vitro* by Con A or lymphokine did not show an increased degree of susceptibility to the action of chrysotile (Table 1).

Ingestion of crocidolite, compared to control, stimulated an increased release of glucosaminidase in all cell populations ($p < 0.01$). An even more considerable release of this enzyme in all populations of cells followed chrysotile ingestion ($p < 0.01$) (Figs. 4 and 5). The level of release of LDH was lower than that observed for the glucosaminidase, although the asbestos-induced populations released a greater quantity of LDH than the unstimulated, saline and *C. parvum*-induced populations ($p < 0.025$).

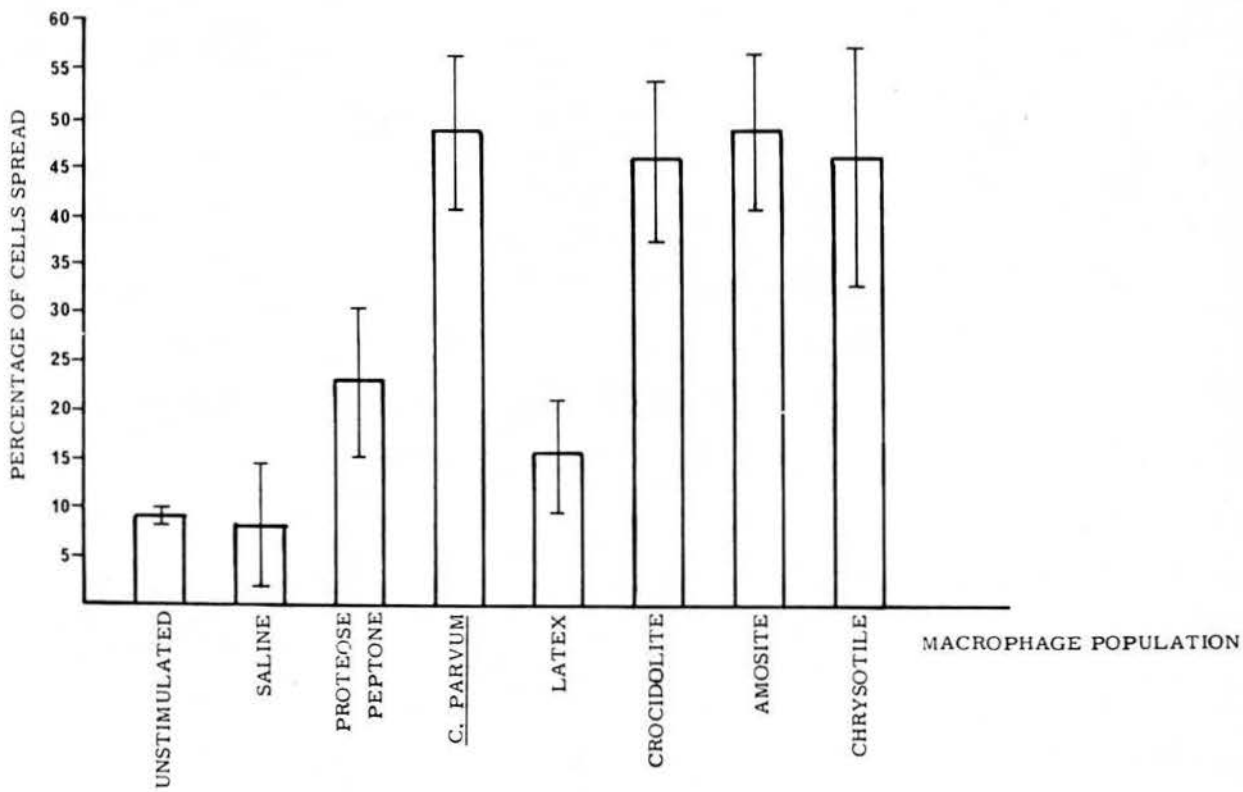


FIGURE 1. Relative degree of activation of macrophage populations according to spreading assay. Calculated as % cells spread (number of cells spread/total number of cells counted) × 100. Results are means of at least three experiments ± SD.

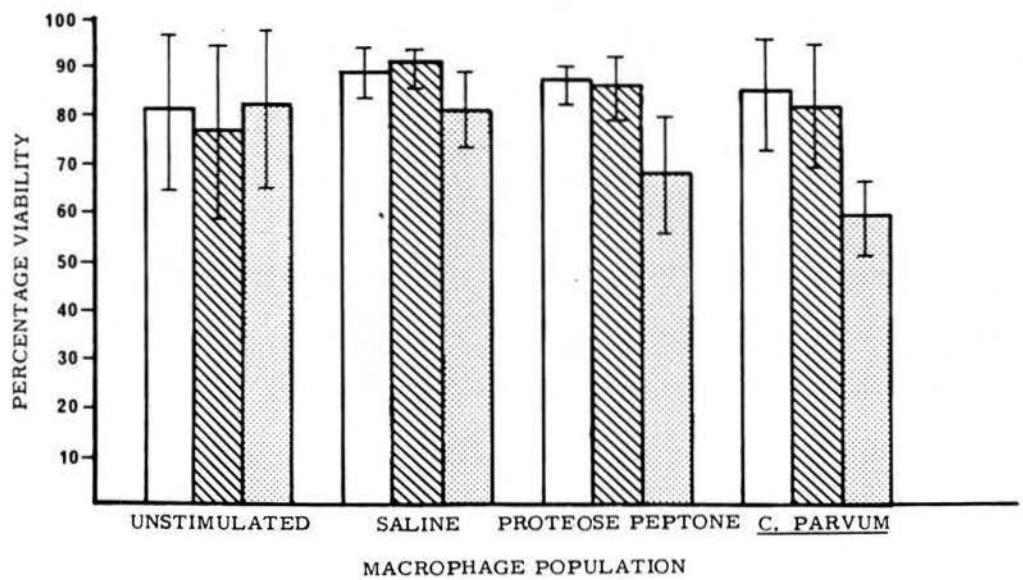


FIGURE 2. Percentage viability of macrophage populations after 24-hr exposure to asbestos *in vitro*. Treatment *in vitro*: □ crocidolite; ▨ amosite: ⊠ chrysotile. Macrophages unstimulated or stimulated with saline, protease peptone and *C. parvum*. Viability calculated as % viability = (number of viable cells on treated plate/number of viable cells on control plate) × 100. Results are means of at least three experiments ± SD.

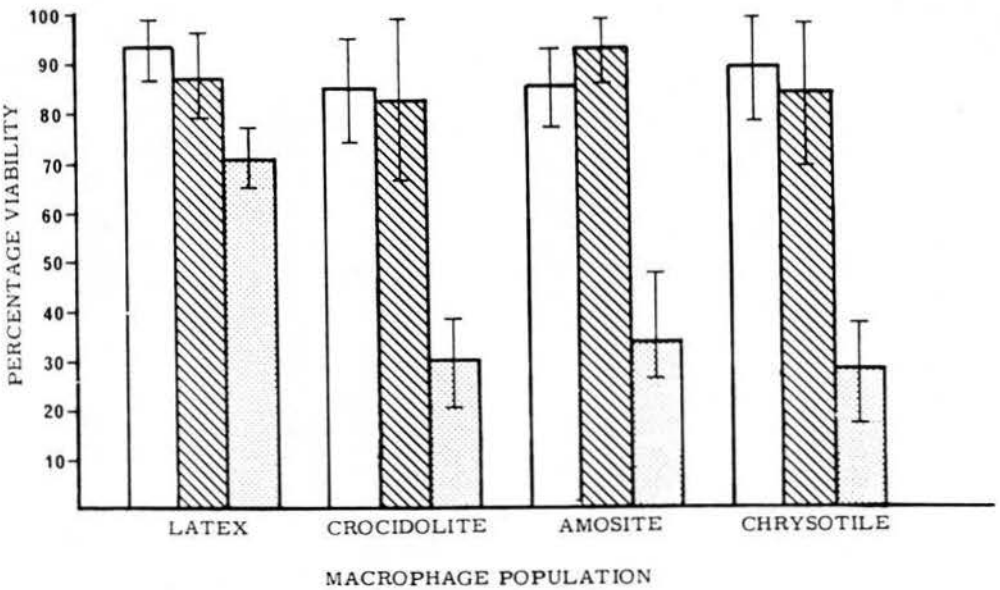


FIGURE 3. Percentage viability of macrophage populations after 24-hr exposure to asbestos *in vitro*. Treatment *in vitro*: □ crocidolite; ▨ amosite; ▩ chrysotile. Macrophages stimulated with latex and asbestos. Results are means of at least three experiments ± SD.

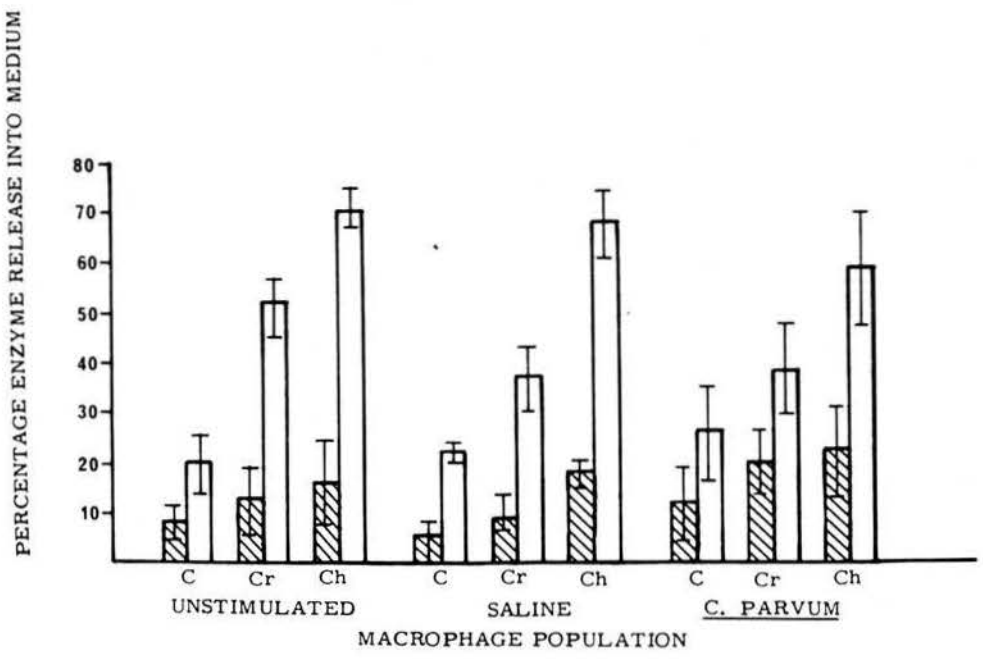


FIGURE 4. Enzyme release into medium after 24-hr exposure to asbestos. Enzyme: ▨ LDH; glucosaminidase. Treated with: C = control; Cr = crocidolite; Ch = chrysotile. Results are means of three experiments ± SE.

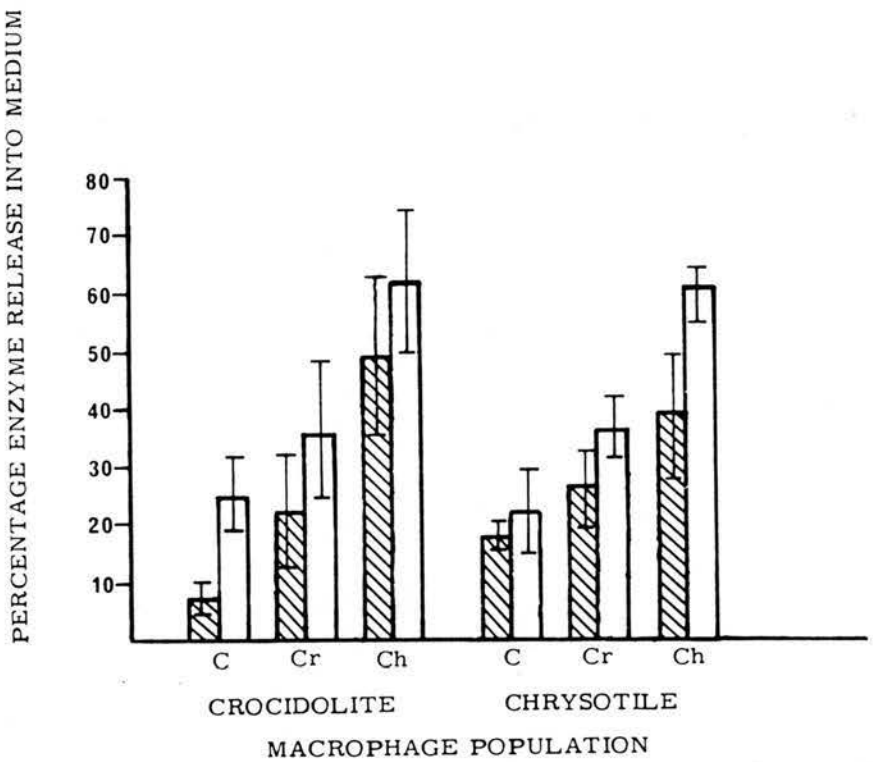


FIGURE 5. Enzyme release into medium after 24-hr exposure to asbestos. Enzyme: ▨ LDH; □ glucosaminidase. Treated with: C = control; crocidolite; Ch = chrysotile. Results are means of three experiments.

Table 1. Percentage viability of *in vitro* activated macrophages following 24-hr treatment with chrysotile.

In vitro treatment of saline-induced macrophages	% viability following ingestion of chrysotile[a]
Untreated control	67.2 ± 7.0
Con A supplemented medium	69.5 ± 8.7
Lymphokine	69.8 ± 9.2

[a] % viability = (number of viable cells on chrysotile treated plate/number of viable cells on control plate) × 100. Results are means $\bar{X}$ of three experiments ± SD.

Discussion

Peritoneal macrophages can be obtained in a variety of states of activation possessing a variety of altered properties (*18*). In general, activated macrophages are larger, have more granules, spread to a greater extent on glass and have a greater capacity to kill microorganisms and tumor cells than unstimulated, resident cells (*19*). The degree of activation of the macrophage can vary considerably, depending on the nature of the stimulating agent; and this is illustrated in Figure 1 by using a single parameter for activation assessment. Studies (*6*, *9*) have shown that intraperitoneal injection of asbestos can produce a population of viable macrophages with characteristics consistent with cellular activation. In the present study, crocidolite, amosite and chrysotile have all induced intraperitoneal populations of cells both viable and apparently activated to a degree similar to *C. parvum*-induced macrophages.

All the populations of cells showed a similarly high rate of phagocytosis, regardless of the activation state, and no cell death was observed because of ingestion of nontoxic latex beads alone. The amphiboles displayed a similar level of low cytotoxicity toward all types of macrophages (Figs. 2 and 3). These cells, however, showed a diverse response to chrysotile. The nonstimulated and saline-induced macrophages appeared resistant to the cytotoxic action of the dust, whereas the more activated populations showed an increased susceptibility, the asbestos-induced cells proving the most sensitive. These results agree with those of McGee and Myrvik (*10*), in that activated macrophages tend to lose viability more rapidly than nonstimulated cells upon phagocytosis of a toxic agent. The cells activated by lymphokine *in vitro* (Fig. 4) did not display an increased sensitivity to the action of chrysotile, thus suggesting that macrophages activated *in vivo* probably possess differing properties to those activated *in vitro*. It is of interest to note that, while the amphiboles—crocidolite and amosite—appeared relatively inert *in*

vitro, both types of fiber have the capacity *in vivo* to induce macrophages that show a high sensitivity to the action of a cytotoxic dust. This is not due simply to an *in vivo* stimulating activity of particulate alone, as latex-induced macrophages did not display a high sensitivity to chrysotile.

The data regarding enzyme release from the macrophages (Figs. 4 and 5) agree with the finding of Hamilton (*6*), in that asbestos-induced macrophages secreted a similar quantity of lysosomal enzyme into the culture medium to that seen for the nonstimulated cells. Phagocytosis of crocidolite induced a slight release of glucosaminidase by all populations; however, chrysotile stimulated a large release of enzyme similar to that seen in other reports (*20*). This large release of lysosomal enzyme was not accompanied by a corresponding release of cytoplasmic LDH for unstimulated, saline and *C. parvum*-induced macrophages. However, an increased release of LDH was observed for the asbestos activated populations, corresponding to the increased loss of viability illustrated in Figure 3.

In conclusion, this report illustrates that asbestos-induced macrophages, upon phagocytosis of a second dose of dust, do not respond in a manner similar to that observed for other types of macrophage populations. This must be taken into consideration when investigating the effect of inhaled particles on macrophages in the lung. Such cells may already have received prior stimulation by other toxic agents or pathogens and also persistent exposure to different dusts, rather than the single dose often used in the *in vitro* situation.

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Chemiluminescence of asbestos-activated macrophages

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Summary. Chemiluminescence, a measure of reactive oxygen species release by phagocytes, was compared in peritoneal exudate macrophages elicited with chrysotile asbestos, *Corynebacterium parvum* and saline. Chrysotile asbestos- and *C. parvum*-activated macrophages produced significantly more chemiluminescence than saline-elicited macrophages. In a second series of experiments the ability of opsonized chrysotile asbestos to act as a trigger for the release of chemiluminescence was tested. Opsonized chrysotile asbestos produced a dose-related release of chemiluminescence from activated macrophages except at the highest dose where chemiluminescence was reduced due, possibly, to a toxic effect of chrysotile during the assay. Opsonized latex also triggered a dose-related chemiluminescent response from activated macrophages. The potential role of toxic reactive oxygen species, released from macrophages, in the development of asbestos-related pulmonary inflammation and fibrosis are discussed.

Keywords: chemiluminescence, asbestos-activated macrophages

Pulmonary interstitial fibrosis (asbestosis) is a major disease associated with asbestos inhalation (Becklake 1976; Selikoff & Lee 1978) and is typified by increased deposition of interstitial collagen and fibroblast proliferation. Fibrosis is a common sequel to chronic inflammation and the ability of asbestos to generate inflammation in the lung has been discussed as a factor in the aetiology of asbestos-associated pulmonary interstitial fibrosis (Hamilton 1980; Miller 1978). The central role of the alveolar macrophage in interacting with inhaled particles makes it likely that alteration in the activities of these cells in the lungs of individuals inhaling asbestos could be important in the pathogenesis of asbestos-related diseases. Evidence that the macrophage may play a central role in asbestos inflammation has come from studies demonstrating macrophage-activating properties of asbestos (Davies *et al.* 1974; Hamilton *et al.* 1976; Miller & Kagan 1976; Sirois *et al.* 1980; Donaldson *et al.* 1982, 1983*a,b*). Macrophages from inflammatory sites have been found to be activated and capable of releasing an extensive array of biologically-active substances (Allison *et al.* 1978; Nathan *et al.* 1980). One group of these secreted molecules, the reactive oxygen species (ROS): hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical ($OH\cdot$) and singlet oxygen ($^1O_2$), have been implicated in the tumoricidal and microbicidal functions of leucocytes (Nathan 1982) and in tissue damage at inflammatory foci (Fantone & Ward 1982).

In the present study we assessed the release of ROS from asbestos- and *Corynebacterium parvum*-elicited mouse peritoneal macrophages by measuring lucigenin-amplified chemiluminescence in response to a variety of stimuli including asbestos. Lucigenin-amplified chemiluminescence in leucocytes is a measure of superoxide release

(Allen 1981: Williams & Cole 1981*b*). In previous studies we have characterized the asbestos-elicited mouse peritoneal macrophage as a model asbestos-activated macrophage (Donaldson *et al.* 1982, 1983*a.b*).

Materials and methods

Mice. Outbred CF1 mice 6–8 weeks of age at the time of injection were used throughout.

Peritoneal exudate cells. Peritoneal exudate cells (PEC) were harvested 5 days after intraperitoneal injection of 2.5 mg chrysotile asbestos (Union International Contre Cancer sample) in 0.5 ml Dulbecco phosphate-buffered saline A (DulA), 0.5 ml DulA or 0.2 ml (0.4 mg) *Corynebacterium parvum* (heat-killed: Wellcome). Mice were killed by ether inhalation and the cells were harvested by three 2-ml washes with DulA containing 10 units/ml Heparin (Pularin) into ice-cold plastic tubes. The cells were washed, counted and maintained in Hams F_{10} medium containing 10% heat-inactivated fetal calf serum (FCS) (GIBCO) on ice until assay when they were transferred to Hanks solution without phenol red.

Neutrophil depletion. Neutrophil depletion was by centrifugation through sodium metrizoate: 10×10^6 PEC in 5 ml of F_{10} and 10% FCS were layered on top of sodium metrizoate (Lymphoprep: Nyygaard) in a 10-ml siliconized centrifuge tube and centrifuged at 400 *g* for 30 min. The cells at the interface were retrieved and washed before use.

Characterization of PEC. To determine the cellular composition of the PEC, cytospin preparations were made, stained with Wright stain and differential counts carried out.

Triggers of ROS release. The following triggers for the stimulation of ROS release were used. (a) Opsonized zymosan: zymosan was opsonized by incubation at 37°C for 30 min in 10% Human AB serum, washed and adjusted to 10 mg/ml in Hanks solution without phenol red. (b) Opsonized latex: latex particles (Sigma, 1.09 μm diameter) were opsonized as above and adjusted to final concentrations of 1, 5 or 10%. (c) Opsonized chrysotile asbestos: chrysotile asbestos (UICC) was opsonized as above, washed and adjusted to final concentrations of 1, 5, 10 and 20 mg/ml.

Lucigenin-amplified chemiluminescence. Chemiluminescence (CL) was measured directly in mV in an LKB 1250 luminometer. Cells were prepared at 5×10^6/ml in cold Hanks and maintained on ice. Two hundred microlitres of cells were transferred to polystyrene cuvettes (LKB) followed by 200 μl of lucigenin (10^{-3}M). The cuvettes were placed in the luminometer and the baseline CL was allowed to develop. When this had plateaued 500 μl of trigger were added. Following addition of trigger, CL was monitored until it peaked.

Viability assay. To determine the extent of any toxic effect of asbestos on PEC, 50-μl samples of cells were removed from a typical CL assay (*C. parvum* PEC: 10^{-3}M lucigenin 10 mg/ml chrysotile) and percentage viability determined by trypan blue exclusion for 200 cells.

Use of enzymes. In order to determine the role of different reactive oxygen species in CL superoxide dismutase (Sigma: 50 μg/ml) and catalase (Sigma: 25 μg/ml) were included in two separate experiments with opsonized zymosan- and *C. parvum*-elicited PEC.

Statistics. The statistical significance of replicate experiments was analysed using Student's *t*-test and analysis of variance.

Results

Characteristics of CL

Baseline CL. As discussed by Williams & Col

(1981*a*) the addition of PEC and lucigenin to the polystyrene cuvettes resulted in a release of CL. Trigger was added after this reached a plateau. In each experiment this baseline CL was subtracted from the final peak CL to obtain the true peak CL due to the trigger. The magnitude of the baseline CL varied but was always low for the saline-induced PEC (0.4 ± 0.2: mean $\pm$ SD) and was increased with *C. parvum*- (7.2 ± 6.5) and chrysotile asbestos- (5.4 ± 6.0) elicited PECs.

Effect of enzymes on CL. Fig. 1 shows that CL is inhibited by superoxide dismutase (SOD) and unaffected by catalase. The same result was obtained in two separate experiments.

Role of polymorphonuclear neutrophils (PMN) in CL by peritoneal exudate cells. In order to determine the role of PMN in CL by activated PEC the PEC were centrifuged through lymphoprep to reduce the proportion of granulocytes. The cells at the interface had a mean reduction in PMN of 40% but, as shown in Table 1, this had virtually no effect on peak CL when the same number of cells were compared. It was therefore assumed that PMN and macrophages from the activated PEC used in these experiments released approximately equal quantities of CL. Consequently the proportion of CL due to PMN in any activated PEC was approximately equal to the percent PMN present (Table 2) and no further attempt was made to separate macrophages from neutrophils.

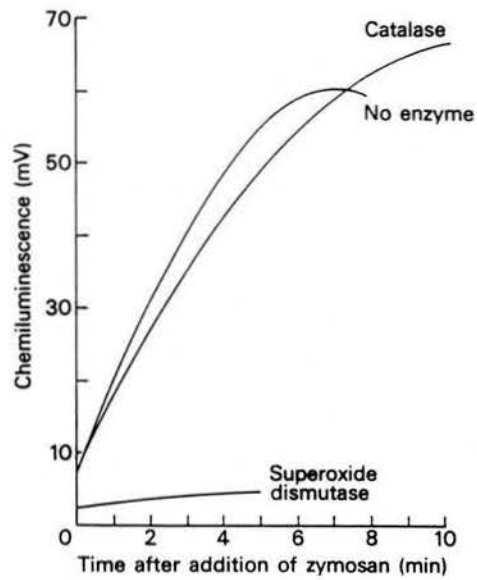


Fig. 1. Effect of superoxide dismutase (50 μg/ml) and catalase (25 μg/ml) on opsonized zymosan-triggered chemiluminescence of *C. parvum*-elicited PEC (500 μl of 10 mg/ml opsonized zymosan added at time 0). The same result was obtained in two separate experiments.

Table 1. Effect of separation on sodium metrizoate on proportion of polymophonuclear neutrophils and chemiluminescence of *C. parvum*- and chrysotile-elicited peritoneal exudate cells: opsonized zymosan as a trigger

Agent eliciting peritoneal exudate cells	Experiment no.	Polymorphonuclear neutrophils (%)		Reduction in polymorphonuclear neutrophils (%)	Change in peak chemiluminescence (%)
		Before	After		
C. parvum	1	23.1	12.2	47.2	−2.7
	2	18.4	8.3	54.9	−11.2
Chrysotile	1	15.7	10.7	31.8	−4.0
	2	31.8	23.3	26.7	+3.1
All experiments	—			40.6 ±13.9	−3.1 ±6.8

Table 2. Percent macrophages and polymorphonuclear neutrophils in various peritoneal exudate cells Remainder of cells were lymphocytes with low (< 5%) numbers of eosinophils and basophils

Agent eliciting peritoneal exudate cells	Polymorphonuclear neutrophils (%)	Macrophages (%)	No. of experiments
Saline	5.8 ± 2.4	70.8 ± 16.2	3
C. parvum	15.2 ± 5.4	69.7 ± 6.2	5
Chrysotile	21.4 ± 7.3	55.8 ± 6.3	4

Results are mean ± SD.

CL responses of different PECs

Fig. 2 shows the pooled results of all experiments measuring the CL response, to opsonized zymosan, of PEC elicited with saline, *C. parvum* or chrysotile asbestos. Initial analysis of the peak CL data from all experiments (Table 3) suggested approximately equal variance in the scale of logarithm mV and analysis of variance was therefore carried out in this scale. Two effects were apparent from the analysis of variance: Firstly, small but significant systematic variation in the absolute values of peak CL between experiments. The highest and lowest values differed by a factor of 1.6 and it was not possible to identify, retrospectively, the source of this variation. Secondly, taking into account this temporal variation between experiments there were highly significant differences between the different PECs with *C. parvum* PEC being greater than chrysotile PEC by a factor of 1.3 and chrysotile PEC greater than saline PEC by a factor of 2.0.

CL in response to asbestos and latex

Fig. 3 shows a typical dose–response of CL to opsonized latex with 5-day *C. parvum*-elicited PEC. Fig. 4 shows the response of the same cells to increasing doses of opsonized chrysotile asbestos and a clear dose–response is evident over 1, 5 and 10 mg/ml: at 20

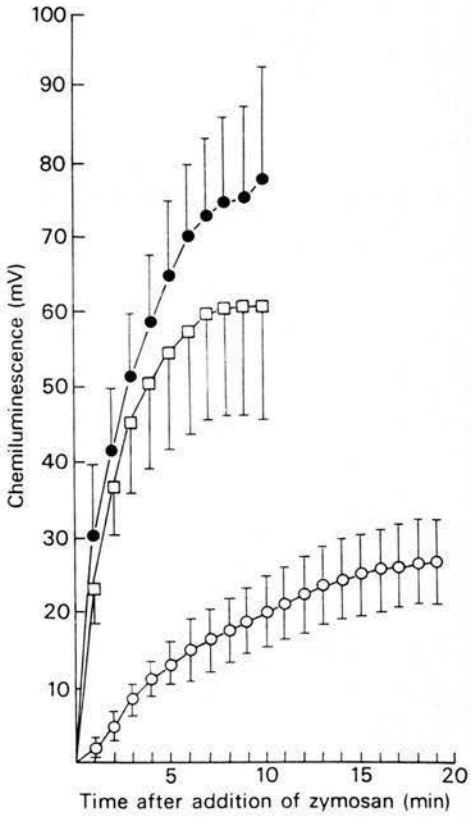


Fig. 2. Chemiluminescence response of peritonea exudate cells elicited with saline (○), *C. parvu* (●) or chrysotile asbestos (□) to opsonized zymo san (500 μl of 10 mg/ml opsonized zymosa added at time 0). Mean ± standard deviation three (saline), four (chrysotile) and five (*C. par vum*) separate experiments.

Table 3. Combined results of replicate experiments to measure the peak chemiluminescence response of saline-, *C. parvum*- and chrysotile-elicited peritoneal exudate cells in response to opsonized zymosan

Agent eliciting peritoneal exudate cells	Peak chemiluminescence	No. of experiments
Saline	27.4 ± 5.5	3
C. parvum	66.1 ± 14.2	5
Chrysotile	54.5 ± 12.7	4

Results are mean ± SD.

Analysis of variance showed significant differences between peritoneal exudate cells elicited by the three different agents: *C. parvum* > chrysotile > saline (see results).

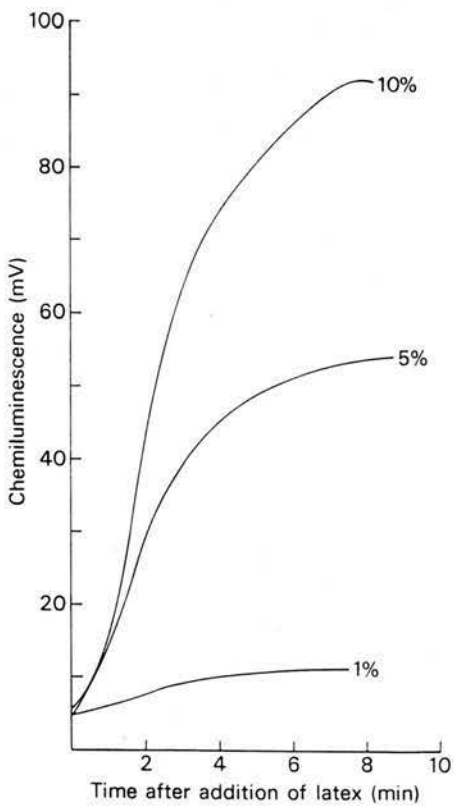


Fig. 3. Chemiluminescence response of 5-day *C. parvum* PEC to opsonized latex at 1, 5 and 10% (500 μl of latex at the stated concentrations added at time 0). The same result was obtained in two separate experiments.

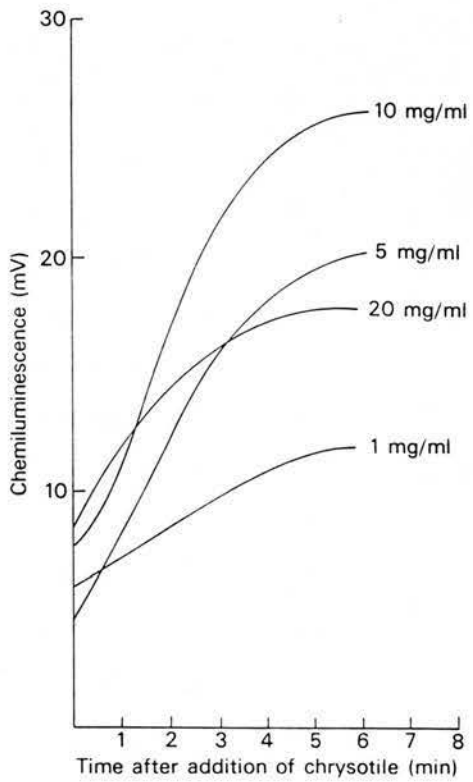


Fig. 4. Chemiluminescence response of 5-day *C. parvum*-elicited PEC to opsonized chrysotile at 1, 5, 10 and 20 mg/ml (500 μl of opsonized chrysotile, at the stated concentrations added at time 0). The same result was obtained in two separate experiments.

mg/ml, however, the response is reduced to less than that found with 10 mg/ml.

In order to determine whether toxicity might be a factor in the loss of dose–response at high opsonized chrysotile concentration, a time course of cell viability was taken during a CL assay (*C. parvum* PEC: 10^{-3}M lucigenin: 10 mg/ml opsonized chrysotile). As shown in Fig. 5 there is 30–40% toxicity to cells within the timespan of a normal assay (10–20 min).

Table 4 shows the pooled peak CL response from replicate experiments using *C. parvum*-elicited PEC with opsonized zymosan and opsonized chrysotile as triggers. It is evident that opsonized zymosan elicited a significantly greater CL response than opsonized chrysotile on an equal-mass basis.

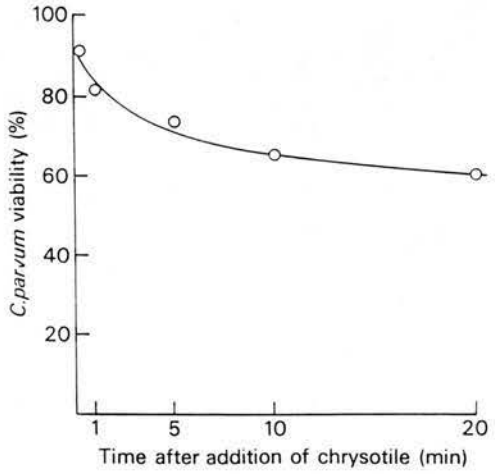


Fig. 5. Change in viability of *C. parvum* PEC during a chemiluminescence assay with 10 mg/ml opsonized chrysotile as trigger added at time 0.

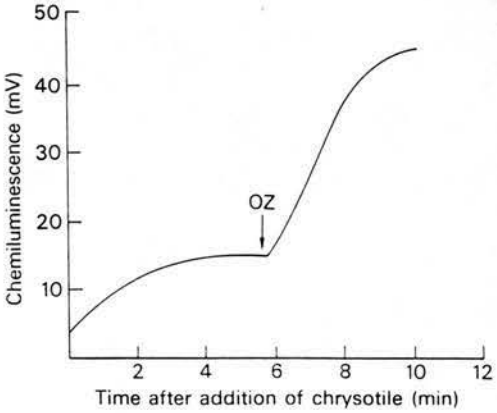


Fig. 6. Chemiluminescence response of 5-day C *parvum* PEC to opsonized chrysotile added as 50(μl of 10 mg/ml at time 0: on reaching a plateau opsonized zymosan was added (OZ); typical result

Table 4. Effect of different triggers on the peak chemiluminescence response of *C. parvum* peritoneal exudate cells

Trigger	Peak chemiluminescence	No. of experiments
Opsonized zymosan	66.1 ± 14.2*	5
Opsonized chrysotile	14.2 ± 7.3*	5

Results are mean ± SD.
* Significance of difference between zymosan and chrysotile: $P < 0.001$.

Figure 6 shows that on reaching a plateau of CL in response to 10 mg/ml opsonized chrysotile the cells are still sufficiently viable to respond to a further stimulus from opsonized zymosan.

Discussion

In the present study we set out to determine whether asbestos could elicit activated macrophages with increased ability to release reactive oxygen species (ROS) and also whether asbestos could act as a trigger fo the release of ROS from activated macro phages. Activated peritoneal macrophage elicited by asbestos injection were used sinc these have been previously characterized while saline- and *C. parvum*-elicited macro phages were used as unactivated and acti vated controls respectively (Donaldson *et a* 1982; Cullen 1978). As a measure of RO release. lucigenin-amplified chemilumines cence (CL) was used. Chemiluminescence i correlated with other indices of the respira tory burst including O_2 consumption. hexos monophosphate shunt activity. H_2O_2 releas and O_2^- release (Johnston *et al.* 1980) whic are induced in phagocytes by phagocytic c membrane-perturbing stimuli (Schadelin *al.* 1980). Lucigenin-amplified chemilu minescence is correlated with O_2^- release i macrophages (Williams & Cole 1981*b*) and reaction sequence for the interaction of O with lucigenin in the generation of CL ha been described (Allan 1981). In preliminar experiments we gained support for the in volvement of O_2^- in CL by demonstrating th superoxide dismutase-dependent abolition CL while catalase had no effect on C response.

In the first set of experiments reported here the chrysotile asbestos-elicited PEC were shown to release significantly more CL than saline-elicited PEC in response to the conventional trigger of ROS release—opsonized zymosan. *C. parvum*-elicited PEC, however, released significantly more CL than chrysotile-elicited PEC and phorbol myristate acetate (PMA), a non-particulate membrane-perturbing agent, produced the same pattern of CL response from the three different PECs (data not shown). As discussed below the macrophage component of the PEC contributed the major part of the CL response.

Asbestos is thus similar to other macrophage-stimulating agents such as casein, *C. parvum*, BCG and virus infections, endotoxin and thioglycollate in causing increased chemiluminescence or ROS release in response to membrane perturbation or phagocytosis (Nathan & Root 1977; Schleupner & Glasgow 1978; Johnston *et al.* 1980).

The finding that the asbestos-elicited macrophages used here show increased CL is consistent with other markers of activation which have been reported for macrophages treated with asbestos *in vivo* and *in vitro* (Davies *et al.* 1974; Hamilton *et al.* 1976; Miller & Kagan 1976; Sirois *et al.* 1980; Donaldson *et al.* 1982, 1983*a*,*b*) and was predicted in a previous study on the basis of increased lectin-induced capping in asbestos-activated macrophages (Donaldson *et al.* 1983*a*).

From the outset of these studies we were primarily interested in the macrophage component of the PEC although substantial numbers of PMNs could be present in some preparations. Depletion of PMNs from within activated PEC samples, by centrifugation through lymphoprep, resulted in an average reduction in PMNs of 40% with no appreciable reduction in the CL response of these PEC. We therefore concluded that CL of activated macrophages and PMNs was equal in magnitude on a cell-for-cell basis. Consequently the CL due to PMNs in any activated PEC was equivalent to the percentage of PMN present in the PEC. It is important to note, however, that the study of Sykes *et al.* (1982), and our own unpublished data, indicate that pulmonary deposition of pathogenic dust may well result in the presence of PMNs amongst the free alveolar cell population where they could contribute significantly to the accumulation of ROS if appropriately stimulated. Mixed macrophage/PMN preparations therefore constitute a relevant population for study.

The second set of experiments described above confirm that asbestos can act as a trigger for the release of ROS from activated macrophages, as measured by CL, although the response to opsonized chrysotile was much less than that produced by opsonized latex. The dose dependency of the CL response to chrysotile asbestos which was evident at the low and intermediate doses was lost at the highest dose (20 mg/ml) due possibly to a toxic effect of such a large asbestos dose; testing for cell viability during an assay confirmed a toxic effect of asbestos under the conditions of the assay. This toxicity was evident despite the fact that the asbestos fibres were opsonized with human serum and the acute toxic effects of asbestos are reduced by protein coating of the active fibre surface (Miller 1978). However, increased susceptibility of activated macrophages to the toxic effects of asbestos *in vitro*, in the presence of serum, has been previously reported from our laboratory (Wright *et al.* 1983).

We appreciate that the asbestos dose used to trigger CL in the present study was very high and far in excess of those encountered, for example, in the lungs of animals inhaling asbestos. The relatively low sensitivity of the chemiluminescence amplification and detection system, however, necessitated the use of such high doses and we do not believe that this diminishes the potential relevance of the findings.

Two recent studies have reported the effects of asbestos in triggering CL responses in phagocytes; these studies revealed that

peripheral blood PMNs could be stimulated by asbestos to release ROS (Doll *et al.* 1982*a*) while peripheral blood monocytes did not show this response (Doll *et al.* 1982*b*). Our use of activated macrophages as indicator cells in the present study is supported by reports of the macrophage-activating potential of asbestos (Davies *et al.* 1974; Hamilton *et al.* 1976; Miller & Kagan 1976; Sirois *et al.* 1980; Donaldson *et al.* 1982) and the central role of activated macrophages in inflammation (Allison *et al.* 1978).

The in-vivo relevance of the present findings lies in the possibility that activation of macrophages in the lungs of individuals inhaling asbestos could lead to localized accumulation of ROS. We do, however, recognize the metabolic and functional differences between peritoneal and alveolar macrophages although ROS release by alveolar macrophages has been reported (Williams & Cole 1981*b*). In this regard it is notable that Miller & Kagan (1976) have reported evidence of alveolar macrophage activation in the lungs of rats inhaling crocidolite asbestos.

Evidence that ROS could have local toxic and tissue damaging effects is derived from experiments where both cell-free enzyme system-derived, and phagocyte-derived, oxygen radicals have been found to cause endothelial cell damage (Sacks *et al.* 1978), red cell lysis (Kellogg & Fridovich 1977), fibroblast toxicity (Simon *et al.* 1981) and autotoxicity to PMNs (McCord & Wong 1979). These toxic effects are thought to be brought about largely by peroxidation of membrane lipids leading to structural disorganization of the cell membrane (Slater 1979). It is possible therefore that, in the lungs of individuals inhaling asbestos, a factor in the maintenance of an inflammatory response is localized release of increased levels of ROS. The asbestos-activated macrophages could be envisaged as being primed to produce increased amounts of ROS, the release of which could be triggered by contact with any of a number of inhalable particles reported to have this triggering property such as bacteria, yeast (Williams *et al.* 1980), pollen (Lindberg *et al.* 1982) and, as shown in the present study, asbestos itself. If an excess of ROS in the alveolar spaces overcame the normal scavenging systems this could lead to epithelial damage with subsequent activation of inflammatory cascades leading ultimately to fibrosis (Pickrell 1981).

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A Note on

PROTEASES AND OXIDANTS IN INJURY TO ALVEOLAR EPITHELIAL CELLS CAUSED BY LUNG-DERIVED NEUTROPHILS *IN VITRO*

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This contribution concerns leucocyte-mediated injury in the alveolar region of the lung. Inflammatory leucocytes accumulate in this region in a range of chronic and acute conditions [1]. Whilst undoubtedly performing a defensive function in normal inflammatory responses within the lung, persistent or extensive accumulation of activated leucocytes within the fragile structure of the lung parenchyma is associated with damage to the alveolar septa which may be mediated by leucocyte products [2]. The outcome of this damage may be either fibroplasia within the septal interstitium or destruction of septa [1].

We set out to develop a model for detecting injurious effects of inflammatory leucocytes on one element of the alveolar septa, the alveolar epithelial cells. Leucocytes which accumulate in the alveolar spaces during alveolitis [1] are in close contact with these cells, and we sought to reproduce this situation in the assay. Here we describe preliminary results from experiments aimed at elucidating the mechanism of inflammatory cell-mediated injury to epithelial cells *in vitro*. The benefits of the assay system are also discussed.

THE ASSAY SYSTEM

The target cells were a Type II alveolar epithelial cell line A549 [3] which we ascertained by e.m. to be virus- and mycoplasma-free. The cells were cultured under standard conditions in Eagle's Minimum Essential Medium + 10% Foetal calf serum. For assay, 5×10^4 cells were seeded into microtitre plate wells with 100 µl of complete medium, and incubated overnight in the presence of 74 KBq of ^{51}Cr. The cells were washed to remove free ^{51}Cr, and effector cells were added, viz. two different populations of cells obtained by bronchoalveolar lavage of inbred, SPF, PVG rats: (i) control cells (98% alveolar macrophages);

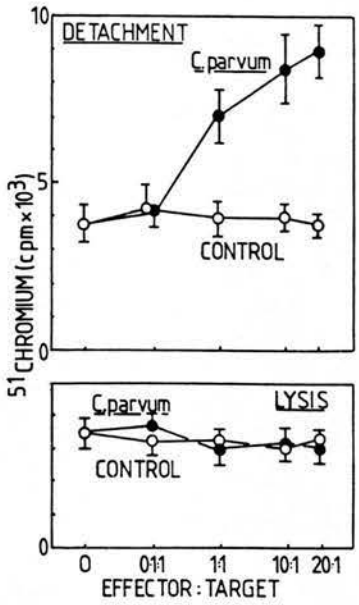


Fig. 1. Dose-dependent detachment of A549 alveolar epithelial cells caused by co-culture with control and inflammatory cells *(see text)*. The latter caused detachment ($P<0.001$) at ratios other than the lowest (*vs.* no effector); none occurred with control cells at any ratio. Neither cell population caused significant lysis. Vertical bars denote S.E.M.'s. Data from triplicate wells in three separate experiments.

(ii) 16 h after instillation of 1.4 mg of heat-killed *Corynebacterium parvum* into the lung (~90% neutrophils). Effectors and targets were co-cultured for 4 h at effector/target ratios of 0.1 to 20. Both lytic injury and non-lethal detachment injury were assessed [4].

OBSERVATIONS ON EPITHELIAL CELL DETACHMENT

Depending on the effector/target ratio, inflammatory leucocytes differed significantly from control bronchoalveolar cells in producing detachment of epithelial cells *in vitro* (Fig. 1). Neither of these populations caused lytic damage to the epithelial cells.

The most obvious candidates for causing epithelial detachment amongst the leucocyte products are oxidants and proteases, both of which have already been implicated in tissue damage [2]. We therefore used exogenous proteases and oxidants for attempted mimicking of the detachment injury caused by inflammatory leucocytes.

Exogenous proteases.- The four proteases used all caused statistically significant dose-dependent detachment (Fig. 2), much more so with trypsin or elastase than with pronase or microbial collagenase. None of the proteases caused lytic damage.

Hydrogen peroxide or superoxide anion.- Dose-dependent detachment of significant extent resulted from H_2O_2 addition (Fig. 2). The decrease in detachment at the highest concentration (500 μM) was due to extensive lysis which decreased the available detachable cells. Increased detachment (215% of control; $P<0.001$) likewise occurred with acetaldehyde/xanthine oxidase, a system which generated superoxide anion (~30 μmol). However, we later discovered that the system also generated 20 μmol of H_2O_2 which itself caused some detachment (Fig. 2).

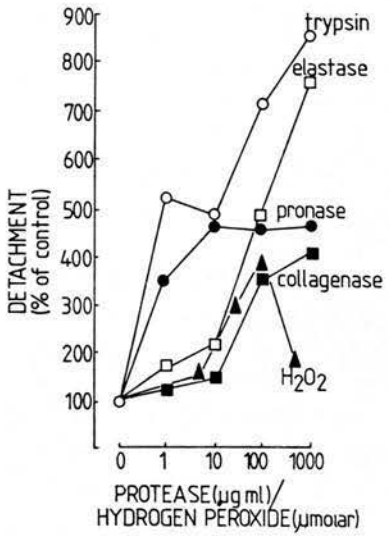


Fig. 2. Dose-dependent detachment of epithelial cells caused by exogenous proteases or H_2O_2. All data normalized to control (100%); error bars omitted for clarity, but S.E.'s were all <10% of the mean. All treatments caused significant detachment ($P<0.05$ to $P<0.001$). No lysis was caused by any treatment except for 500 μM H_2O_2.

Use of anti-oxidants or anti-proteases.- The use of specific inhibitors gave the following results for % inhibition of the detachment caused by inflammatory bronchoalveolar cells: α1-protease inhibitor, 100% ($P<0.001$); catalase, 17.4%; superoxide dismutase, 67.8% ($P<0.001$).

Conclusion.- Evidently the *in vitro* detachment injury caused to alveolar epithelial cells by leucocytes from the bronchoalveolar space of acutely inflamed lung is mediated both by leucocytic proteases and by superoxide anion. These agents could also damage the other cellular or matrix elements of the alveolar septum, and subsequent re-modelling may lead to alveolar fibrosis or destruction of septa.

METHODOLOGICAL CONSIDERATIONS

The co-culture model used has several points of advantage for studies of leucocyte-mediated cellular injury.-

1. **Sub-lethal injury *in vitro*** - The loss of integrity of cellular barriers such as epithelium and endothelium are important factors in many pathobiological processes, but cell killing does not have to occur for the integrity of such a barrier to be threatened. Sub-lethal injury, detectable as loss of attachment to substratum, is detectable in the assay described here, while lytic injury can also be measured if it occurs.

2. **Role of cell attachment factors.-** All cells, and particularly mesenchymal and epithelial cells, have important associations with their surrounding extracellular matrix, and these may be of particular importance during cell injury. Coating of wells with elements of the extracellular matrix before culturing cells on them enables their role in any subsequent injury to be assessed.

3. **Triggering of leucocytes.-** Leucocytes exist in a range of secretory states which can be altered by both exogenous agents and endogenous signals generated during inflammation and immunity. These

triggers can be included in the assay to assess their significance for leucocyte-mediated injury during inflammation or immune responses.

4. **Mechanism of the injury.**- The system easily allows the inclusion of agents which block particular effector pathways (e.g. anti-oxidants or anti-proteases) so that the mechanism of leucocyte-mediated injury can be dissected.

Acknowledgement

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Kinetics of the bronchoalveolar leucocyte response in rats during exposure to equal airborne mass concentrations of quartz, chrysotile asbestos, or titanium dioxide

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ABSTRACT The kinetics of the bronchoalveolar response was assessed in rats exposed, at equal airborne mass concentration (10 mg/m^3), to titanium dioxide—a non-pathogenic dust—and the two pathogenic mineral dusts quartz and chrysotile asbestos. Rats were killed at intervals over a 75 day exposure period and groups of rats exposed for 32 and 75 days after recovery for two months. Bronchoalveolar lavage was carried out and the lavage fluid characterised for cellular content, macrophage activation, and concentrations of free total protein, lactate dehydrogenase, and *N*-acetyl-β-D-glucosaminidase. Inhalation exposure to the two pathogenic dusts resulted in an increased number of leucocytes, macrophage activation, and increased levels of free enzymes and total protein. The pattern and magnitude of the responses to quartz and chrysotile differed. Chrysotile caused less inflammation than quartz, and the main cellular response peaked around the middle of the period of dust exposure whereas the highest levels of enzymes occurred towards the end. The difference in timing suggests that macrophages were not available for lavage towards the end of the exposure, owing to their playing a part possibly in deposition of granulation tissue. Quartz caused a greater cellular and enzyme response than chrysotile, particularly towards the end of the dust exposure phase. There was a noticeable progression of inflammation in the quartz exposed groups left to recover for two months, but not in the chrysotile recovery groups.

Introduction

Prolonged inhalation of asbestos dust may lead to the development of asbestosis (a form of interstitial lung fibrosis), bronchogenic carcinoma, and (with some types of asbestos) mesothelioma.[1] Exposure to quartz produces the fibrotic lung disease silicosis, in which the primary lesion is nodular in contrast to that in asbestosis.[2] Many studies have used bronchoalveolar lavage to examine the pulmonary response in people occupationally exposed to pathogenic dusts[3–6] and in experimentally exposed animals.[7,8]

Bronchoalveolar lavage has been used in many other pulmonary diseases for diagnosis and staging[9] and in the attempt to elucidate the cellular events underlying the pathological changes.[10] Such studies have centred on the leucocytes in bronchoalveolar lavage fluid in view of their prevalence and their key role in inflammation, the immune response, carcinogenesis, and control of mesenchymal cell proliferation. These studies show that the quantity and function of the bronchoalveolar leucocytes may determine current state of disease and subsequent progression.[9,10] We attempted to define differences in the bronchoalveolar leucocyte response to equal airborne mass concentrations (10 mg/m^3) of three dusts of very different pathogenic potential: titanium dioxide, a non-fibrogenic dust commonly used in mineral dust studies as an inert control;[11] chrysotile asbestos, which causes diffuse interstitial lung fibrosis and is a pulmonary carcinogen; and quartz, which causes nodular pulmonary fibrosis.

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Methods

ANIMALS

Male specific pathogen free rats of the *PVG* strain,

inbred at the institute's animal unit, were used; they were 12 weeks old at the start of dust exposure.

DUSTS

The dusts used were titanium dioxide (TiO_2) (rutile—Tioxide UK Ltd, Stockton on Tees), quartz (Sikron F-600, Eurostandard), and chrysotile asbestos (Union Internationale Contre Cancer (UICC) standard sample "A").

GENERATION AND MEASUREMENT OF DUST CLOUDS

Groups of up to 48 rats were exposed to dust clouds of 10 mg/m^3 in 1 m^3 inhalation chambers.[12] The dusts were dispersed into the chambers with Wright dust dispensers[13] for the two non-fibrous dusts and a Timbrell fibrous dust dispenser[14] was used for chrysotile asbestos. The rats were exposed for seven hours a day, five days a week, over a maximum period of 15 weeks (75 days' exposure). The mass concentration of respirable dust was measured daily in each chamber with the Casella MRE 113A sampler. The present legal factory limit for quartz is 0·1 mg/m^3, although this may be exceeded in exposure to mixed dusts, where there may be no legal limit. The present legal limit for chrysotile asbestos is 0·1 fibre/ml and UICC chrysotile at 10 mg/m^3 produces about 2000 fibres/ml. The inert dust titanium dioxide has no specified legal limit.

PROTOCOL

Groups of four rats were removed from the chamber and killed for study after 2, 4, 8, 16, 32, 52, and 75 days' exposure. In addition, groups of four rats were removed from exposure after 32 and 75 days and kept for 62 days before being killed. These latter groups were designated as 32 days or 75 days plus recovery. On each experimental day two control rats, similar in age to the experimental rats, were killed and studied in an identical manner.

BRONCHOALVEOLAR LAVAGE

Rats were killed by intraperitoneal injection of pentobarbitone sodium (Ceva, Watford) and weighed. The lungs were exposed by dissection and exsanguinated while in situ by injecting 30 ml of 0·85% sodium chloride (37°C) slowly through the right ventricle; blanching of the lungs indicated exsanguination. To prevent flow back due to hydrostatic pressure in the pulmonary vasculature, the right auricle was cut before injection of the final 5 ml. The trachea was cannulated with a blunt 16 gauge needle secured with nylon thread. The lungs, heart, and trachea were removed en bloc, weighed, and suspended in 0·85% NaCl at 37°C until they were lavaged. The lungs were placed on a cork mat and 4 × 10 ml aliquots of 0·85% NaCl at 37°C were introduced into the lungs and then gently withdrawn before the next aliquot. After instillation of the first 5 ml of the second lavage the lungs were massaged by gently stroking them outwards towards the tip of each lobe; the remaining 5 ml was then instilled and removed in the usual way; this procedure increases the yield of cells.[15] Each 10 ml of lavage fluid was stored separately in ice cold plastic tubes to allow the soluble elements in the first 10 ml of fluid to be analysed separately without excessive dilution. The heart, the mediastinal lymphoid and adipose tissue, and the trachea plus the cannula were dissected free of the lung and weighed; this weight was subtracted from the initial total weight to give the weight of lung tissue. The lung:body weight ratio (lung index) was calculated for each rat.

DETERMINATION OF CELL NUMBERS

Cells were obtained from the pooled 40 ml of lavage fluid by centrifugation, and counted in a Neubauer chamber after dilution. Cytocentrifuge (Shandon, Runcorn) preparations were made and stained with May-Grünwald-Giemsa stain, and a differential count was performed. The proportions of the following cell types were determined and converted to absolute cell counts: macrophages, neutrophils, lymphocytes, monocytes, binucleate macrophages, and multinucleate macrophages.

DETERMINATION OF SOLUBLE ELEMENTS

The concentrations of the following proteins in the first 10 ml of lavage fluid were assessed as described: total protein by the Coomassie Blue method (BioRad, Bradford), the cytoplasmic enzyme lactate dehydrogenase,[16] and the lysosomal enzyme *N*-acetyl-β-D-glucosaminidase.[17]

ASSAYS OF MACROPHAGE ACTIVITY

Phagocytosis was assessed on days 8, 32, and 75. Triplicate coverslip cultures of macrophages were prepared by incubation of 100 μl of lavaged cells (10^6 cells/ml of F10 medium and 10% heat inactivated fetal calf serum (complete medium)) on 6 × 22 mm alcohol cleaned glass coverslips (Chance-Propper, Warley) for one hour at 37°C in a humidified 5% carbon dioxide atmosphere. Coverslips were then washed vigorously in saline to remove non-adherent cells and placed on racks. Fluoresceinated latex beads (Polysciences, Warrington) were diluted 1:40 with complete medium and incubated for one hour at 37°C, and 100 μl was added to each coverslip culture. After 1·5 hours the coverslips were washed in four changes of saline to remove non-attached beads before being fixed in methanol for five minutes and mounted in phosphate buffered saline and glycerol (1:1). One hundred random cells were counted on each coverslip and scored as phagocytic if seen to contain three or more latex beads when viewed in an ultraviolet microscope with a × 40 objective.

Macrophage spreading was assessed by preparing macrophages on coverslips in complete medium at 10^6/ml as described above and incubating the coverslips for one hour at 37°C in a humidified 5% carbon dioxide atmosphere to allow adherence and spreading. The coverslips were fixed for five minutes in methanol and then stained with Diff-Quik. The mean diameter of 200 macrophages was assessed with a microscope interfaced with a digitising computer.[18] Occasional adherent neutrophils were clearly distinguishable from macrophages and were not measured.

HISTOLOGY

Lungs lavaged in the latter part of each exposure regimen were fixed in formalin processed to paraffin wax, and sections stained with haematoxylin and eosin.

ANALYSIS OF RESULTS

Results were obtained from four experimental rats and two control rats on each day. The initial assessment of the control data showed the anticipated day to day variation but no significant difference from TiO_2 exposed rats; in the case of spreading, macrophages from TiO_2 exposed rats showed a 0·5–1 μm increase over control macrophages, which was not significant. All data were logarithmically transformed, values from exposed animals were corrected by subtracting control values on that day to obtain a "standardised" figure, equivalent to the ratio dust exposed:control, for each rat for each assay. This figure was used to compare TiO_2 exposed rats with rats exposed to the pathogenic mineral dusts by means of analysis of variance with the Genstat computer statistical package. Where differences were seen in the analysis of variance, paired comparisons using a *t* test were made at each time point between the three groups and values of $p < 0{\cdot}01$ were taken to indicate a significant difference. For a variable for which seven time points are used this is equivalent to $p < 0{\cdot}07$ overall, and for a variable where nine time points are used to $p < 0{\cdot}09$. No data were available for TiO_2 exposed rats on days 4 and 8.

Results

DUST EXPOSURE TARGETS

The airborne concentrations of each dust for each day were within ± 30% of the target mean concentration in 212 out of 225 results. At the end of 75 days of dust exposure the mean daily dust concentrations in each chamber were: titanium dioxide 9·9 mg/m^3, chrysotile 10·0 mg/m^3, and quartz 10·1 mg/m^3.

MACROPHAGE NUMBERS

The predominant cell types in lavage fluid were macrophages in control rats and macrophages plus neutrophils in rats exposed to quartz and chrysotile. Basophils and eosinophils never exceeded 1% and lymphocytes never exceeded 10% of the total cells. With chrysotile macrophages were twice as numerous as in control samples by day 32 (fig 1) but had returned to control levels by 75 days. With quartz macrophage numbers remained at around control levels initially but then increased fourfold between days 52 and 75. In rats treated with titanium dioxide, macrophage numbers remained at control levels throughout the period of dust exposure. Rats treated with quartz for 32 or 75 days and allowed to recover showed an increase in macrophage numbers during the recovery period. With animals exposed for 75 days and examined two months later, macrophage numbers were 10 times higher than in control animals. Animals exposed to

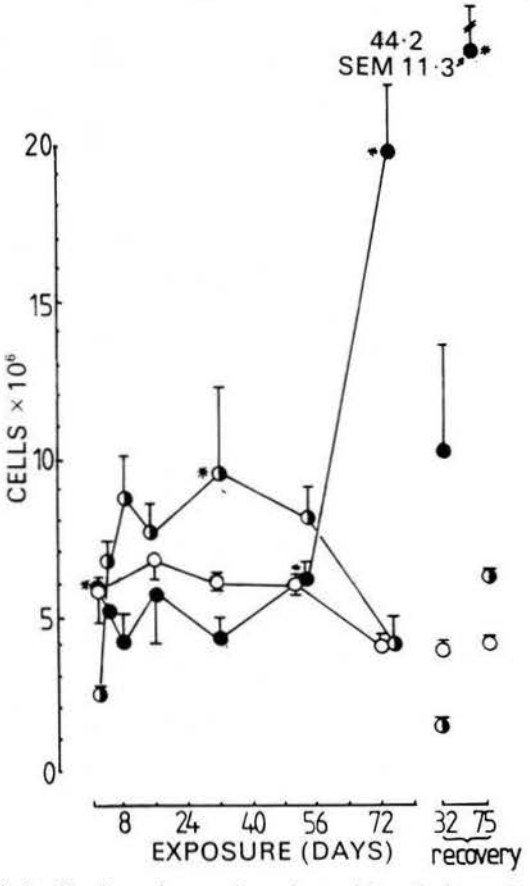


Fig 1 *Numbers of macrophages lavaged from the lungs of rats exposed to airborne titanium oxide (TiO_2), quartz, or chrysotile asbestos (means with standard errors). An asterisk denotes a significant ($p < 0{\cdot}01$) difference from the TiO_2 exposed group.* ○—○ *TiO_2;* ●—● *quartz;* ◑—◑ *chrysotile.*

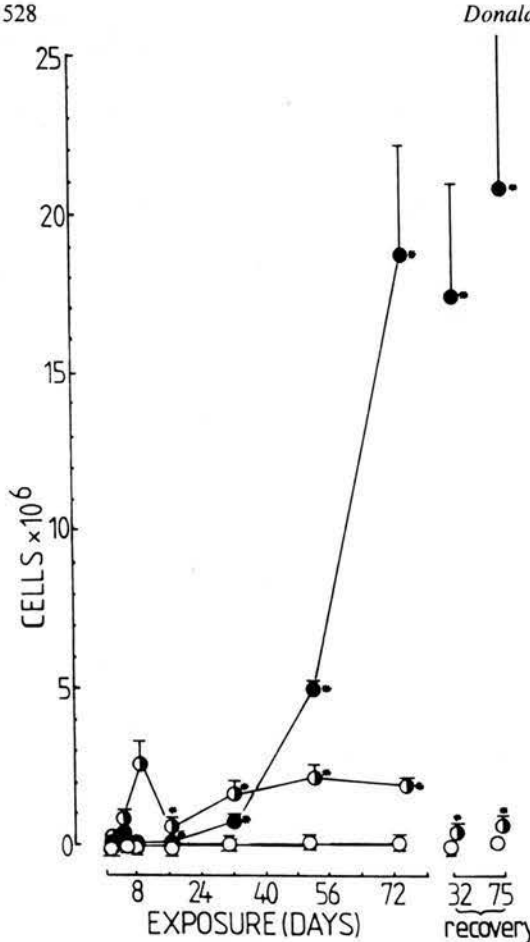


Fig 2 *Numbers of neutrophils lavaged from the lungs of rats exposed to airborne titanium oxide (TiO_2), quartz, or chrysotile asbestos (means with standard errors). An asterisk denotes a significant ($p < 0.01$) difference from the TiO_2 exposed group. ○—○ TiO_2; ●—● quartz; ◑—◑ chrysotile.*

chrysotile showed no increase in macrophages during recovery periods.

In the untreated and TiO_2 treated rats the number of mononuclear cells identified morphologically as monocytes never exceeded $0{\cdot}1 \times 10^6$ cells/rat and was frequently zero. With chrysotile exposure the mean (SEM) number of monocytes was significantly greater than with TiO_2 exposure on days 32 and 52 (0·15 (0·07) and 0·25 (0·08) $\times 10^6$ respectively). With quartz exposure a rise in monocytes did not occur until 75 days (0·23 (0·22) $\times 10^6$) but, by contrast with chrysotile exposure, this persisted in both the 32 and 75 day recovery animals (0·39 (0·12) and 0·35 (0·45) $\times 10^6$).

The number of binucleate macrophages was extremely low in the untreated and TiO_2 exposed rats ($< 0{\cdot}32 \times 10^6$). The numbers were significantly higher in chrysotile exposed rats from day 16 to day 75 and during recovery (range 0·39–0·83 $\times 10^6$). With quartz there was also an increase in the number of binucleate cells but not until day 75 (0·41 (0·12) $\times 10^6$); the numbers were particularly high in the 75 day recovery group (1·58 (1·05) $\times 10^6$).

Multinucleate macrophages were absent or very few in the untreated and TiO_2 exposed groups (range 0–0·02 $\times 10^6$). The numbers were significantly increased in the later two thirds of the chrysotile exposure period (range 0·12–0·29 $\times 10^6$) and on day 75 with quartz exposure (1·69 (0·77) $\times 10^6$). Macrophages showing mitosis were rare in both the control and the pathogenic dust exposed groups (range 0–0·04 $\times 10^6$); only with 75 day quartz exposure was there a significant increase over TiO_2 (0·05 (0·08) $\times 10^6$), and this was not maintained in the 75 day recovery group.

NEUTROPHIL NUMBERS

No neutrophils were present in lavage fluid from untreated or TiO_2 exposed rats at any time (fig 2). Exposure to both chrysotile and quartz resulted in significant increases in numbers of neutrophils by comparison with TiO_2 from 16 days onwards, much larger numbers being seen with quartz. On days 52 and 75 with quartz the numbers of neutrophils were 5·1 and 18·8 $\times 10^6$. The number of of neutrophils was greater in the 32 days plus recovery group (17·6 $\times 10^6$) than in the 32 day group (0·8 $\times 10^6$). With chrysotile neutrophil numbers never exceeded 3 $\times 10^6$ and in the latter two thirds of the exposure mean values remained at 1–2 $\times 10^6$ cells per rat. In the chrysotile recovery groups neutrophil numbers were greater than in the TiO_2 controls but (by contrast with the quartz group) smaller than in the equivalent treatment groups that had not been allowed to recover.

LYMPHOCYTE NUMBERS

Lymphocyte numbers were low, though the percentage was greater in the chrysotile exposed rats (mean 8·4 (SEM 5·5)) than in the quartz (4·8 (2·7)) or TiO_2 exposed (3·8 (2·6)) rats.

MACROPHAGE FUNCTION

The ability of macrophages to spread after culture for one hour on glass, a marker of macrophage activation, was assessed at all time points (fig 3). Rapid macrophage activation was seen at the start of exposure to chrysotile, and this continued throughout the exposure period, although there was an unexpectedly low level of spreading at 75 days. The fact that the 75 day plus recovery cells had spreading similar to that present from day 8 to day 52 suggests that an unidentifiable technical error may have been responsible. With quartz no great increase in spreading

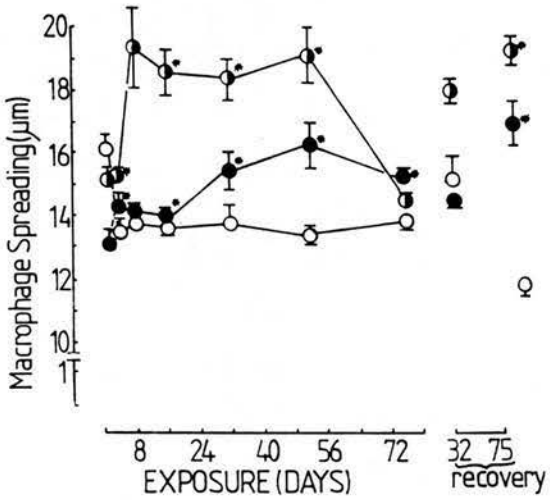


Fig 3 *Spreading activity of macrophages lavaged from the lungs of rats exposed to airborne titanium oxide (TiO_2), quartz, or chrysotile asbestos (means with standard errors). An asterisk denotes a significant ($p < 0{\cdot}01$) difference from the TiO_2 exposed group. ○—○ TiO_2; ●—● quartz; ◑—◑ chrysotile.*

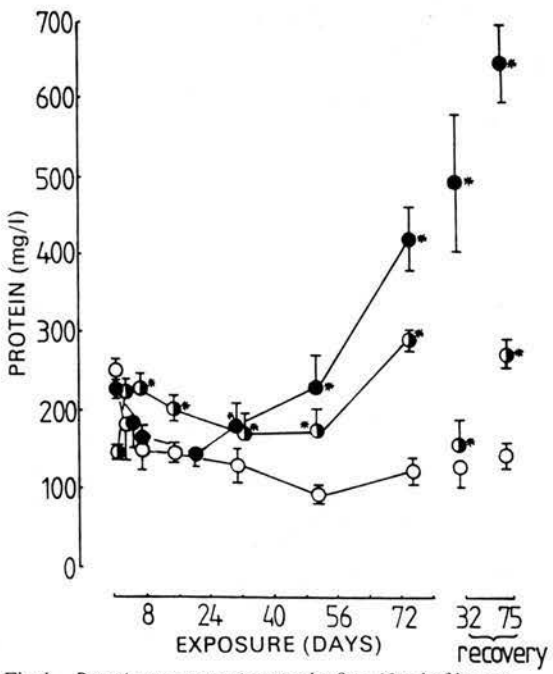


Fig 4 *Protein concentrations in the first 10 ml of lavage fluid obtained from rats exposed to airborne titanium oxide (TiO_2), quartz, or chrysotile asbestos (means with standard errors). An asterisk denotes a significant ($p < 0{\cdot}01$) difference from the TiO_2 exposed group. ○—○ TiO_2; ●—● quartz; ◑—◑ chrysotile.*

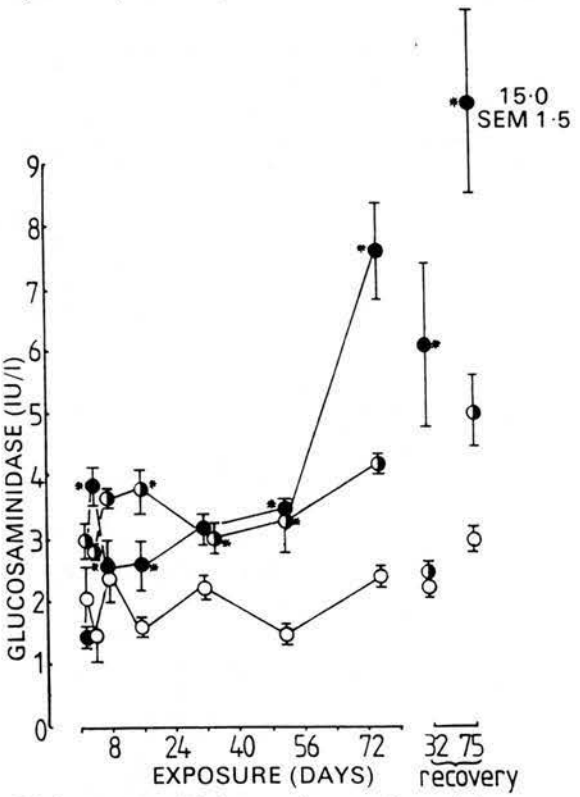


Fig 5 *Activity of the lysosomal enzyme N-acetyl-β-D-glucosaminidase in the first 10 ml of lavage fluid obtained from rats exposed to airborne titanium oxide (TiO_2), quartz, or chrysotile asbestos (means with standard errors). An asterisk denotes a significant ($p < 0{\cdot}01$) difference from the TiO_2 exposed group. ○—○ TiO_2; ●—● quartz; ◑—◑ chrysotile.*

was observable until 32 days, but this macrophage activation was maintained throughout the dust exposure period in the 75 day recovery group, though not in the 32 day recovery group. The proportion of macrophages able to phagocytose fluoresceinated latex beads was constant throughout the exposure regimen, being in the range 89–95% for all three dusts.

SOLUBLE COMPONENTS

Protein concentrations in lavage fluid from the control rats were initially high (days 2 and 4) for unknown reasons. Subsequent concentrations were around 150 mg/ml (fig 4). In the TiO_2 exposed groups the level was slightly lower than in the controls throughout most of the exposure period, but both chrysotile and quartz exposure produced significantly more protein in the bronchoalveolar space than TiO_2. This effect was evident from eight days with chrysotile and from 32 days with quartz. During both recovery periods after quartz exposure there was a further increase in protein

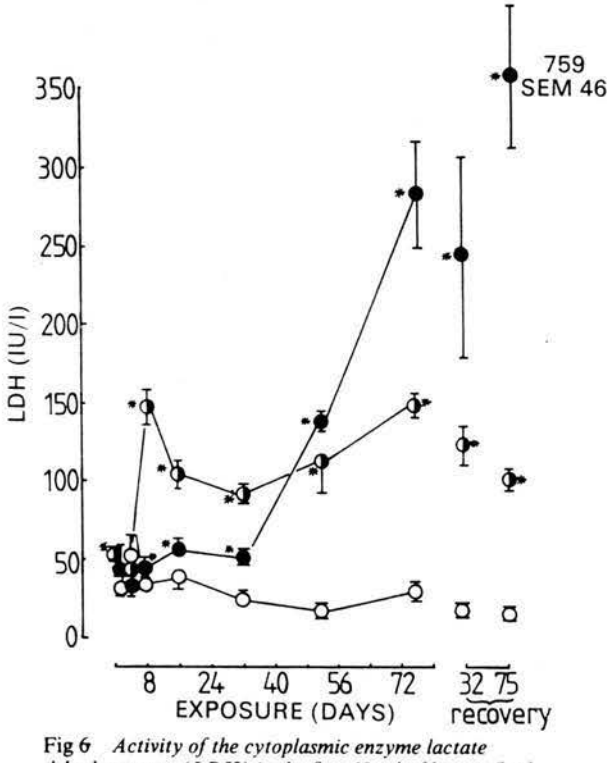


Fig 6 *Activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the first 10 ml of lavage fluid obtained from rats exposed to airborne titanium oxide (TiO_2), quartz, or chrysotile asbestos (means with standard errors). An asterisk denotes a significant ($p < 0{\cdot}01$) difference from the TiO_2 exposed group. ○—○ TiO_2; ●—● quartz; ◑—◑ chrysotile.*

concentration, particularly in the 32 day exposed animals—from 182 mg/ml at the end of exposure to 492 mg/ml after two months of recovery. With chrysotile there was no change in protein concentrations during recovery. The two enzymes measured in the lavage fluid (figs 5 and 6) showed similar patterns, both being unaltered with TiO_2 exposure. Chrysotile exposure caused early increases, which were maintained throughout the middle part of the exposure period, and rose further towards the end of exposure. Quartz caused modest early increases, with high levels of free enzyme towards the end of exposure. During recovery the concentrations of both enzymes increased in the quartz groups but with chrysotile the concentrations of enzyme remained the same as at the end of exposure.

LUNG INDEX

The lung index (lung weight:body weight × 10^4) gave conflicting results, with some early increases in the chrysotile and quartz exposed groups that did not persist, although there were increases at some of the later time points.

HISTOLOGY

Diffuse fibrosis of the lungs developed in the chrysotile exposed rats and nodular fibrosis in the quartz exposed rats after prolonged exposure.

Discussion

In this study rats were exposed to equal airborne respirable mass concentrations (10 mg/m^3) of quartz, chrysotile asbestos, and titanium dioxide. Differences were detected in the bronchopulmonary lavage fluid profile between the three dust types in terms of the kinetics of cell accumulation and the presence of soluble factors considered important in inflammation.

Titanium dioxide is an amorphous particulate, which in the rutile form used in the present study is employed in the paint, cosmetic, and food industries. Epidemiologically, TiO_2 is largely non-pathogenic in the lungs of occupationally exposed individuals.[19,20] Exposure of laboratory animals to TiO_2 also failed to induce a fibrogenic response,[11,21] although pathological changes occurred at a very high dose (250 mg/ml).[22] In our unpublished studies we have found TiO_2 to be only slightly cytotoxic to alveolar macrophages and much less toxic than chrysotile or quartz; it also causes negligible inflammation when injected into the peritoneal cavity of mice[23] or the lungs of rats. The bronchoalveolar response to medium term exposure to 10 mg/m^3 airborne TiO_2 in the present study was consistent with its non-reactive nature. Alveolar macrophages phagocytosed the TiO_2 extensively, as shown by particle laden macrophages predominating in the lavaged cell population, but there was no increase in lactate dehydrogenase activity suggesting cell death. There was, however, evidence of a degree of macrophage activation in the slight but persistently increased spreading in TiO_2 exposed macrophages, but no increase in glucosaminidase was seen in the lavage fluid, which suggested no release of lysosomal enzymes. There was also no evidence of increased mitosis in macrophages or giant cell formation as exposure progressed, and no neutrophil influx to TiO_2 exposed lung.

In contrast to this lack of response with TiO_2, both chrysotile asbestos and quartz, mineral dusts whose pathogenic potential is well known, caused pulmonary inflammation. The macrophage increases, found predominantly in the middle of the exposure phase with chrysotile asbestos, were modest, as found in other studies exposing animals to asbestos by inhalation,[24,25] and was not matched by a rise in monocytes, which, although significantly increased in

number, still formed a very small proportion of the macrophage population.

Phagocytosis of asbestos causes macrophage activation and formation of multinucleate giant cells, a process probably stimulated by the inability of single macrophages to engulf the longer fibres completely. In the chrysotile exposed animals macrophage, multinucleate giant cell, and neutrophil numbers all peaked around the middle of the dust exposure phase and tended to undergo a slight decline towards the end of the exposure regimen. In contrast, the levels of free lactate dehydrogenase, glucosaminidase, and protein were rising towards the end of dust exposure. Rising levels of enzyme release indicate progressive pulmonary inflammation, and one explanation of the contradictory cell and enzyme data is that macrophages that have phagocytosed asbestos tend to be taken up in deposits of granulation tissue around the terminal and respiratory bronchioles,[27] thus making them unavailable for lavage. Such lesions were evident in sections of asbestos exposed lung. The inflammatory indices taken together, however, show that chrysotile does not produce the extent or progression of inflammation found with quartz.

The sudden peak of lactate dehydrogenase activity, indicative of cell death, from day 4 to day 8 of chrysotile exposure may be related to the concurrent neutrophil influx and could be a result of chrysotile cytotoxicity to macrophages, with release of lactate dehydrogenase and macrophage derived neutrophil chemotactic factor[28] from dying cells. The lactate dehydrogenase may, however, be derived from alveolar epithelial cells injured by the products of inflammatory leucocytes.[29] Support for the latter possibility comes from the increased total protein in the lavage fluid, indicating increased permeability at the endothelial-alveolar epithelial barrier.

Rats exposed to quartz for up to 12 days showed no cellular or humoral changes except for low level neutrophil influx, but by 52 days there was a five fold increase over the 32 day level, with an accompanying increase in total protein, suggesting increased permeability of the epithelial barrier, and raised lactate hydrogenase activity, suggesting cell death. This was followed towards the end of the exposure phase by massive macrophage and neutrophil influx with associated increases in free enzyme and protein. As with chrysotile, the increase in macrophage number was not met by substantial increase in mitotic macrophages or monocytes and so presumably was due to emigration of interstitial macrophages into the alveolar space. Macrophages produce a neutrophil chemotactic factor in response to quartz[30] and this may have induced the large neutrophil influx. There was a concurrent notable increase in free lactate hydrogenase on day 52, suggesting cell death in the bronchoalveolar region. Macrophage breakdown products have been reported to possess powerful activity in causing inflammatory cell recruitment,[31] and this could have been a factor causing further accumulation of leucocytes in quartz exposed lung.

The recovery experiments showed that the intensity of the quartz induced alveolitis increased during the two months in which the animals breathed normal air. This progression was particularly noticeable in the 32 day exposed group. With the 75 day exposed group there was also evidence of progression, although this was generally less, perhaps because by 75 days of quartz exposure there was a near maximum response.

It has been suggested that contaminating minerals, including aluminium, may protect against quartz toxicity during exposure to mixed dusts, with progression of disease when this protection is lost at cessation of exposure.[32] Our data, obtained with a highly pure quartz standard, indicate that contaminating minerals are not a prerequisite for progression of pulmonary inflammation on cessation of exposure to quartz. With chrysotile there was no evidence of progression of inflammation in animals allowed to recover after exposure.

The phagocytic activity of macrophages did not differ between the three dust types, although the assay does not detect the total phagocytic potential of the population, which may have altered, but provides information only on the cells able to phagocytose, which approached 90% in all cases. The lung weight:body weight ratio indicated significant differences between lungs exposed to TiO_2 and to pathogenic dusts; but these were not consistent and we conclude that the method is not sufficiently sensitive.

There was a modest rise in the proportion of lymphocytes in the lavage fluid from the chrysotile exposed rats by comparison with the rats that had the other two treatments. This is in keeping with the increased number of lymphocytes reported in bronchoalveolar lavage fluid from patients with asbestosis[3] and suggests that some immune phenomenon may be occurring.

The difference in the kinetics of the alveolitis produced by asbestos and by quartz may indicate different responses to the dusts. Chrysotile produces an early response, with an influx of inflammatory cells in as little as eight days; but many of the cells that have phagocytosed dust may become bound up in the lung tissue. Once the inflammatory response to quartz has started, its progression is consistent with the known highly toxic nature of this material. Quartz particles not cleared from the lung retain their toxicity, probably killing generations of macrophages and thus stimulating the recruitment of greater numbers of leucocytes. The lack of enhanced leucocyte response to inhaled quartz during the early phase of dust inhala-

tion has been reported previously[33] and in vitro studies may provide an explanation. These have shown that, although quartz is more toxic than chrysotile, both dusts kill most macrophages within 48 hours.[34] Equal masses of the two dust types would therefore have been expected to produce similar levels of pulmonary inflammation with recruitment of inflammatory cells. In vitro studies use a great excess of dust, with many dust particles available for each cell to phagocytose. After dust inhalation, however, the dose each macrophage receives is likely to be considerably less owing to the filtration in the airways. Macrophages containing only a small amount of quartz may therefore survive for a long time before being killed or being activated to release chemoattractants. With chrysotile, however, many fibres will be incompletely phagocytosed causing chemoattractants and enzymes to be released more quickly, although in the long term the toxic effect may be less than with quartz.

In addition, the data from the macrophage spreading assay suggest that the activated macrophages produced by quartz and by chrysotile are phenotypically distinct, at least with regard to the membrane functions concerned with spreading on glass. Possibly the effector functions of the two macrophage populations are also different, and this may be expressed in the different kinetics of the inflammation produced by the two dusts and the different types of lesions found in quartz and in asbestos exposed lungs.

These results support the findings of several studies on human quartz and asbestos exposure. Macrophage and neutrophil alveolitis with increased lymphocytes was reported in asbestosis,[1 4] similar to that described above. With quartz and coal dust containing quartz human bronchoalveolar lavage has shown a great increase in macrophages with only a modest increase in neutrophils.[5 6] This is in contrast to our findings of large scale neutrophil recruitment as a result of quartz inhalation. This may be due to the higher dose used experimentally.

The kinetics of the bronchoalveolar leucocyte response to quartz and chrysotile asbestos delivered by inhalation exposure show important differences from those found with intratracheal instillation in previous studies. After intratracheal instillation of 5 mg of silica into mice a rapid (within one week) increase occurred in lavage cell numbers, which plateaued throughout the remaining 12 weeks of the study.[35] Silica (2 mg) injected into mouse lungs caused a similar rapid rise in bronchoalveolar leucocytes; by eight weeks this had fallen to almost control levels but by 20 weeks, the end of the observation period, the response was increasing again.[36] Lugano *et al*[37] described the cellular response after instillation of 50 mg of silica into guinea pig lungs, where a sudden rise and decay in neutrophil response was followed by a slow rise in macrophage numbers up to 14 days after injection. Intratracheal instillation has also been used to study pulmonary responses to asbestos, where a very late onset alveolitis occurred 16 months after a cumulative dose of over 2 g of chrysotile by repeated instillation in sheep.[38] Lemaire,[7] using a single 5 mg injection of chrysotile into rat lungs, obtained a maximal neutrophil response at one day followed by a decline in neutrophil numbers, which was replaced gradually by macrophage alveolitis. Similar treatment of hamsters[39] resulted in an influx of neutrophils at day one, which remained at much the same level (about 20%) 180 days after instillation.

The patterns of bronchoalveolar leucocyte response obtained in these studies with quartz and asbestos are complicated by differences in dose and species but are all very different from those described in the present study, where rats were exposed to respirable dust by inhalation. Because it is easy to use and relatively inexpensive, intratracheal instillation must continue to be a valuable tool for studying cellular responses within the lung to toxic agents, but its limitations are evident and have been studied.[40] The particular course of the bronchopulmonary response obtained after inhalation exposure to respirable dust is clearly an important element in understanding the pathological outcome of exposure to any toxic dust.

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The ability of inflammatory bronchoalveolar leucocyte populations elicited with microbes or mineral dust to injure alveolar epithelial cells and degrade extracellular matrix *in vitro*

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Summary. Inflammatory cells are recruited to the parenchyma of the lung in a range of conditions where they are considered to have the ability to exert damaging effects on elements of the alveolus. The injurious effects of rat bronchoalveolar-derived inflammatory cells on an alveolar Type II epithelial cell line were therefore assessed. Inflammatory populations produced by intratracheal injection of *Corynebacterium parvum* or quartz caused non-lethal detachment injury to the epithelial cells on co-culture whereas control bronchoalveolar cells had no effect on epithelial cells. The pathogenic mineral dusts quartz and chrysotile asbestos caused increased detachment injury when added to co-cultures of epithelial cells and bronchoalveolar leucocyte populations; neither titanium dioxide, a control mineral dust, nor zymosan were active in this respect. Detachment injury was particularly marked when quartz was added to co-cultures of epithelial cells and inflammatory bronchoalveolar cells from quartz treated lung. On the basis of anti-protease and anti-oxidant studies, the detachment injury was found to be mediated by protease alone in the case of quartz cells and protease plus oxidant in the case of *C. parvum* cells. The two inflammatory bronchoalveolar cell populations were found to have increased proteolytic activity, compared to control bronchoalveolar cells, as shown by increased ability to degrade fibronectin, laminin and denatured collagen. Inflammatory bronchoalveolar cells therefore have the potential to attack elements of the septal extracellular matrix as well as to compromise the integrity of the alveolar epithelium.

Keywords: bronchoalveolar, macrophages, neutrophils, epithelial cells, mineral dust

Alveolar inflammation is a feature of many pulmonary diseases and considerable effort has been expended in elucidating the role of inflammatory cells in mediating alveolar damage. In interstitial lung disease, alveolitis is a prominent feature (Crystal *et al.* 1981), and activated macrophages and neutrophils (PMN) have been implicated in promoting further inflammation and causing proteolytic and oxidative damage to the parenchyma leading to interstitial fibrosis (Hunninghake & Moseley 1984). In smokers, increased

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numbers of PMN are present in the alveolar region and have been firmly implicated in the pathogenesis of emphysema (Hoidal & Niewoehner 1983). In mineral dust-related lung disease, increased numbers of neutrophils and macrophages are found in the bronchoalveolar lavage of occupationally-exposed humans (Gellert *et al.* 1985*a*; Voisin *et al.* 1985) and experimentally-exposed animals (Begin *et al.* 1983; Lugano *et al.* 1982). Dust-exposed leucocytes have been found to possess a number of properties potentially important to the development of the pneumoconioses (Doll *et al.* 1983). The adult respiratory distress syndrome (ARDS) is characterized by massive recruitment of neutrophils to the lung parenchyma and these cells are considered to be major aetiological factors in the respiratory impairment and parenchymal damage which ensues (Weiland *et al.* 1986).

One obvious target for harmful effects of activated inflammatory cells in the alveolar region is the alveolar epithelial cells with their associated matrix components. We set out to determine whether inflammatory cells derived from the bronchoalveolar spaces of laboratory rats treated with agents causing pulmonary inflammation, could cause injury to cells of an alveolar epithelial cell line *in vitro*. The consequences of such injury, in terms of proteolytic attack on the septal extracellular matrix, were also investigated.

Materials and methods

Special reagents. Alpha-1-protease inhibitor, soybean trypsin inhibitor, alpha-2-macroglobulin, catalase, superoxide dismutase, zymosan, phorbol myristate acetate, collagen (rat Type I) and fibronectin (human plasma) were purchased from the Sigma Chemical Co., Poole, Dorset. Ethylene diamine tetra-acetic acid (EDTA) was obtained from BDH, Poole, Dorset. ^{51}Cr ($NaCrO_3$) and ^{125}I (NaI) were purchased from Amersham International, Aylesbury, Buckinghamshire. *Corynebacterium parvum* (heat killed) was obtained from Wellcome Reagents Limited, Hither Green, London. The standard mineral dust samples used were titanium dioxide (rutile) obtained from Tioxide UK Limited, Stockton-on-Tees, Cleveland; UICC chrysotile asbestos 'A'; and DQ_{12} quartz. Laminin (mouse) was purchased from Gibco, Paisley, Renfrewshire.

Animals. SPF, syngeneic, PVG rats, aged 10 weeks or greater at the time of use, were obtained from the Institute of Occupational Medicine Animal Unit.

Intratracheal injection. Ether-anaesthetized rats had their trachea exposed and a blunt ended needle was introduced into the trachea through a small incision. A volume of 0.5 ml sterile phosphate buffered saline (PBS) containing 1.4 mg of *Corynebacterium par-*

Table 1. Cellular composition of bronchoalveolar cells from control rats and rats injected intratracheally with *C. parvum* or quartz*

Bronchoalveolar lavage population	Macrophages (%)	Neutrophils (%)	Lymphocytes (%)	Total numbers of cells retreived
Control	98.5 ± 1.9	0	< 2	6.6 ± 2.3
C. parvum	11.0 ± 8.2	89.8 ± 6·7	< 2	43.5 ± 11.9
Quartz	55.0 ± 2.6	45.0 ± 6.7	< 2	22.7 ± 7.6

* Data derived as mean ± s.e.m. of five separate rats.

vum, or 1 mg of quartz was injected and the skin closed with metal clips.

Bronchoalveolar lavage. Rats were killed 16 h after *C. parvum* injection or 5 days after quartz injection by overdose with Nembutal. The lungs were dissected free of the thoracic cavity and lavaged with 4 sequential 10 ml volumes of saline at 37°C. Lavaged cells were stored on ice until counting and preparation of cytocentrifuge smears.

Alveolar epithelial cell line. A549 cells are Type II-like cells derived from a human lung tumour (Lieber *et al.* 1976) and were obtained from the Pneumoconiosis Research Unit, Penarth, Wales. By electron microscopy examination these cells were virus- and mycoplasma-free with prominent lamellar bodies. They were maintained by culturing in MEM plus 10% heat-inactivated foetal calf serum (complete medium) (GIBCO, Paisley, Renfrewshire).

Epithelial cell lysis and detachment assay. A549 epithelial cells were plated on to microtitre plate wells (Falcon, Becton Dickinson, Cowley, Oxford) as 5×10^4 cells/100 μl complete medium and incubated overnight in 74KBq ^{51}Cr/well. Monolayers were washed three times with PBS and effector bronchoalveolar lavage leucocytes were added in 200 μl of serumless medium (GIBCO) in varying numbers to obtain the required effector:target ratios of 0.1:1, 1:1, 10:1 and 20:1. Wells were incubated at 37°C in 5% CO_2 for 4 h. In experiments where the effects of triggers (zymosan, PMA, TiO_2 chrysotile asbestos and quartz) were to be included, these were added in 10 μl of PBS to obtain the required concentration. At the end of the 4 h incubation period soluble ^{51}Cr, a measure of cell lysis, was obtained by aspirating 50 μl of supernatant and counting in a gamma counter. This figure was multiplied by four to give total free counts. Spontaneous release of ^{51}Cr was assessed by incubating labelled cells in medium alone and 100% lysis was obtained by lysing

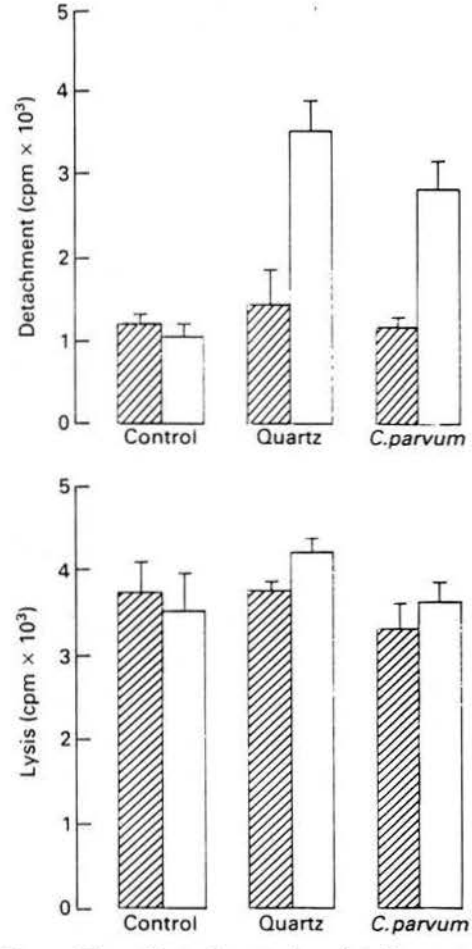


Fig. 1. The effect of control and inflammatory bronchoalveolar cell populations on detachment and lysis of alveolar epithelial cells *in vitro*. Results represent the mean ± s.e.m. of four replicate wells in three separate experiments. Significant ($P < 0.001$) increase in detachment on addition of quartz and *C. parvum* bronchoalveolar cells. Effector:target ratio: 10:1. ■, Epithelial cells only; □, epithelial cells plus BAL cells.

labelled cells with Triton-X-100 (Sigma) (1%); spontaneous lysis at 4 h was always 10–20% of the maximum value. Detachment of epithelial cells was assessed by removing the remaining 150 μl of supernatant, with accompanying detached cells, washing the wells with 2×200 μl volumes with PBS and pooling these for counting. The counts due to cell lysis in 150 μl of superna-

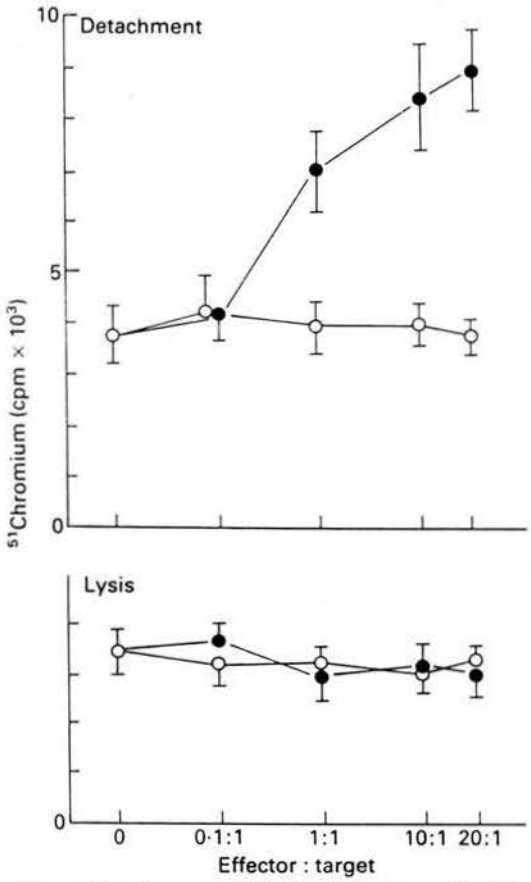


Fig. 2. Detachment and lysis of alveolar epithelial cells co-cultured with control or 16 h *C. parvum* bronchoalveolar cells at increasing effector : target ratio. Results represent mean ± s.e.m. of four replicate wells in three separate experiments. ●, *C. parum*; ○, control.

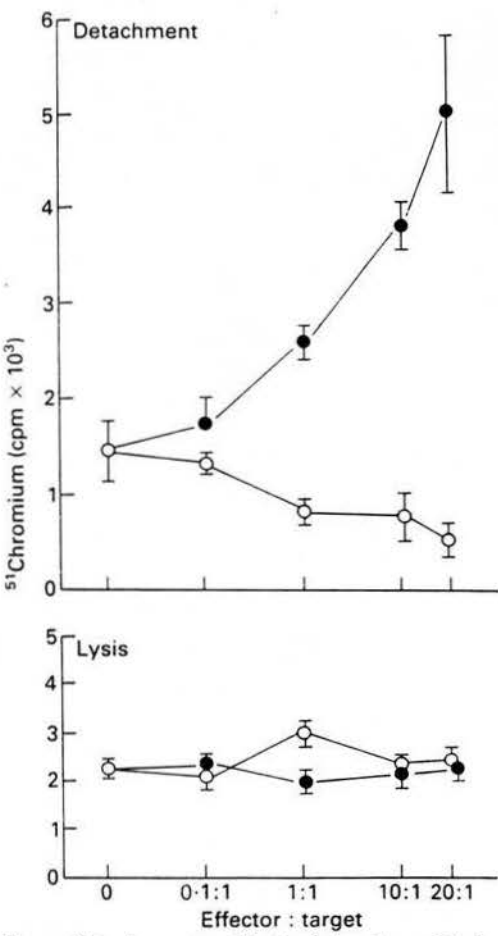


Fig. 3. Detachment and lysis of alveolar epithelia cells co-cultured with control or 5 d quart bronchoalveolar cells at increasing effector : targe ratio. Results represent mean ± s.e.m. of fou replicate wells in two separate experiments. ● Quartz; ○, control.

tant was subtracted from the pooled total to yield the counts in detached cells alone. In experiments to assess the effect of particulates on A549 cells alone, bronchoalveolar leucocytes were omitted but otherwise the protocol remained the same. To test the effect of anti-protease and anti-oxidant on leucocyte-mediated epithelial injury the following agents were added to wells as 10 μl of stock solution to obtained the indicated concentrations: alpha-1-protease inhibitor (1 mg/ml); soybean trypsin inhibitor (1 mg/ml); alpha-2-macroglobulin (0.1 mg/ml); EDTA (1mM); rat serum 5% v/v); catalase (50 μg/ml superoxide dismutase (50 μg/ml).

Assessment of lung permeability. Lung per meability was assessed by measuring th albumin concentration in the first 10 ml o lavage fluid. Albumin was assessed usin Albumin Colour Reagent (Sigma) accordin to the suppliers instructions.

Assessment of protease activity of bronchoalve lar cell population. Fibronectin, collagen ar

laminin were labelled with ^{125}I using the method of McConahey and Dixon (1966) modified by substituting L-cysteine for sodium metabisulphite. Following elution on a Sephadex-G25 column (PD10 Pharmacia, Milton Keynes, Buckinghamshire) to remove unbound ^{125}I, the fractions of the labelled protein peak were pooled and showed activities of $5-25 \times 10^6$ cpm/μg protein. ^{125}I collagen was diluted in 0.1 M acetic acid and 100 μl (10 000 cpm) were dried on to the bottom of microtitre plate wells at 45°C, a temperature at which collagen is denatured to gelatin. ^{125}I laminin was diluted in PBS and 100 μl (10 000 cpm) were similarly dried on to the bottom of microtitre plate wells. Diluted ^{125}I-fibronectin was added as 100 μl (10 000 cpm) to wells and incubated at 37°C for 2 h before aspirating the supernatant and allowing wells to dry at 45°C. Immediately prior to use, wells were incubated for 2 h in serumless medium and washed twice with two 200 μl volumes of PBS. For assay of proteolytic activity of bronchoalveolar cell populations, 10^5 cells were added to triplicate wells in 100 μl of serumless medium and incubated for 4 h. The level of proteolysis was assessed by counting free ^{125}I-labelled degradation products in 50 μl of supernatant. Control wells containing serumless medium alone were always included. Inclusion of proteases such as trypsin in these assays resulted in release of three to five times the activity released with medium alone.

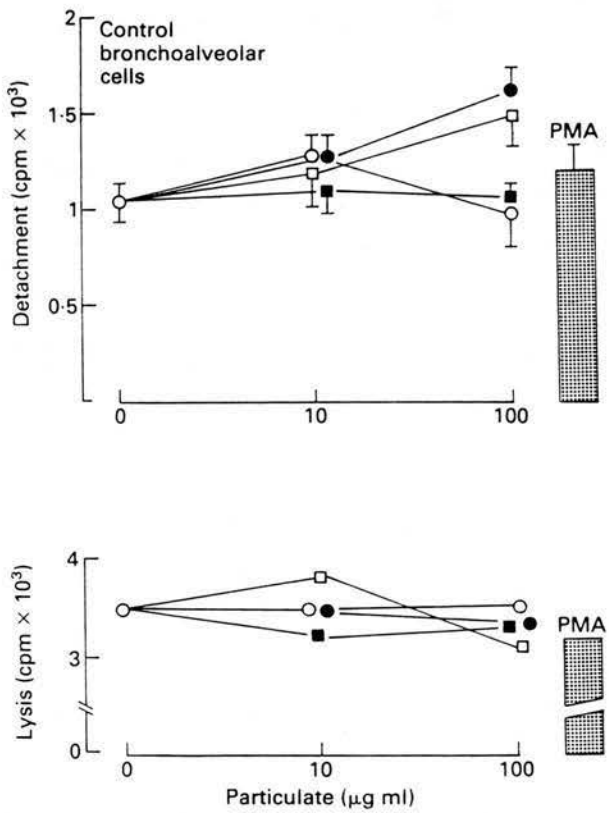


Fig. 4. The effect of TiO_2 (○) quartz (●), chrysotile (□), zymosan (■) and PMA (▣) on detachment and lysis of alveolar epithelial cells co-cultured with control bronchoalveolar cells. Significant ($P<0.001$) increases in detachment caused by chrysotile and quartz at 100 μg/ml. Results represent mean ± s.e.m. of four replicate wells in three separate experiments. s.e.m. omitted on lysis panel for clarity. Effector:target ratio 10:1.

Statistical analysis. Results were analysed using analysis of variance and differences in means of treatment groups were assessed for significance using a Student's *t*-test.

Results

Composition of the different bronchoalveolar cell populations

Table 1 shows the composition of cells obtained from control, 16 h *C. parvum* and 5 day quartz treated rats obtained by differential counts of stained cyto-centrifuge preparations.

Detachment of alveolar epithelial cells caused by co-culture with inflammatory bronchoalveolar cell populations

Figure 1 shows the activity of the three bronchoalveolar cell populations when co-cultured with alveolar epithelial cells. In no case was there any evidence of lytic damage to epithelial cells with any of the three bronchoalveolar cell populations but the two

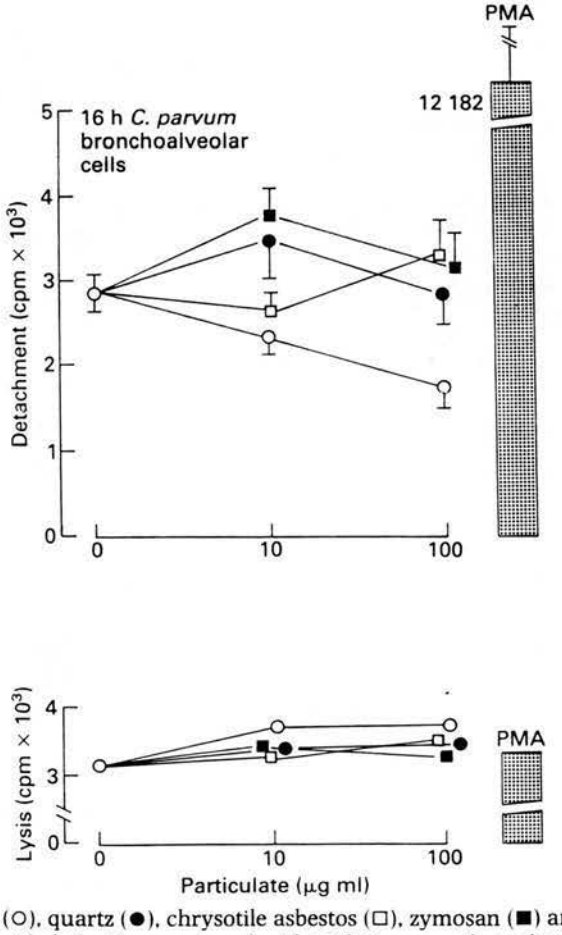


Fig. 5. The effect of TiO_2 (○), quartz (●), chrysotile asbestos (□), zymosan (■) and PMA (▨) on lysis an detachment of alveolar epithelial cells co-cultured with 16 h *C. parvum* bronchoalveolar cells. Significar ($P<0{\cdot}001$) increase in detachment produced by 10 μg/ml quartz and chrysotile asbestos and by PM Results represent mean ± s.e.m. of four replicate wells in three separate experiments. SEM omitted fro lysis panel for clarity. Effector:target ratio = 10:1.

inflammatory populations caused detachment ($P<0.001$) while the control bronchoalveolar cells did not. Figures 2 and 3 show the effect of increasing effector:target ratio on detachment.

The effect of mineral dusts, zymosan and PMA on the epithelial detachment caused by inflammatory bronchoalveolar populations

Preliminary experiments were carried out to determine the effect of mineral dusts, zymosan and PMA on detachment and lysis of epithelial cells in the absence of bronchoalveolar cells. Preliminary experiments showed that no significant detachment or lysis was caused by any of the four particulates alone but PMA caused some detachment: untreated 812 ± 106: PMA 1 μg/ml 2193 ± 92 ($\bar{x}\pm$s.e.m. of four replicates in three separate experiments) but no lysis. The addition of the three mineral dusts or zymosan to co-cultures of bronchoalveolar cells and epithelial cells had different effects depending on the origin of the bronchoalveolar cells. Although there was evidence of

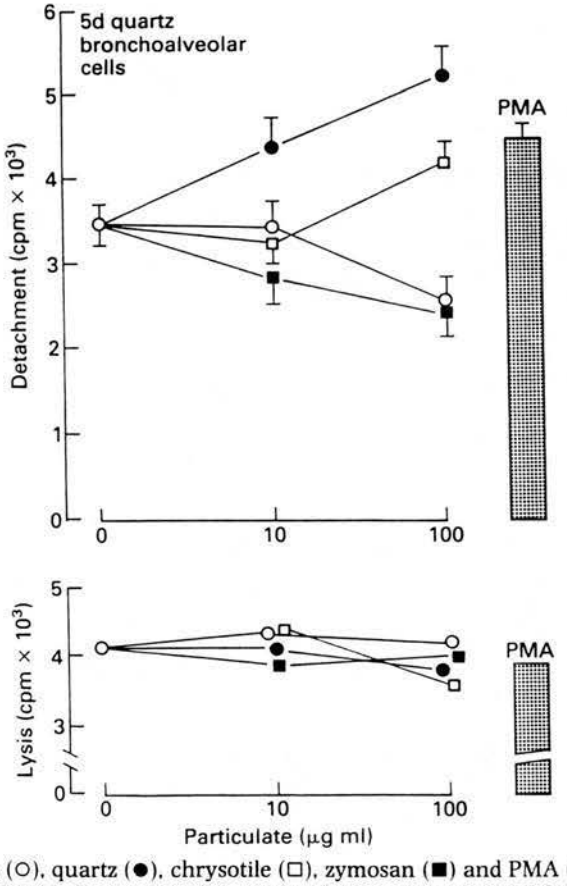


Fig. 6. The effect of TiO_2 (○), quartz (●), chrysotile (□), zymosan (■) and PMA (▨) on detachment and lysis of alveolar epithelial cells co-cultured with 5 d quartz bronchoalveolar cells. Significant ($P<0.001$) increase in detachment caused by quartz at 10 μg/ml and 100 μg/ml, chrysotile at 100 μg/ml and by PMA. Results represent mean ± s.e.m. of four replicate wells in three separate experiments. s.e.m. omitted from lysis panel for clarity. Effector:target ratio = 10:1.

Table 2. Comparison of the inhibitory effect of anti-proteases and anti-oxidants on epithelial cell detachment caused by the two inflammatory bronchoalveolar cell populations

	inhibition of detachment (%)*	
Puative inhibitor	*C. parvum* bronchoalveolar cells	Quartz bronchoalveolar cells
Alpha-1-protease inhibitor	100†	100
Soybean trypsin inhibitor	89.7	100
EDTA	0	61.2
Alpha-2-macroglobulin	71.9	24.2
Serum	100	100
Catalase	19.6	6.8
Superoxide dismutase	76.8	11.9

* Calculated by obtaining the mean from three separate experiments (s.e.m. $< 15\%$ of the mean) and inserting the mean cpm into the equation:

$$100 - \frac{\text{detachment in presence of inhibitor} - \text{spontaneous detachment}}{\text{detachment in absence of inhibitor} - \text{spontaneous detachment}} \times \frac{100}{1}$$

† When spontaneous detachment was inhibited, as in the case of alpha-1-protease inhibitor, the apparent inhibition was $> 100\%$; when this occurred the per cent inhibition is given in the Table as 100%.

Table 3. Proteolytic activity of *C. parvum* bronchoalveolar cells

		Bronchoalveolar population	
Substrate	Medium alone	Control	*C. parvum*
^{125}I. fibronectin	1079 ± 163*	2105 ± 183†	4382 ± 601
^{125}I. laminin	575 ± 48	1111 ± 62	2568 ± 312
^{125}I. denatured collagen	708 ± 98	999 ± 119	2318 ± 303

* Results represent cpm of solubilized substrate in 50 μl of supernatant.

† Significantly more proteolysis by control BAL than medium alone ($P < 0{\cdot}001$) and by *C. parvum* compared with control BAL ($P < 0{\cdot}001$). Results represent mean $\pm$ s.e.m. of four replicate wells in three separate experiments.

Table 4. Proteolytic activity of quartz bronchoalveolar cells

Substrate	Medium alone	Bronchoalveolar population	
		Control	Quartz
^{125}I. fibronectin	644±92*	935±216	2124±93
^{125}I. laminin	466±25	776±63	1359±33
^{125}I. denatured collagen	430±38	628±42	1036±67

* Results represent cpm of solubilized substrate in 50 μl of supernatant. Significantly more proteolysis by control BAL cells than medium alone ($P<0.001$) and by quartz BAL cells compared to control BAL cells ($P<0.001$). Results represent mean±s.e.m. of four replicate wells in three separate experiments.

stimulation of a modest degree of detachment with addition of asbestos and quartz to both control and *C. parvum* bronchoalveolar populations (Figs 4 & 5), the most striking effect was that of quartz in stimulating detachment of epithelial cells when co-cultured with quartz-elicited bronchoalveolar cells (Fig. 6); titanium dioxide and zymosan consistently failed to trigger substantial detachment injury.

Mechanism of detachment

A series of experiments were carried out using inhibitors to determine the nature of the detaching activity produced by *C. parvum* and quartz bronchoalveolar cells. Preliminary experiments showed that none of the inhibitors had any effects in causing lysis or detachment of epithelial cells alone. Table 2 describes the effects of the inhibitors on detachment caused by the two inflammatory bronchoalveolar cell populations.

Quartz and *C. parvum* bronchoalveolar cells produced different patterns of inhibition, the major differences between the inhibitor profiles obtained for the two populations being in the effects of EDTA, alpha-2-macroglobulin and superoxide dismutase. EDTA was ineffective against *C. parvum* bronchoalveolar cell-mediated detachment but substantially inhibited quartz bronchoalveolar cell-mediated detachment. Both alpha-2-macroglobulin and superoxide dismutase were highly active in inhibiting *C. parvum* cell-mediated detachment but were much less active against the quartz bronchoalveolar cells.

Permeability of control and inflamed lung

Permeability of control, *C. parvum* and quartz treated lung was assessed by measuring the levels of albumin present in the bronchoalveolar lavage fluid. This revealed the following amounts of total albumin (mg) in the first 10 ml of lavage fluid (mean±s.e.m. of five rats) control 0.4±0.1, quartz-treated 1.5±0.2, *C. parvum*-treated 2.6±0.3; both treatments were significantly ($P<0.001$) different from control.

Proteolytic activity of control and inflammatory bronchoalveolar populations

The activity of the three bronchoalveolar leucocyte populations in causing proteolysis of pure preparations of fibronectin, laminin and denatured collagen (gelatin) are described in Tables 3 and 4.

Discussion

The demonstration here of epithelial cell detachment in the absence of lysis, mediated by inflammatory bronchoalveolar cell populations, is similar to that shown by peripheral blood PMN in several previous studies as will be discussed. Experiments with anti-protease and anti-oxidants revealing that, in our model, protease and superoxide anion were involved in the detachment injury are also supported by previous studies. In two of these, non-lytic detachment injury was reported when peripheral blood PMN were the effectors and HeLa cells, fibroblasts (Taubman & Cogan 1975), A459 cells (Ayars *et al.* 1984) and endothelial cells (Harlan *et al.* 1981) were the targets; in all cases the effector molecules were proteases. In contrast to the findings of the present study, however, these workers reported that PMN alone had no effect and expression of the proteolytic injury required stimulation with known leucocyte 'triggers' in two cases while the remaining study utilized PMN granule contents directly.

In three further studies (Sacks *et al.* 1978; Suttorp & Simon 1982; Martin 1984) endothelial cells were the targets, and once again unstimulated PMN were inactive but triggering in these cases caused oxidant-mediated lysis of the endothelial target cells. A recent study by Sugahara *et al.* (1986) also showed that induction of peripheral blood PMN-mediated epithelial permeability required a phagocytic stimulus. In the present study, utilising PMN from inflamed lung, the ability to cause detachment injury was spontaneous, protease and oxidant mediated, and did not require triggering. This latter effect can be assumed to be due to the recent activating effect of soluble mediators such as complement and phagocytosis in the inflammatory milieu of the alveolitis (Baggiolini & Dewald 1985).

Aveolar inflammation in humans is likely to be considerably less intense than the inflammation produced here experimentally, except possibly in the case of ARDS, and the burden of inflammatory cells may be insufficient for such an extreme detachment response as demonstrated here *in vitro*. The potential to cause injury may also be ameliorated by anti-protease and anti-oxidant defence mechanisms in the lung parenchyma. Under these conditions low level attack on the epithelial cells could still lead to the increased permeability of the alveolar membrane described in situations where inflammatory cells are present in the alveoli (Weiland *et al.* 1986; Rinderknecht *et al.* 1980; Gellert *et al.* 1985*b*). Increased permeability accompanied alveolar inflammation in the present study as shown by raised albumin levels in the lavage fluid of the *C. parvum* and quartz-injected rats.

Pathogenic mineral dusts did cause stimulation of detachment injury in both control and inflammatory populations but the effect was modest with the exception of quartz treatment of the quartz-elicited inflammatory cells which produced large scale increases in detachment. *In vivo* parallels of this effect exist as evidence of intense alveolar epithelial injury following quartz exposure manifest as Type II epithelial cell hyperplasia (Lugano *et al.* 1982), lipoproteinosis (Heppleston 1975) and evidence of desquamation both experimentally following quartz-exposure (Miller *et al.* 1978) and in bronchoalveolar lavage studies in silicotics (Schuyler *et al.* 1980). The difference between the *C. parvum* and quartz inflammatory bronchoalveolar populations in their relative ability to be stimulated by quartz to cause detachment suggested that the dusted macrophages which comprised about half of the quartz population, could be of particular importance. It is not possible, however, to discoun

differences between the two PMN populations as also being of relevance. It was noteable that asbestos, another pathogenic dust which causes alveolitis, stimulated detachment by quartz cells.

The evidence, from blocking studies, implicating protease in the detachment injury, was supported by experiments which showed that both inflammatory populations possessed much more proteolytic activity against fibronectin, laminin and denatured collagen than the control bronchoalveolar leucocyte population. In the case of the *C. parvum* bronchoalveolar cells, the proteolytic activity was that of a serine protease while in the case of the quartz population the inhibitor profile suggested that activation of a metallo-protease (possibly macrophage collagenase) by a neutrophil-derived serine protease (Werb 1982) was occurring. Separation of macrophages and neutrophils is, however, required to determine the actual role of the two cell types and these experiments are in progress.

The finding of incomplete inhibition by alpha-2-macroglobulin in the case of the quartz cells may be explained as retention of activity against small molecular weight substrates by alpha-2-macroglobulin-bound enzyme (James 1980). The effector proteases produced by the quartz bronchoalveolar leucocytes may retain affinity for small molecular weight cell attachment molecules when bound to alpha-2-macroglobulin.

Oxidants were not involved in the detachment injury caused by quartz bronchoalveolar inflammatory cells but through the use of superoxide dismutase it was revealed that superoxide anion was involved in the epithelial injury caused by *C. parvum* cells. We therefore conclude that superoxide anion and protease can act together to cause detachment injury with some inflammatory leucocyte populations possibly by oxidant rendering the cells more susceptible to proteolytic attack; in this regard we note that oxidants have been reported previously to attack extracellular matrix (Riley & kerr 1985).

The demonstration of increased proteolytic activity against elements of the extracellular matrix by the same inflammatory populations which caused detachment, confirms a previous report that inflammatory leucocytes cause cellular detachment *in vitro* by digesting extracellular matrix components such as fibronectin (Harlan *et al.* 1981).

Thus in the alveolar region of lungs inflamed by the presence of mineral dust or microorganisms, inflammatory leucocytes accumulate which have the ability to injure alveolar epithelial cells and degrade extracellular matrix elements of the basement membrane and interstitium. These effects may be important in long-term remodelling or destruction of the lung parenchyma following inflammation.

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TOXICITY OF CERAMIC AND ARAMID FIBRES IN ANIMAL AND *IN VITRO* STUDIES

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Abstract *— We have examined the toxicity of ceramic aluminium silicate glass fibre (ceramic fibre) and an aramid fibre for short and long-term toxicity in animal models. Comparisons were made with the non-toxic dust titanium dioxide and the pathogenic fibrous dust chrysotile asbestos. In a short-term assay of ability to cause inflammation in the mouse peritoneal cavity, both ceramic and aramid showed marked activity. On long-term exposure to airborne, respirable ceramic fibre, a substantial number of rats developed lung tumours and fibrosis. Intra-peritoneal injection studies with both aramid and ceramic fibre produced small numbers of mesotheliomas. Three different* in vitro *assays were also utilised and these produced conflicting data as to the toxicity of the two test dusts with the only unequivocal evidence of toxicity being shown by the aramid sample in the macrophage cytotoxicity assay. These studies have therefore revealed that (i) both ceramic and aramid fibre may have the potential to cause lung disease in humans (ii) the toxicity of ceramic and aramid fibre, evident in the* in vivo *assays, was not reliably detected in the 3* in vivo *assay systems used.*

INTRODUCTION

Evidence that asbestos fibres cause disease in humans and in experimental animals has stimulated a search for other fibrous materials with the advantages of asbestos but which are less hazardous. Reason dictates that these substances should themselves be comprehensively tested for their ability to cause disease. Amongst the man-made fibrous materials which have some of the useful properties of asbestos are ceramic aluminium silicate glass fibres (ceramic fibres) and aramid fibres. Both of these materials are made by industrial processes whose details are given elsewere[1][2]. At the Institute of Occupational Medecine we have tested these materials in short and long-

(1) Preston J., 1978. Aramid Fibres. In: Encyclopaedia of Chemical Technology, Vol. 3. John Wiley & Sons Inc. New York, 213-242.

(2) Miller W.C., 1982. Refractory Fibres. *In* Encyclopaedia of Chemical Technology, Vol. 20. John Wiley & Sons Inc. New York, 65-77.

MATERIALS AND METHODS

PREPARATION OF MINERALS

Ceramic aluminium silicate glass fibrous insulation material (Triton, Kaowool, Morganite) was received as a bulk sample and passed through steel rollers to disaggregate it. This material was then dispersed as an airborne cloud, using a Timbrell dust generator, passed through a cyclone system to obtain respirable fibres and then into an inhalation exposure chamber at 10 mg/m^3 (for full details see Davis et al 1984)[3]. For injection and *in vitro* studies airborne dust from the chamber was collected on the filters of NCB, MRE dust sample.

Aramid fibre (Kevlar, DuPont) was received as a bulk sample of the pulp. Due to difficulties encountered in generating a respirable cloud from the bulk sample, no inhalation studies were undertaken with the Kevlar aramid. Instead, a portion of the bulk pulp was suspended in sterile saline and disaggregated by utilising the cavitational forces generated by a tissue homogeniser (for details see Davis et al 1987)[4]; the aramid sample used was not representative of that which individuals might be exposed to by inhalation. In long-term *in vitro* assays (V79/4 and A549 assays) the aramid fibre was found to be microbially contaminated; aramid samples were therefore autoclaved (130°C, 10 mins) or irradiated (150K RAD) prior to use in these assays.

CHARACTERISATION OF THE MINERALS

The size distribution of the ceramic dust, as collected from the chamber air, is given in **Figures 1** & **2.** These reveal that whilst there were few long, thin fibres there were large numbers of short, thin fibres (90% fibres <3μm long and <0.3 μm diameter).

In the case of the aramid sample it was not possible to carry out a length distribution since the disaggregation process was complete and many fibres were incompletely separated from tangles of fibre. Diameter estimates were however possible (**Figure 3**) and these showned that 50-60% of fibres had diameter of <0.25 μm.

For comparision with the ceramic and aramid fibres we used UICC 'A' standard chrysotile asbestos as a positive control and titanium dioxide (Rutile;

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(3) Davis J.M.G., Addison J., Bolton R.E., Donaldson K., Jones A.D., Wright A., The Pathogenic Effects of Fibrous Ceramic Aluminium Silicate Glass Administered to Rats by Inhalation or Peritoneal Injection. *In* Biological Effects of Man-Made Mineral Fibres - Proceedings of a WHO/IARC Conference, Copenhagen, April 1982.

(4) Davis J.M.G., 1987. Carcinogenicity of Kevlar Aramid Pulp Following Intraperitoneal Injection into Rats. Institute of Occupational Medicine, Report No. TM/87/12.

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(4) Davis J.M.G., 1987. Carcinogenicity of Kevlar Aramid Pulp Following Intraperitoneal Injection into Rats. Institute of Occupational Medicine, Report No. TM/87/12.

4. *In Vitro* assays —

(i) Cytotoxicity to rat alveolar macrophages

The ability of ceramic and aramid fibres to damage rat alveolar macrophages was assessed using a ^{51}Cr release assay[6]. In this assay freshly harvested rat alveolar macrophages are pre-labelled with ^{51}Cr and the ability of test dusts to cause injury is assessed by release of ^{51}Cr following incubation with the cells for 24 hours.

(ii) V79/4 assay

Details of this and the A549 cell assay are given in Brown et al 1986[7]. Briefly, dusts at various doses are cultured with a lung fibroblast cell line - V79/4, for 6 days and the number of colonies present at the end of this time is assessed by staining and visual counting.

(iii) A549 cell assay

In this assay dusts are assessed for their ability to cause increases in the size of the cells of an alveolar epithelial cell line - A549. Different concentrations of dust are incubated with cells for 4 days, the cells detached and their diameter assessed. Previous studies have demonstrated the activity of fibrous, carcinogenic dusts in the V79/4 and A549 assay systems[7] while the toxic, non-carcinogenic dust quartz has low activity.

STATISTICAL ANALYSIS

Results were examined by analysis of variance and differences in treatment groups assessed using a 't' test.

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(6) Donaldson K., Brown G.M., 1987. Assessment of Mineral Dust Cytotoxicity towards Rat Alveolar Macrophages Using a ^{51}Cr Release Assay. Toxicol. Appl. Pharmacol. (in press).

(7) Brown G.M., Cowie H., Davis J.M.G., Donaldson K., 1986. In Vitro Assays for Detecting Carcinogenic Mineral Fibres: a Comparison of Two Assays and the Role of the Fibre Size. Carcinogenesis 1971-1974.

RESULTS

INFLAMMATION IN THE PERITONEAL CAVITY

These results, shown in **Figure 4** reveal that the titanium dioxide sample caused no substantial inflammation in the peritoneal cavity at the doses used. The UICC chrysotile sample produced a marked burst of inflammation which was sustained throughout the 8 days of the experiment. Ceramic and aramid fibre produced quite different responses, both substantially more than the titanium dioxide control. Compared to the UICC chrysotile the two test dusts produced inflammatory responses which were low in magnitude in the early stages but which progressed and were still present to a considerable degree by day 8.

INFLAMMATION IN THE RAT LUNG

We have observed that the number of cells recovered from the lungs following injection of fibrous dusts is low and this effect was evident with both the ceramic and aramid fibre samples. However, assuming that the cells which are obtained by bronchoalveolar lavage are representative of the cells within the lung the percentage of neutrophils gives an indication of the level of inflammation. In the alveolar spaces of normal rat lung there are 0% neutrophils and with titanium dioxide injection there is a slight neutrophil influx (**Table 1**). It is evident from **Table 1** however that UICC asbestos, ceramic and aramid fibres all produced more marked inflammatory responses as judged by the neutrophil levels.

PATHOLOGY

Table 2 shows the number of lung tumours produced by inhalation of airbrone ceramic fibre or chrysotile asbestos at 10 mg/m^3 airborne concentration. In addition both dusts cause marked parenchymal fibrosis.

Table 3 demonstrates the ability of the aramid, ceramic and chrysotile samples to produce mesotheliomas following intraperitoneal injection in the rat. Fibrosis was evident in the peritoneal cavity of rats injected with aramid fibre.

IN VITRO STUDIES

(a) Cytotoxicity to alveolar macrophages

Figure 5 shows that ceramic fibre is not substantially cytotoxic to rat alveolar macrophages compared to TiO_2 while aramid and autoclaved aramid both showed significant toxic effects as assessed by release of ^{51}Cr after 24 hours. The toxic effect was, however, less marked than that chrysotile or quartz.

(b) V79/4 cell assay

This assay, (results shown in **Figure 6**), as expected from previous studies, revealed quartz to be low in activity and chrysotile to be very active in inhibiting colony formation. Ceramic fibre and the two aramid samples were virtually inactive in this assay system.

(c) A549 cell assay

As shown in **Figure 7** mean size of A549 cells and percentage of cells greater than 36 μm diameter was low in control and with TiO_2 or quartz treatment and increased with chrysotile treatment. With ceramic, and both Kevlar samples, cell size was moderately but significantly increased, with the aramid sample appearing to be slightly more active.

DISCUSSION

This study has demonstrated that both ceramic fibre and aramid fibre have activity in both long and short-term assays of toxicity in animals. Both dusts caused significantly more inflammatory response following injection into mouse peritoneal cavity and the rat lung, than titanium dioxide which has low activity in causing lung damage except at a very high dose[8]. The pattern of the inflammatory response in the peritoneal cavity with ceramic and aramid was different to that produced by chrysotile asbestos and was still present 8 days post-injection. The significance of the difference in pattern of inflammatory response from that produced by UICC chrysotile is not clear.

In terms of long-term pathology following exposure, differences between dusts is complicated by the different exposure regimes used. Ceramic fibre could be prepared as an airborne cloud so rats were exposed by inhalation. Administered in this way ceramic fibre showed a marked ability to produce both tumours and fibrosis in the lung. With aramid, the failure to generate respirable clouds precluded its use in inhalation studies. Both aramid and ceramic were utilised in intraperitoneal injection studies where both were found to cause few tumours at the high dose used (25 mg), a dose at which chrysotile asbestos produced tumours in virtually all exposed animals. With aramid the low number of tumours obtained may be explained on the basis that there were not very many small fibres present due to the problems with the efficiency of the disaggregation process; thus the dose of fibres in the size range known to be optimal in causing mesotheliomas (see discussion in Brown et al.)[7] was likely to be quite small in terms of the total mass injected. The net

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(8) Lee K.P., Trochimowicz, Reinhardt C.F., 1985. Pulmonary Responses of Rats Exposed to Titanium Dioxide (TiO_2) by Inhalation for Two Years. Toxicol. Appl. Pharmacol. 79: 179-192.

result is, therefore, that activity of a truly respirable sample of aramid is likely to be greater than that indicated in these experiments.

The *in vitro* assay of alveolar macrophage cytotoxicity revealed ceramic fibre to be very low in toxicity; aramid fibre however was markedly cytotoxic as has been shown by Dunnigan et al.[9] using a similar assay system. In the V79/4 and A549 *in vitro* assay systems which are sensitive to fibrous dust and may reflect their carcinogenic potential[7] the results were inconsistent. The V79/4 assay showed no activity while the A549 assay was suggestive of mild toxic potential in both ceramic and aramid fibres. These findings are in agreement with the problem of occasional anomalous results in these assays which we have described previously[7] and confirms that, although *in vitro* assays may be useful indicators, they cannot at present replace *in vivo* testing of asbestos substitutes.

The relative activities of ceramic and aramid fibre compared to titanium dioxide and chrysotile in the assays used here are shown, in summary, in **Table 4.**

The findings presented here on the biological activity of aramid are in agreement with those of several previous studies which have demonstrated toxic effects. Pott et al.[10] reported a 12.9% incidence of tumours in the peritoneal cavity following injection of Kevlar aramid into 31 rats. Lee et al[11] exposed rats for only 2 weeks to airborne Kevlar aramid and found septal thickening and granuloma formation. Dunnigan[9] et al. using a small fibre size fraction found aramid to be highly cytotoxic to alveolar macrophages *in vitro* as assessed by release of enzymes. Clearly the present and previous studies, with the exception of Lee et al.[11], used non-inhalation exposures and samples of dust prepared in various ways which make them of questionable direct relevance to inhalation exposure in humans. However, previous work with asbestos and other toxic dusts have shown that injection and *in vitro* assays have given an indication of the ultimate toxicity of dusts and on the basis of this some caution on the use of aramid is warranted.

In the case of ceramic fibre, Pott et al.[10] once again found tumours following injection into the peritoneal cavity of rats with two different ceramic samples. We know of no published studies other than our own on the use of ceramic fibre in short-term animal assays or *in vitro* assays. The results so far, however, also suggest that ceramic fibre should be treated as a potential risk until shown otherwise.

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(9) Dunnigan J., Nadeau D., Paradis D., 1984. Cytotoxic Effects of Aramid Fibres in Rat Pulmonary Macrophages: Comparision with Chrysotile Asbestos. Toxicology Letters 20: 277-282.

(10) Pott F., Ziem V., Reiffer F.J., Huth F., Ernst H., Mohr V., 1987. Carcinogenicity Studies on Fibres, Metal Compounds and Some Other Dusts in Rats. Exp. Pathol. (in press).

(11) Lee K.P., Kelly D.P., Kennedy G.C., 1983. Pulmonary Response to Inhaled Kevlar Aramid Synthetic Fibres in Rats. Toxicol. Appl. Pharmacol. 71: 242-253.

ACKNOWLEDGEMENT

The authors acknowledge the financial assistance of the Brithish Asbestosis Research Council.



TABLE 1

Proportions of leukocytes in the bronchoalveolar lavage 3 days after intratracheal injection of 2.5mg of the indicated dusts: x(SD) of 3 rats

DUST	MACROPHAGES	NEUTROPHILS	LYMPHOCYTES
TiO_2	86.3 (5.5)	6.7 (4.7)	7.0 (1.0)
UICC chrysotile	74.3 (11.9)	23.7 (12.7)+	1.7 (0.6)
Ceramic	76.3 (4.7)	21.0 (4.6)+	2.0 (1.0)
Aramid	75.7 (7.6)	21.3 (7.1)+	2.7 (3.0)

+ significant increase over TiO_2 $P<0.01$

TABLE 2

Numbers of tumours produced following inhalation exposure of rats to ceramic fibre or chrysotile asbestos at 10 mg/m^3 airborne dust concentration

NUMBER OF RATS	DUSTS	NUMBER OF LUNG TUMOURS
48	Ceramic	8
40	UICC Chrysotile	15*
40 + 20	Control	0

*Results from Davis J.M.G. et al., Br J. Cancer 37: 673, 1978.

TABLE 3

Development of tumours following intraperitoneal injection of aramid fibre or chrysotile asbestos into the peritoneal cavity of rats

DOSE(mg)	DUST	NUMBER OF RATS	NUMBER OF TUMOURS (MESOTHELIOMAS)
25.0	Aramid	32	2
25.0	Ceramic	32	3
25.0	UICC chrysotile'A'	31	30*

* Data from Bolton R.E. et al., Ann. Occup. Hyg. 26: 569, 1982.

TABLE 4

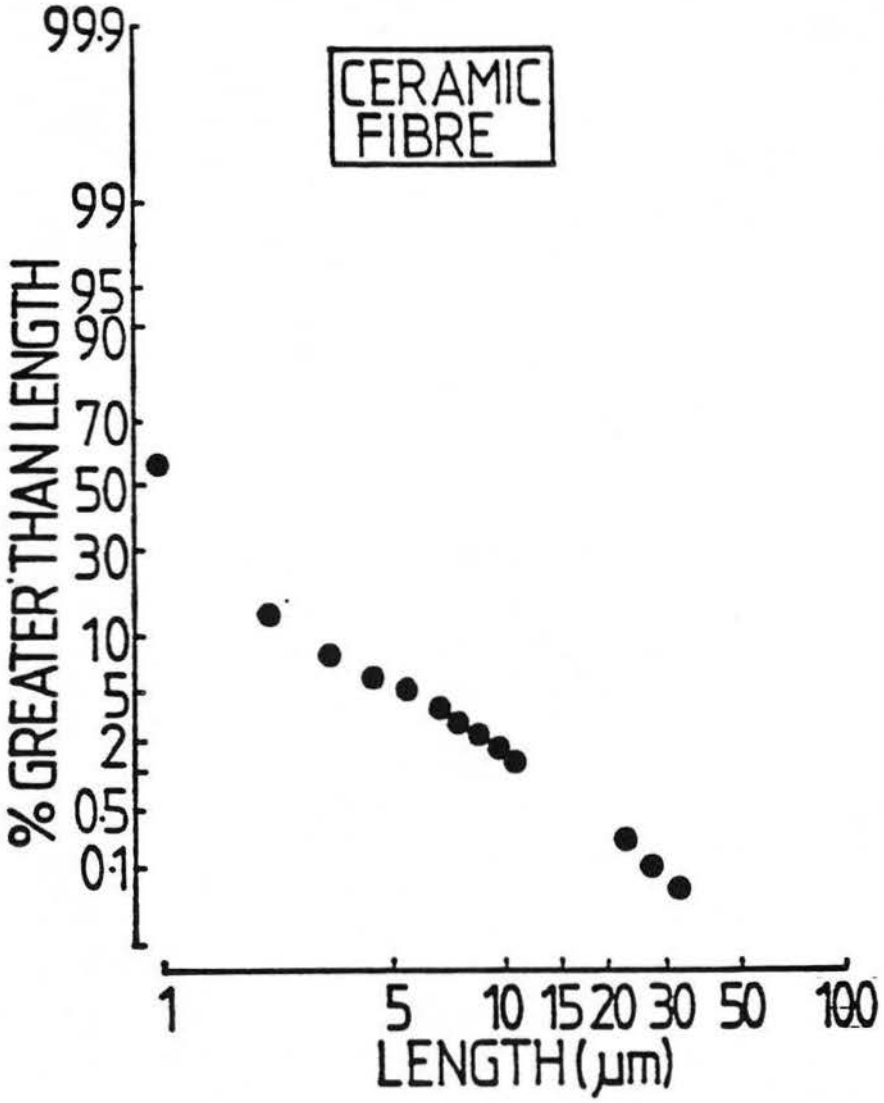
Summarised activity of different dust samples in assays of biological response carried out at the Institute of the Occupational Medicine

DUST	INFLAMMATION ASSAY PERITONEAL CAVITY	INFLAMMATION ASSAY LUNG	IN VITRO ASSAYS	LUNG PATHOLOGY FIBROSIS	LUNG PATHOLOGY TUMOURS	PERITONEAL MESOTHELIO
TiO2	0	0/+	0	ND	ND	ND
UICC chrysotile	+++	++	+++	+++	+++	+++
Ceramic	++	++	0/+	++	++	+
Aramid	++	++	+	ND	ND	+

ND = not done

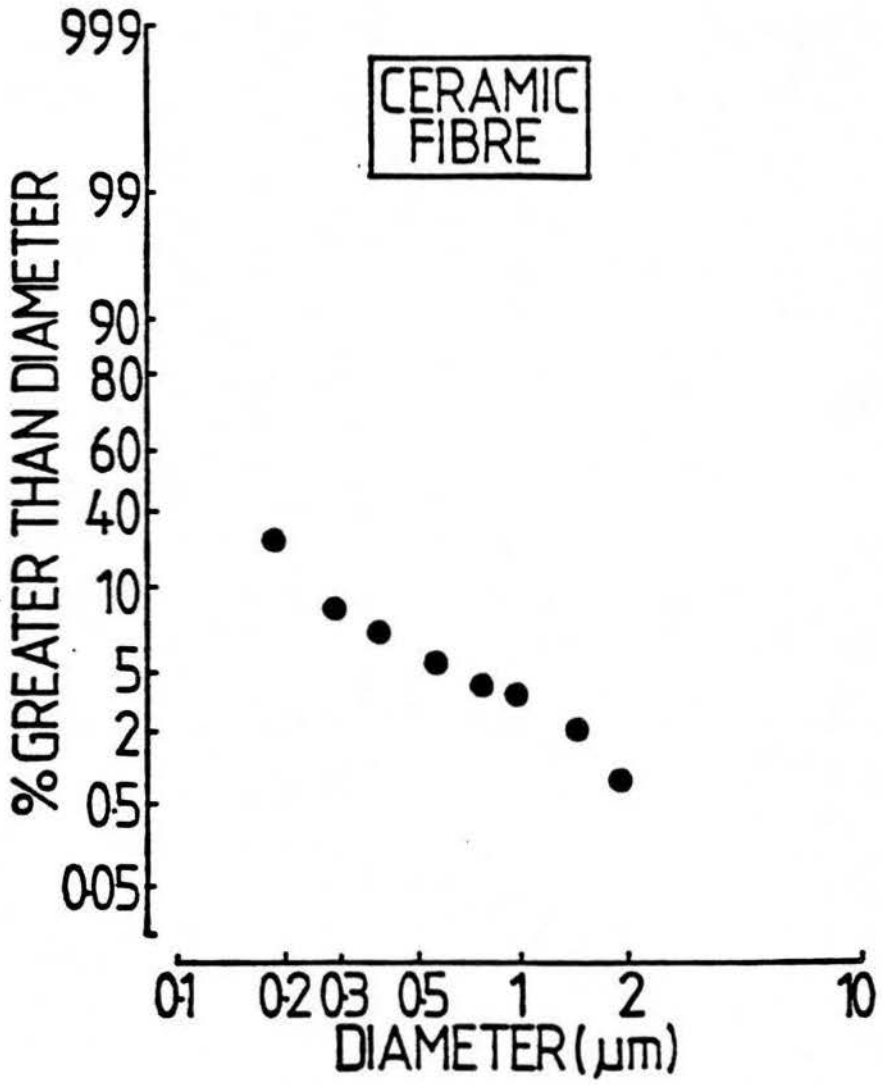
Scale of activity: -+++ = marked —> 0 = minimal.

FIGURE 1



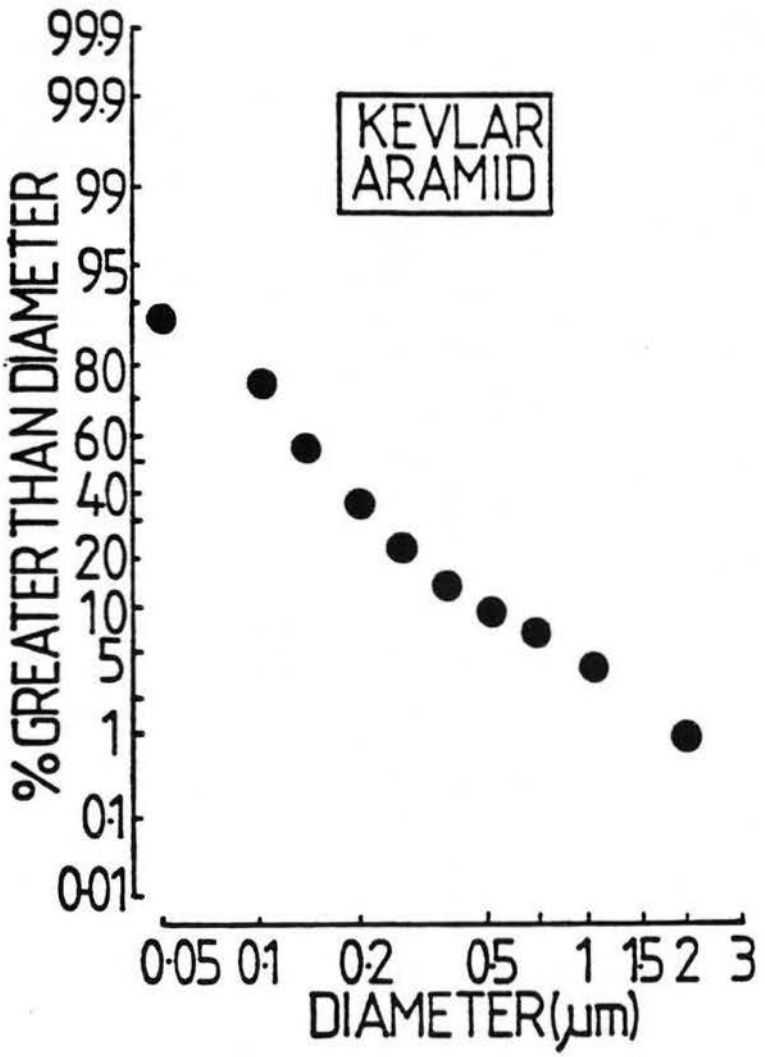
Fibre length distribution of ceramic fibre used the study; 900 fibres counted at a magnification of 10,000x by Scanning Electron Microscopy.

FIGURE 2



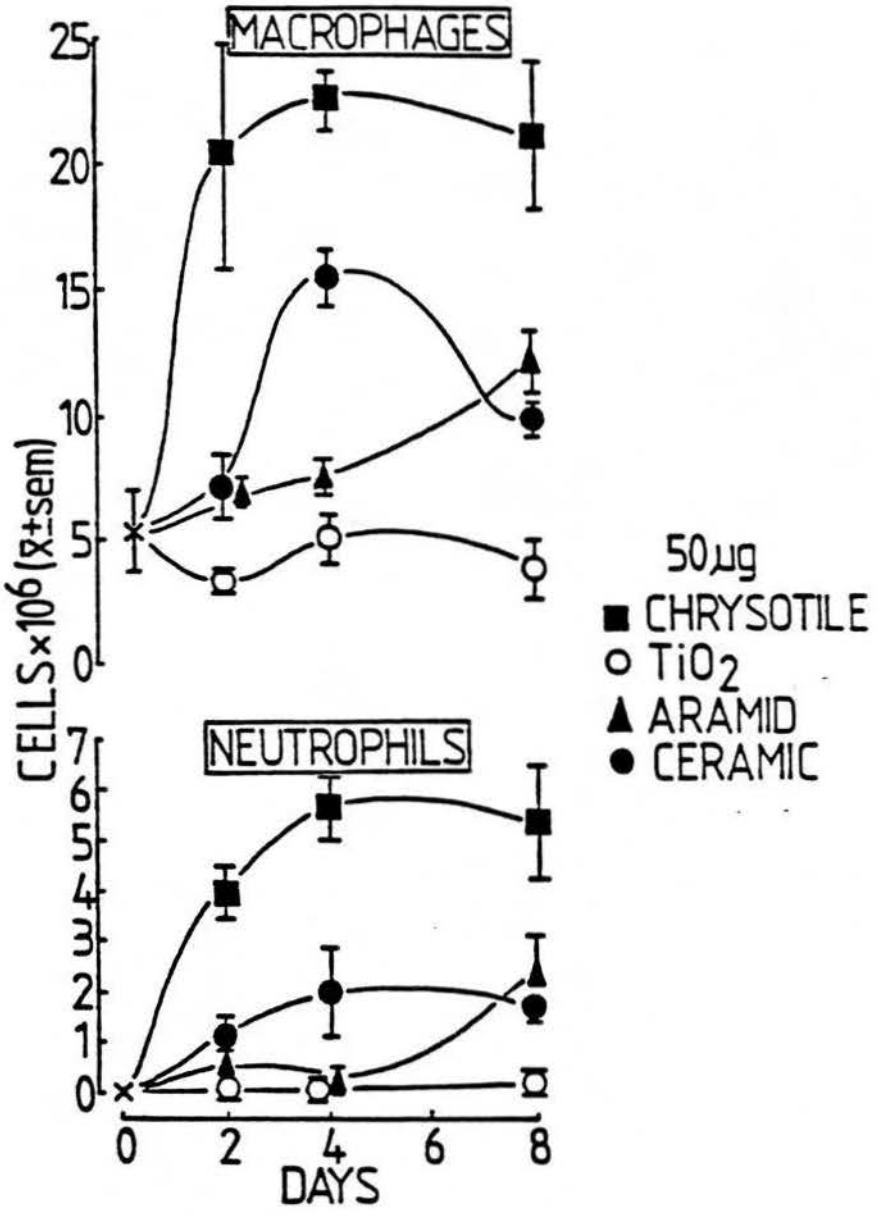
Fibre diameter distribution of ceramic fibre used in the study; 900 fibres counted at a magnification of 10,000x by Scanning Electron Mircroscopy.

FIGURE 3



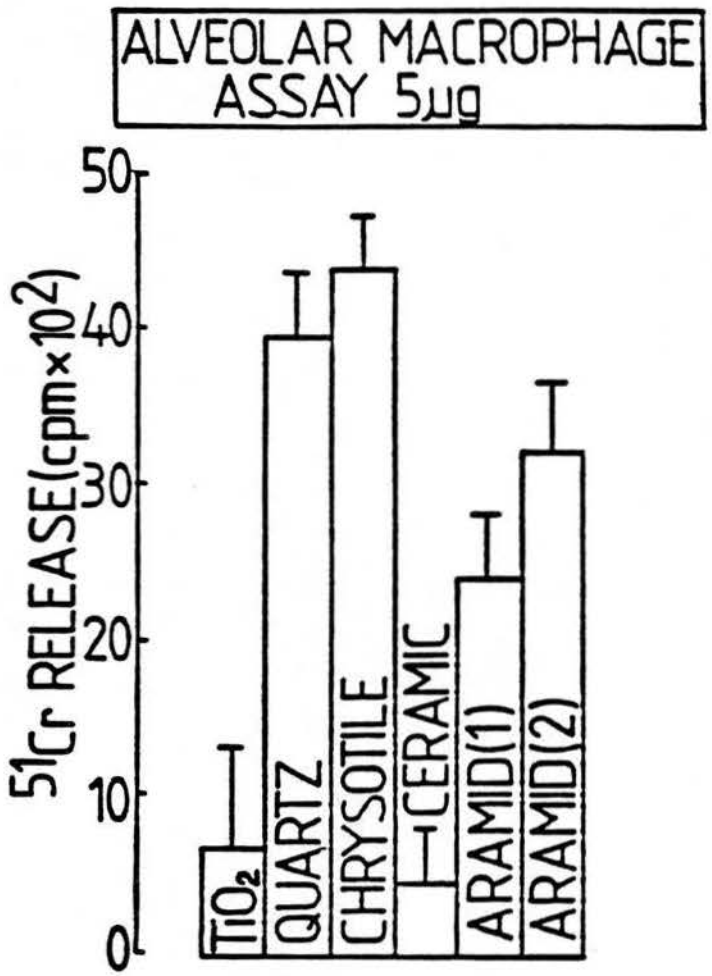
Fibre diameter of the aramid fibre sample used in the study; 900 fibres counted at a magnification of 10,000x by Scanning Electron Microscopy.

FIGURE 4



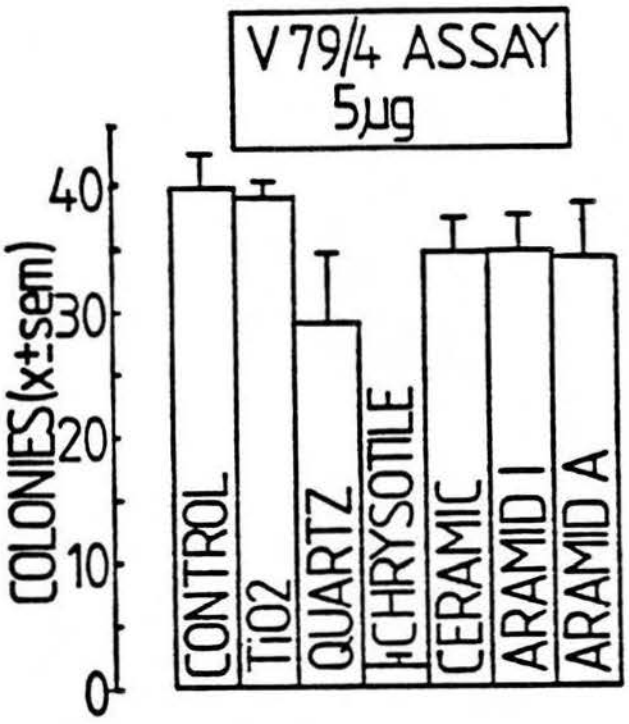
Recruitment of neutrophils and macrophages into the mouse peritoneal cavity following injection of 50 µg of the indicated dusts. Each point represents mean ± SEM of 3 mice. Significant ($P<0.05$ - $P<0.001$) increases with all dusts compared to TiO_2.

FIGURE 5



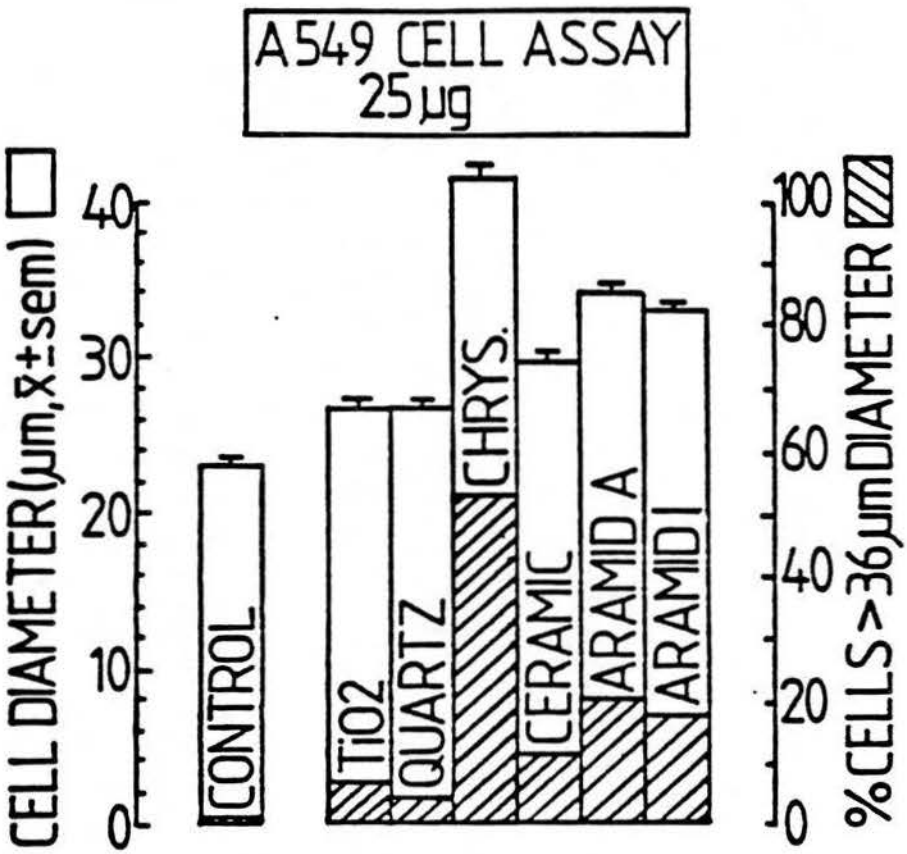
Cytotoxicity of the indicated dusts as assessed by release of ^{51}Cr from pre-labelled rat alveolar macrophages - dose 10µg. TiO_2 = Titanium dioxide; Aramid 1 = non-autoclaved; Aramid 2 = autoclaved Aramid. Mean ± SEM of 3 replicates. Significant ($P<0.01$-0.001) increases over TiO_2 with all dusts except ceramic.

FIGURE 6



Number of V79/4 cell colonies present after 6 days of culture with 5 µg of the indicated dusts. Aramid A = autoclaved; Aramid 1 = irradiated; mean ± SEM of 3 replicates. Significant difference for control with chrysotile, ($P<0.001$).

FIGURE 7



Diameter (open bars) and percentage of cells greater than 36 µm (hatched bars) of A549 cells treated with the indicated dusts. Aramid A = autoclaved; Aramid 1 = irradiated; mean ± SEM of 200 cells.

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INFLAMMATORY CELL RECRUITMENT AS A MEASURE OF MINERAL DUST TOXICITY

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Abstract—The cellular inflammatory response has been assessed following intra-peritoneal injection of mineral dusts in mice. To calibrate the system, dusts of known pathogenicities were injected at 50, 500 or 2500μg/animal, and the inflammatory response assessed at 2, 4 and 8 days; inflammation was measured as total cells recovered by peritoneal lavage, differential cell count and macrophage activation status. The cellular response was found to be highly sensitive, since a transient response could be detected following injection of saline alone. Using standard dust preparations, titanium dioxide was found to be relatively inactive while quartz and chrysotile asbestos induced a marked inflammatory response. There was some evidence that the cell yield was influenced by macrophage adherence and possibly direct cytotoxicity, and the dose response was consequently not linear. Further work is planned to systematically examine this aspect. Two other particulates were examined in the test system — a respirable coal mine dust and an aramid fibre preparation. The response to the coal mine dust was small while aramid fibre induced a considerable sustained inflammatory reaction. We conclude that the mouse peritoneal cavity can provide a rapid, simple and reliable *in vivo* test of the potential pathogenicity of particulate material.

INTRODUCTION

THE INFLAMMATORY response is a concerted reaction of the host to tissue injury and is now well characterised (RYAN and MAJNO, 1977). One of the earliest manifestations of inflammation is the accumulation at the site of injury of leukocytic inflammatory cells; in the case of a non-antigenic injurious agent such as mineral dust these comprise principally activated macrophages and neutrophils.

Recruitment of both neutrophils and macrophages has been demonstrated following experimental pulmonary deposition of several mineral dusts including quartz (SYKES *et al.*, 1983) and asbestos (LEMAIRE, 1985) and different aspects of dust induced inflammatory reactions have been described (HAMILTON, 1980; DONALDSON *et al.*, 1982). The peritoneal cavity of experimental animals is a site which can be quickly and accurately dosed with known amounts of dust and this has led to its use in studies on the response to particulates. Using this approach it has been possible to obtain large numbers of inflammatory cells recruited in response to dust (HAMILTON, 1980; DONALDSON *et al.*, 1982; MILLER, 1978) and to study the tumorigenicity of mineral dusts (BOLTON *et al.*, 1982).

In the course of our studies into the inflammatory response we noted that a highly repeatable cellular reaction occurred in the peritoneal cavity and we set out to systematically examine this reaction in the hope that it might provide a means for accurately assessing the inflammatory potential of dust. We report here the preliminary results of these experiments on the magnitude and duration of the inflammatory response to two dusts of known pathogenic potential, quartz and chrysotile asbestos; and a relatively inactive control dust, titanium dioxide. We also

present the results of experiments undertaken using the same assay system with two test particulates – an aramid fibre sample and a respirable coal mine dust sample.

MATERIALS AND METHODS

Animals

Syngeneic C57B16 mice aged 10–14 weeks were used throughout in groups of 3 animals per test condition.

Dusts

The dusts used were: a rutile titanium dioxide sample (TiO_2), chrysotile asbestos (UICC sample 'A'), DQ_{12} quartz, Min-U-Sil quartz, Sikron-F-600 quartz, respirable coal mine dust (quartz content 5.4%) and an aramid fibre sample. All of the dusts, apart from the aramid fibre preparations, were taken from bulk samples and suspended in Dulbecco's saline (Gibco) to 5, 1 or 0.1mg/ml. In the case of the aramid fibre it proved difficult to obtain an adequately dispersed preparation from the bulk material. Accordingly, a known mass of the fibre was made to 10mg/ml in saline and disaggregated for 5 minutes in a rotating turret homogeniser to obtain a sample for injection. The particulates were administered intra-peritoneally as 0.5ml of dust suspension into conscious, restrained, unanaesthetised mice at total doses of 50, 500 or 2500μg per mouse; the effects of each dose were examined 2, 4 and 8 days after injection.

Cells

Mice were killed by ether overdose and the peritoneal cavity lavaged with 3 × 2ml washes of heparinised (1IU/ml, Leo Laboratories) Dulbecco saline. The cells were stored on ice in plastic tubes at all times. The cells were washed and resuspended in Ham's F10 medium (Gibco) + 10% foetal calf serum (Gibco) and then counted. Differential counts were obtained from cyto-centrifuge preparations stained with Diff Quik. The total number of cells of each type was calculated from the differential and the total counts. It was found that the great majority of cells were macrophages or neutrophils, lymphocytes rarely exceeding 10% and eosinophils only occasionally being seen. The extent of macrophage activation was estimated using the macrophage spreading assay as previously described (DONALDSON *et al.*, 1984).

Statistics

The results were examined initially using analysis of variance and differences in means of treatment groups were tested using a test statistic analagous to the usual '*t*' test but applicable when the populations to be compared are of unequal variance (SNEDECOR and COCHRAN, 1980). Details of statistical significance are presented in the figures; except where stated otherwise the comparisons made are with saline injected mice lavaged at the same time points.

RESULTS

The effect of saline

The injection of saline alone caused a significant reduction in the number of cells lavaged from the mouse peritoneal cavity 2 and 4 days later (Fig. 1). There was no

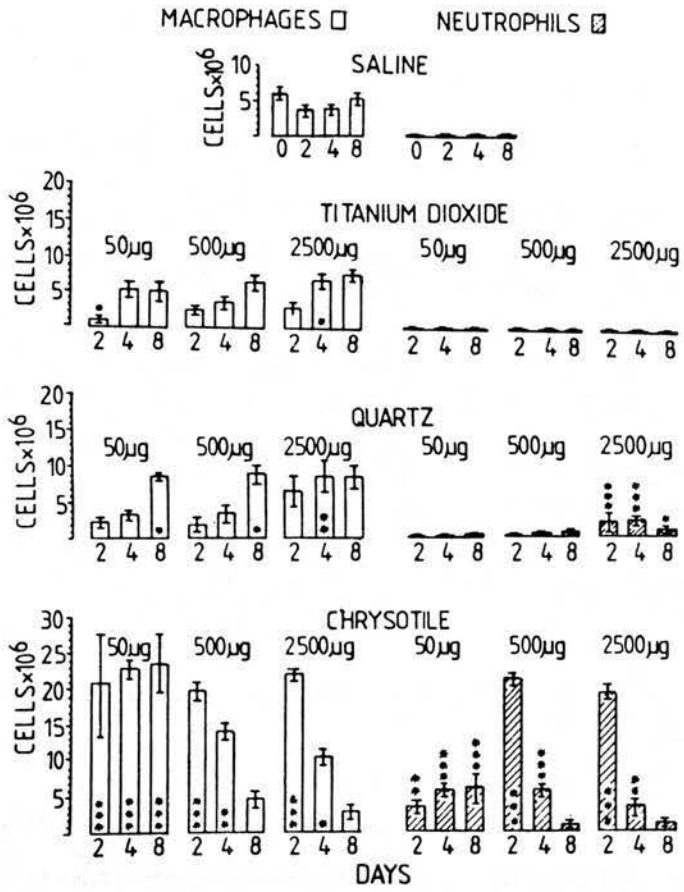


FIG. 1. Number of cells lavaged from the peritoneal cavity of mice injected with saline, titanium dioxide, quartz or chrysotile asbestos at the doses indicated, lavaged on the days shown. Left hand side (open columns) macrophages; right hand side (hatched columns) neutrophils. Mean ± standard error. All statistical comparisons made with saline on the appropriate days. $*P<0.05$, $**P<0.01$, $***P<0.001$.

detectable effect on neutrophil numbers but transient macrophage activation was detected on day 2.

The inflammatory response to titanium dioxide, quartz and chrysotile asbestos

Figure 1 details the inflammatory response to the 3 dusts used in the first part of the study. It is evident that TiO_2 had relatively little effect even over the fifty-fold dose range used. There was no significant neutrophil influx and only at the highest dose was there a modest but significant increase in macrophage numbers. DQ_{12} quartz on the other hand, produced a detectable increase in neutrophil numbers at both the 500 and 2500μg doses at all the time points, although statistical significance was confined to the 2500μg dose. There were no systematic increases in macrophage numbers with dose or time. As can be seen from Fig. 1, chrysotile asbestos caused increases in lavageable neutrophils at all doses; the macrophage response showed the same pattern of increase as was found with neutrophils. Using macrophage spreading as an index of cellular activation, Fig. 2A reveals that the TiO_2 treated animals showed a transient increase in macrophage activational state but only at the 2 day time point with the highest dose. DQ_{12} quartz caused increased spreading with the 50 and 2500μg doses on day 2 and with all doses on day 4. Chrysotile treated mice, however, produced the greatest evidence of macrophage activation with 8 out of the 9 treatment combinations

providing evidence of activation. Figure 3 compares the response of the mouse peritoneal system to 2500μg of three varieties of quartz: DQ_{12}, Sikron-F-600 and Min-U-Sil, at the 4 day time point. It can be seen that the inflammatory response was effectively similar for all 3 dusts, with significant increases in both macrophages and neutrophils.

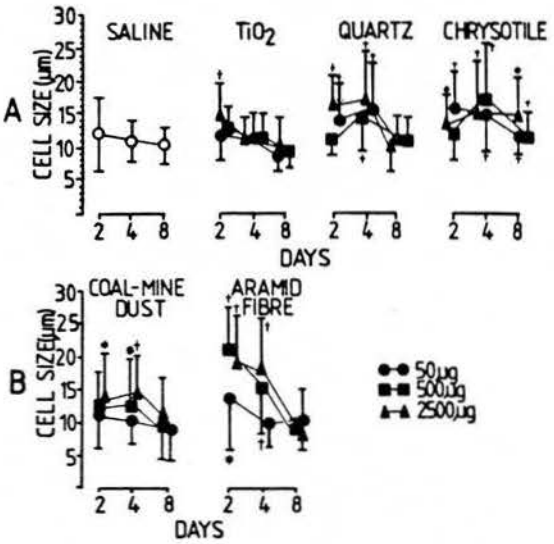


FIG. 2. Macrophage spreading assays results. Peritoneal macrophages collected from mice injected with saline or particulates at the indicated doses and lavaged on the days shown. Each point denotes mean ± 1 standard deviation. All statistical comparisons are made with saline injected on the appropriate day: $^{*}P<0.01$, $^{+}P<0.001$

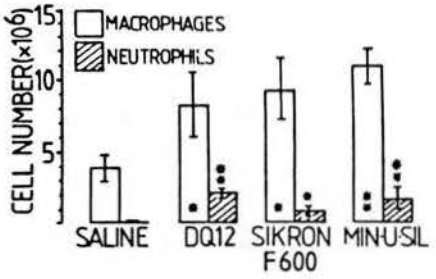


FIG. 3. Numbers of cells lavaged from the peritoneal cavity of mice injected with saline or 2500μg of the quartz samples indicated. Open columns: macrophages. Hatched columns: neutrophils. Mean ± standard error. All statistical comparisons made with saline. $^{*}P<0.05$, $^{**}P<0.01$.

The inflammatory response to coal mine dust and aramid fibre

A trend of increasing macrophage number, with increasing coal-mine dust dose was evident and this relationship was linear when dose was plotted on a log scale. This dose effect was highly significant (Fig. 4). The number of neutrophils lavaged was increased at the higher doses of coal-mine dust. With aramid fibre there were substantial increases in both macrophages and neutrophils with all doses, persisting throughout the 8 day experimental period. The macrophage spreading assay results presented in Fig. 2B show increased macrophage activity consistent with the extent of inflammation assessed by cell recruitment described above.

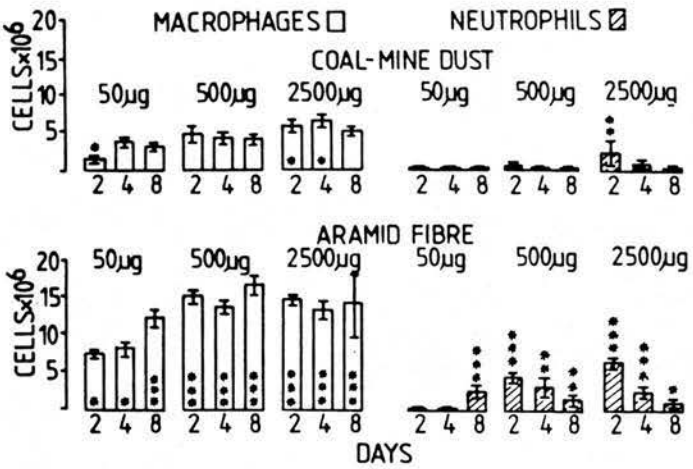


FIG. 4. Numbers of cells lavaged from the peritoneal cavity of mice injected with coal mine dust or aramid fibre. Particulates injected at the doses indicated and lavaged on the days shown. Left hand side (open columns): macrophages. Right hand side (hatched columns) : neutrophils. Mean ± standard error. All statistical comparisons made with saline: $*P<0.05$, $**P<0.01$, $***P<0.001$. Highly significant ($P<0.001$) dose effect of coal-mine dust on macrophage number.

DISCUSSION

In the present study we set out to examine recruitment of inflammatory cells to the mouse peritoneal cavity in response to mineral dusts. The study initially revealed the mouse peritoneal cavity to be very sensitive to insult. This was evident as a marked decrease in the number of lavageable cells up to 4 days after injection of saline alone. This effect can be explained since cells activated during inflammatory responses may be retained at the inflammatory focus by alterations in macrophage secretion. In the peritoneal cavity this response is reflected in decreased number of lavageable cells (HASKILL and BECKER, 1985); this effect is discussed in greater detail below. The results from the injection of saline alone, which caused significant reduction in macrophage number up to 4 days after injection, strongly suggest that increased adherence is a factor in the peritoneal model. This was further confirmed by harvesting cells 2 hours after injection of saline and noting that the number of lavageable cells had dropped to less than one sixth of the resting level found in untreated mice (data not included). Further evidence of the sensitivity of the system was provided by the finding of transient macrophage activation 2 days after injection of saline, which had subsided by day 4.

Of the 3 dusts used in the first part of the study, TiO_2 was found to be virtually inactive. Although transient macrophage activation was present at 2 days with the higher dose, no neutrophils were present in the lavage following any of the TiO_2 treatment combinations. DQ_{12} quartz, in contrast, produced evidence of dose dependent neutrophil influx, although the numbers were not statistically significant with the lower doses. Increases in macrophage number were not clearly dose-dependent. Two other quartz samples, Min-U-Sil and Sikron-F-600 produced similar effects to DQ_{12} when assessed at the 4 day time point using a 2500μg dose. Chrysotile asbestos was markedly more active than quartz in increasing yields of both macrophages and neutrophils. In general, macrophage activation data confirmed the differences in inflammatory activity of the 3 dusts indicated by the cell counts described

above. The results of these tests of the inflammatory capacity of the 3 different dusts are in agreement with their known toxicities *in vivo* and *in vitro* (CHRISTIE *et al.*, 1963; GORMLEY *et al.*, 1979; SYKES *et al.*, 1983; SEEMAYER and MANOJLOVIC, 1980; LEMAIRE, 1985; WRIGHT *et al.*, 1983).

However, while clear differences were found between the 3 different dust types, with the two pathogenic dusts causing neutrophil recruitment and macrophage activation in the peritoneal cavity, the dose responses were not linear. There were also very contrasting responses to quartz and chrysotile although both have been found to be highly cytotoxic *in vitro*. There are at least two major factors which, in the face of leukocyte recruitment, could act to decrease the number of cells lavaged—cytotoxicity and increased adherence. The cytotoxicity of mineral dusts such as quartz and chrysotile is well documented and we have previously shown that activated macrophages from inflamed peritoneal cavity are more susceptible to cytotoxic damage than resting cells (WRIGHT *et al.*, 1983). Thus in the case of chrysotile and quartz there could be an as yet undetermined depletion of cells in the peritoneal cavity due to cytotoxicity with some or all of the doses. An alternative explanation for these dose response characteristics is increased adherence of inflammatory cells. The 'macrophage disappearance reaction' is a well documented phenomenon (NELSON and BOYDEN, 1963) which occurs when macrophages become activated during immune responses in the peritoneal cavity. The sudden reduction in numbers of lavageable macrophages has been shown to be due to increased macrophage adherence to the peritoneal surfaces. This occurs because the macrophages are activated to release increased amounts of coagulation factors and as a result they are eventually trapped and immobilised in a fibrin meshwork (HOPPER *et al.*, 1981). In the present study the decrease in cell numbers found with saline alone strongly suggests that further insult by a toxic dust could cause increased amounts of coagulation factors and as a result they are eventually trapped and immobilised in a fibrin meshwork (HOPPER *et al.*, 1981). In the present study the decrease in cell numbers found with saline alone strongly suggests that further insult by a toxic dust could cause increased adherence and thereby affect the number of lavageable cells. Experiments are in progress to examine the influence of these factors on the inflammatory response to mineral dust in the peritoneal cavity.

The two additional dusts which were tested in the assay system, coal mine dust and aramid fibre, both induced inflammation in the peritoneal cavity. Coal mine dusts are complex and variable mixtures of minerals many of which have been found, using *in vitro* techniques, to be relatively low in toxicity compared to pure quartz (GORMLEY *et al.*, 1979; SEEMAYER and MANOJLOVIC, 1980). This was confirmed in the mouse peritoneal assay system, where the coal mine dust sample produced a greater inflammatory response than TiO_2 but less than quartz. Aramid fibre is a relatively new, man-made material which is currently being tested for its potential toxicity. We found the aramid fibre to be highly active in producing inflammation; its activity was greater at equal masses than that of quartz, and approached that of chrysotile asbestos. These results are in agreement with those of DUNNIGAN *et al.* (1984) who reported its ability to cause alveolar macrophage cytotoxicity. The present study has shown that the mouse peritoneal cavity is a very sensitive site for studying the effects of mineral dusts. Measurement of the inflammatory response has provided a means of assessing the relative in vivo toxicities of several particulates on the basis of their ability to recruit neutrophils and macrophages. We are currently examining several aspects of the inflammatory cell recruitment process in more detail.

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Degradation of connective tissue components by lung derived leucocytes in vitro: role of proteases and oxidants

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ABSTRACT Inflammatory leucocytes are implicated in connective tissue damage during chron inflammatory lung disease. In an investigation of the role of leucocytes in connective tiss derangements in the lung, inflammatory leucocytes were generated in rat lungs by intratrach instillation of inflammatory agents and retrieved by bronchoalveolar lavage. The proteoly activities of control macrophages and of two inflammatory cell populations were compar iodinated collagen, laminin, and fibronectin matrices were used. The inflammatory cells caus consistently and substantially more degradation of the matrices than the controls on a per cell ba The oxidant scavengers superoxide dismutase and catalase did not inhibit matrix degradation, bu protease inhibitor and α_2 macroglobulin were inhibitory. It is concluded that matrix damage in t assay is enhanced by inflammatory cells and is mediated principally by serine protease activity.

Accumulation of inflammatory leucocytes in the alveolar region of the lung is characteristic of disorders leading to fibrosis or destruction of lung parenchyma; inflammatory macrophages and polymorphonuclear leucocytes have been implicated in the pathogenesis of both types of disease.[1] In the adult respiratory distress syndrome large numbers of polymorphonuclear leucocytes (neutrophils) accumulate in the alveoli and are believed to have a major role in the disease process.[2] In interstitial lung fibrosis alveolitis is the initiating event in a process that leads, ultimately, to remodelling of the lung parenchyma.[3] Inflammatory cells are also implicated in the degradation of lung tissue and subsequent loss of alveoli in emphysema.[4]

Polymorphonuclear leucocytes and activated macrophages secrete proteases and reactive oxygen intermediates at sites of inflammation[5,6] and, although beneficial during short term inflammation, the persistence of increased numbers of inflammatory cells in the alveolar region may lead to an excessive burden of these products. Indeed, concentrations of proteases are raised in the bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome,[7] idiopathic pulmonary fibrosis,[8] and sarcoidosis.[9]

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Proteolytic enzymes and oxidants can damage conn tive tissue components in vitro[10–13] and in vivo.[14,15] T structural derangements of the lung parenchyma t occur during chronic inflammatory lung disease n be mediated by inflammatory cell derived prote and reactive oxygen intermediates.

In the present study we have measured the abilit lung derived macrophages and polymorphonuc leucocytes to degrade connective tissue component vitro. We prepared iodinated matrices, using con tive tissue components that occur in lung basem membrane and extracellular matrix. Proteol activity was assessed by measuring degradation of matrices by three populations of lung deri leucocytes—control and two populations of infl matory cells. To assess the role of proteases oxidants in matrix degradation, we examined inhibitor profile of matrix destruction caused by three cell populations, using protease inhibitors oxidant scavengers.

Methods

REAGENT

Rat tail (type 1) collagen, human plasma fibronec α_1 protease inhibitor, α_2 macroglobulin, catal superoxide dismutase, and bovine serum albu were obtained from the Sigma Chemical Comp (Poole, Dorset). Mouse laminin, phosphate buff

saline (PBS) and serumless medium (N and T) were purchased from Gibco BRL (Paisley, Renfrewshire). Ethylene diamine tetra-acetic acid (EDTA) was obtained from BDH, Poole, Dorset, and *Corynebacterium parvum* from Wellcome Reagents Ltd (Hither Green, London). Iodine-125 (^{125}I) was purchased from Amersham International (Aylesbury, Buckinghamshire). The quartz used was the DQ_{12} standard sample.

ANIMALS

Specific pathogen free, syngeneic, female *PVG* rats were obtained from the breeding unit of the Institute of Occupational Medicine, Edinburgh.

CELL PREPARATIONS

Production of inflammatory cells was induced in rat lungs by intratracheal instillation of 1 mg *C parvum* or quartz cells were retrieved by bronchoalveolar lavage of the resected lungs 16 hours after *C parvum* or five days after quartz instillation; control cells were obtained similarly from untreated rats. The resected lungs were cannulated with a blunt 16G needle and lavaged with four sequential 8 ml volumes of saline at 37°C. The lungs were gently massaged during each wash and the recovered lavage fluid (5 ml from the first lavage, 6–7 ml thereafter) was pooled in plastic universal containers and placed immediately on ice. The bronchoalveolar lavage cells were pelleted by centrifugation at 800 g, washed once in 30 ml of ice cold PBS and resuspended in the appropriate medium for the assay. To ensure that non-specific activation did not occur, the cells were kept ice cold throughout the preparation procedure and all manipulations were carried out with plastic pipettes and containers.

SUBSTRATE IODINATION AND MATRIX PREPARATION

Collagen, laminin, and fibronectin were iodinated by the method of McConahey and Dixon,[16] except that L-cysteine was substituted for sodium meta-bisulphite. Unbound iodine was removed by chromatography on a Sephadex G25 column (PD10, Pharmacia, Milton Keynes, Bucks). The labelled proteins showed activities of 5–25 × 10^6 cpm/μg protein. Collagen labelled with iodine-125 (^{125}I) was diluted in 0·1 M acetic acid and 100 μl aliquots (10000 cpm) were placed in microtitre removawells and dried on at 45° C, a temperature at which collagen is denatured to gelatin. Laminin labelled with ^{125}I was diluted in PBS and 10000 cpm aliquots were dried on to removawells as above; ^{125}I fibronectin was also diluted in PBS and 40000 cpm aliquots similarly dried onto removawells.

PROTEOLYSIS ASSAY

All assays of fibronectin proteolysis were carried out in N and T medium containing 2% bovine serum albumin; N and T medium without bovine serum albumin was used in the collagen and laminin degradation experiments. To reduce background counts, the removawells coated with ^{125}I substrate were presoaked for two hours immediately before use with 200 μl of assay medium alone and were then washed once with 300 μl of PBS. In all experiments except the dose-response study of fibronectin proteolysis, 1 × 10^5 cells were added to each removawell in a final volume of 200 μl medium. In all but the dose (6 h) and time response of fibronectin proteolysis, matrix degradation was assessed following four hours' incubation at 37° C. To assess release of ^{125}I labelled degradation products, 150 μl of supernatant medium was harvested from each well and counted by gamma counter.

INHIBITORS

The role of oxidants in matrix degradation was assessed in inhibition studies, the hydrogen peroxide scavenger catalase and superoxide dismutase, a scavenger of superoxide anion, being used. Similarly, the role of proteases was assessed by means of the protease inhibitors α_1 protease inhibitor and α_2 macroglobulin. Catalase, superoxide dismutase, and α_1 protease inhibitor were tested at final concentrations of 0·01, 0·1 and 1·0 mg/ml; α_2 macroglobulin was used at 0·005, 0·05, and 0·5 mg/ml.

STATISTICAL ANALYSIS

The effect of inhibitors on fibronectin matrix degradation was analysed by means of the Genstat linear interpolation program.[17] All other results were assessed by analysis of variance; the Minitab statistical package was used. The significance of differences between mean values at specific times and concentrations were tested by Student's *t* test.

Results

BRONCHOALVEOLAR LAVAGE CELL POPULATIONS

Both groups of treated rats showed alveolar inflammation, evidenced by greater numbers of cells in the bronchoalveolar lavage fluid compared with controls and by changes in the proportion of cells present (fig 1). In the control population the average yield of cells per rat was 7·5 (SEM 2·0) × 10^6, of which over 95% were macrophages and the remainder lymphocytes. The population of cells elicited by *C parvum* showed the greatest increase in cell numbers—to 62·6 (22·1) × 10^6, of which over 75% were neutrophils, about 20% macrophages, and about 2% lymphocytes. Cell numbers in the quartz lavage fluid were also greater than in the control lavage fluid—23·78 (7·26) × 10^6, of which

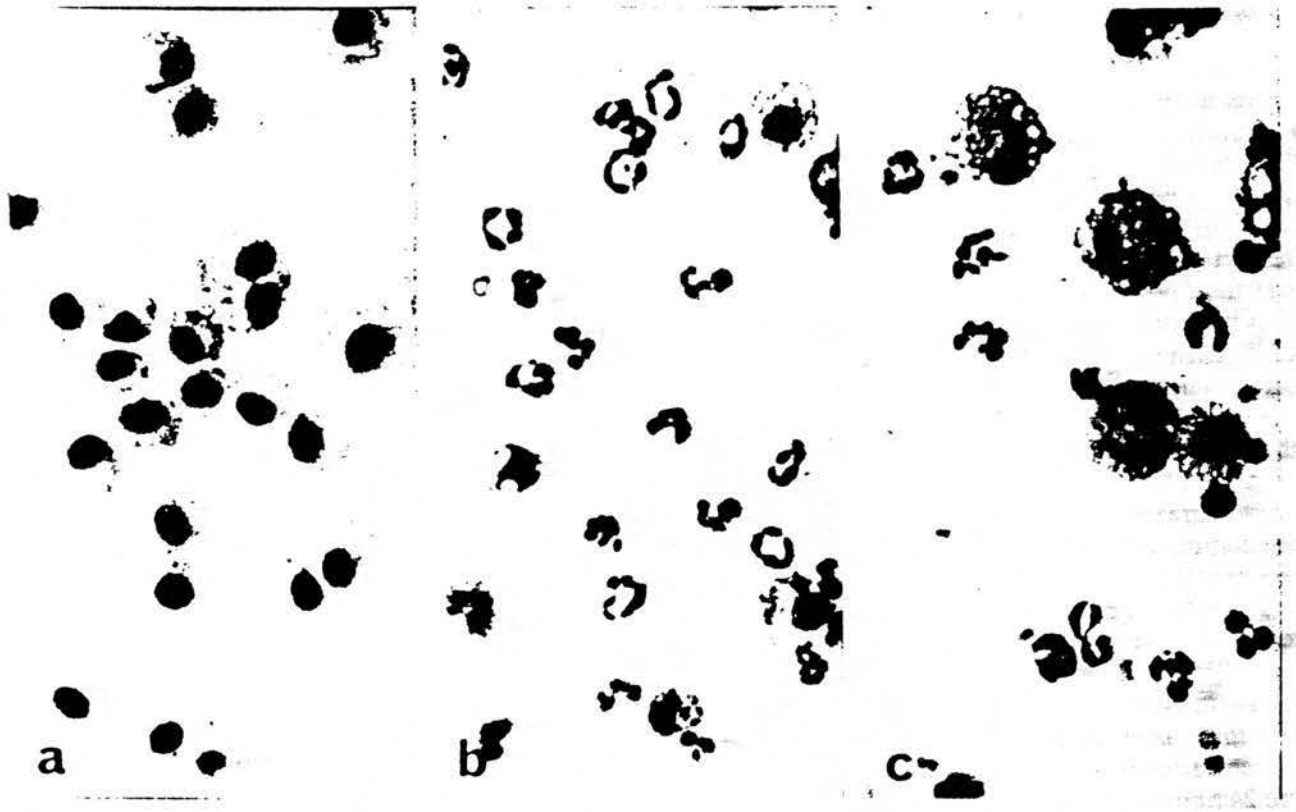


Fig 1 *Bronchoalveolar lavage populations from rat lungs. (a) Control cells (>95% macrophages); (b) Corynebacterium parvum elicited cells (>75% leucocytes, ≃20% macrophages, ≃2% lymphocytes); (c) quartz elicited cells (≃50% macrophage, ≃50% polymorphs).*

about half were neutrophils and about half macrophages.

VALIDATION OF MATRIX DEGRADATION ASSAY

Proteolysis of fibronectin, denatured collagen, and laminin matrices were assessed in preliminary validation experiments with the enzymes trypsin, elastase, and collagenase. The matrices were equally susceptible to dose dependent proteolysis by the three enzymes (results not published).

DOSE-RESPONSE STUDY

The proteolytic activity of each lavage population was assessed at four concentrations (0·001, 0·01, 0·1, and 1·0 × 10^5 cells/well) with an ^{125}I fibronectin matrix, and all showed some ability to degrade the matrix (fig 2).

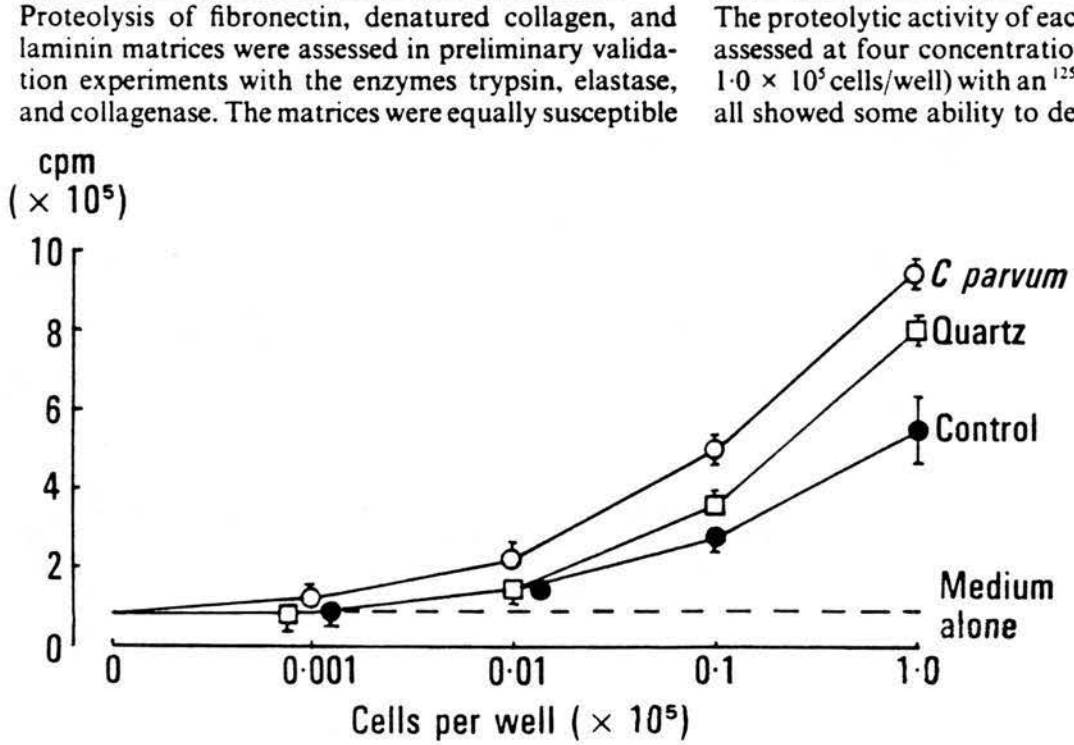


Fig 2 *Dose-response relationship of fibronectin matrix degradation by control cells and cells elicited by Corynebacterium parvum and quartz: counts released into the medium after six hours' incubation. Results are the means and standard errors of three experiments, with triplicate samples in each.*

The degradative capacity of each cell population increased in a dose dependent manner. The release of ^{125}I degradation products by the control and quartz populations was significantly greater than the background level only at concentrations of 0·1 and 1·0 × 10^5 cells/well (controls $p < 0·0025$ and $p < 0·005$ respectively, quartz $p < 0·005$); but at 1 × 10^5 cells/well the amount of radioactivity released by the quartz cells was almost double that of the control cells. The cells elicited by *C parvum* were more actively proteolytic than either the control or the quartz elicited cells, releasing significantly more radioactivity than the background level at 0·01 × 10^5 cells/well ($p < 0·025$). Although the cells elicited by *C parvum* caused greater matrix proteolysis than the quartz population at each cell concentration, the difference between the two populations of inflammatory cells was significant only at a concentration of 0·1 × 10^5 cells/well ($p < 0·005$).

TIME-RESPONSE STUDY

The three lavage cell populations were tested at a concentration of 1 × 10^5 cells/well for periods of 2, 4, 6 and 24 hours with an ^{125}I fibronectin matrix. Each cell population produced significantly more release of radioactivity than medium alone at every time point ($p < 0·025$–$< 0·005$) (fig 3). The proteolytic activity of all three lavage cell populations showed a time dependent increase, which continued up to 24 hours for both inflammatory populations, but plateaued at 6 hours with the controls. Both inflammatory populations were significantly more active than the controls at all time points ($p < 0·01$–$p < 0·005$) and similarly the cells elicited by *C parvum* were significantly more active than the quartz elicited cells at each time point ($p < 0·025$–$< 0·005$).

DENATURED COLLAGEN AND LAMININ

In addition to fibronectin, we tested the proteolytic activity of the lavage cells with two alternative connective tissue components that occur in lung extracellular matrix—laminin and denatured collagen. As with fibronectin degradation, the two inflammatory cell populations caused greater proteolysis of collagen and laminin than control cells, and collagen degradation produced by *C parvum* elicited cells was greater than that produced by quartz elicited cells (fig 4); but there was no difference in laminin degradation between *C parvum* and quartz elicited cells.

ROLE OF PROTEASES AND OXIDANTS

To examine the role of proteases and oxidants in matrix degradation, we studied the effect of protease inhibitors and oxidant scavengers on the proteolysis of a fibronectin matrix produced by control cells and by cells elicited by *C parvum* and quartz. The oxidant scavengers superoxide dismutase and catalase had no effect on the matrix proteolysis caused by any of the lavage cell populations (fig 5). Similarly, the protease inhibitors α_1 protease inhibitor and α_2 macroglobulin had no effect on control cell proteolysis but caused a dose dependent reduction in matrix proteolysis by both inflammatory cell populations (table). α_1 protease inhibitor was equally effective in inhibiting matrix proteolysis by *C parvum* and quartz elicited cells, causing a significant reduction of both cell populations at 0·1 and 1 mg/ml ($p < 0·001$) and at 0·01 mg/ml ($p < 0·01$). α_2 macroglobulin significantly reduced

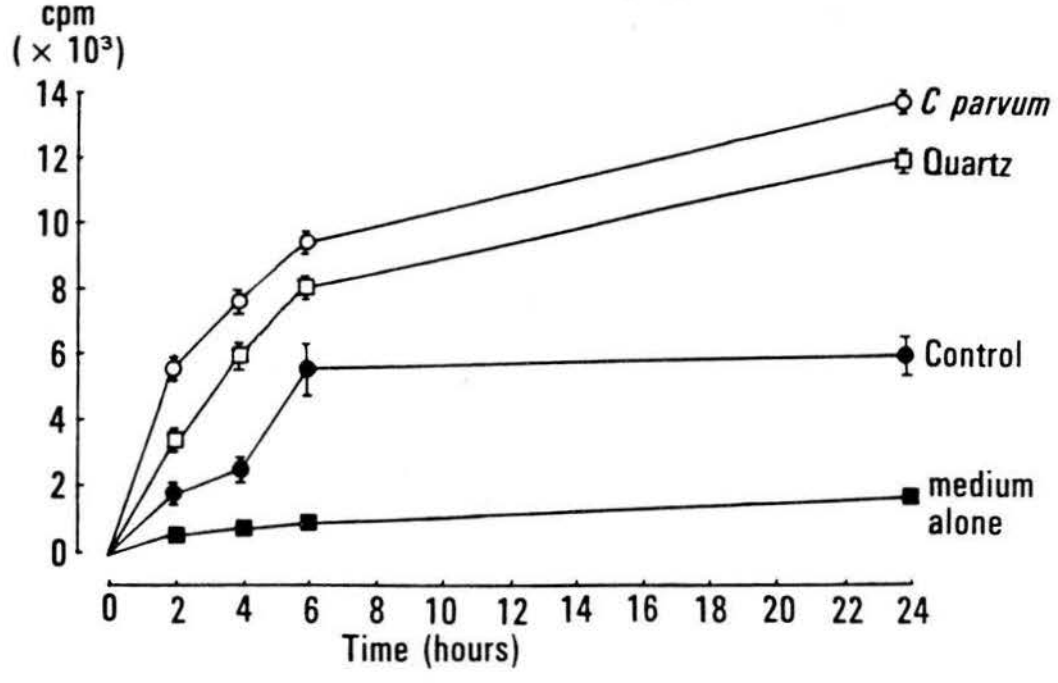


Fig 3 *Time response of fibronectin matrix degradation by control cells and cells elicited by Corynebacterium parvum and quartz: counts released into the medium by 1 × 10^5 cells per well. Results are the means and standard errors of three experiments, with triplicate samples in each.*

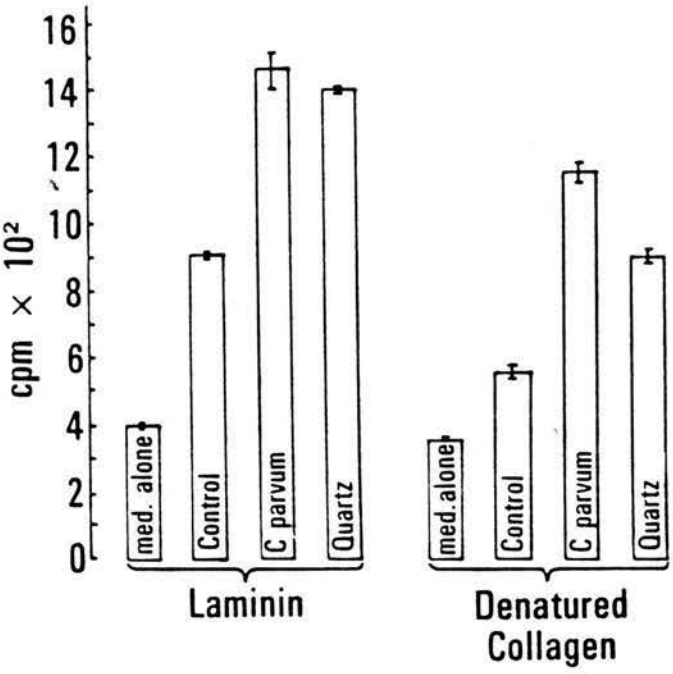


Fig 4 *Degradation of laminin and denatured collagen matrices: counts released into the medium by control cells and cells elicited by Corynebacterium parvum and quartz after four hours' incubation with 1 × 10⁵ cells per well. Results are the means and standard errors of three experiments, with triplicate samples in each.*

fibronectin matrix proteolysis by the quartz elicited cells at 0·05 mg/ml ($p < 0·05$) and 0·5 mg/ml ($p < 0·001$) but was less effective in reducing proteolysis produced by *C parvum* elicited cells, achieving a statistically significant effect only at a concentration of 0·5 mg/ml ($p < 0·001$).

Discussion

The aim of the present study was to assess the role c inflammatory leucocytes in connective tissue damag in the lung and to examine the relative contributions c reactive oxygen intermediates and proteases to th damage.

The connective tissue components collage laminin, and fibronectin occur extensively in the lun parenchyma[18] and have diverse properties, relevant t normal lung functioning, which may be altered durin chronic lung disease.[19 20] Alteration of the conform tion of connective tissue structure leads to the loss c basement membrane[21] and breakdown of cell to ce and cell to matrix interactions,[22-27] which are impo tant in growth control and the maintenance of norm tissue structure. In addition, protease generated co nective tissue fragments are chemotactic f leucocytes.[28 29] Thus proteolytic damage to connecti tissue may enhance and prolong the inflammato process and lead ultimately to fibrosis emphysema.[20]

In this study, we assessed the ability of lung deriv inflammatory cells to damage connective tiss components normally present in lung extracellul matrix and basement membrane. All matrices we susceptible to damage by control and inflammato bronchoalveolar leucocytes, the latter being co sistently and substantially more active. Fibronect degradation by the control cell population plateau by 6 hours, which may indicate a transient activati of the cells during preparation. On a per cell basis, t quartz population produced twice as much neut protease as the controls and the *C parvum* populati three times more than the controls. The increas

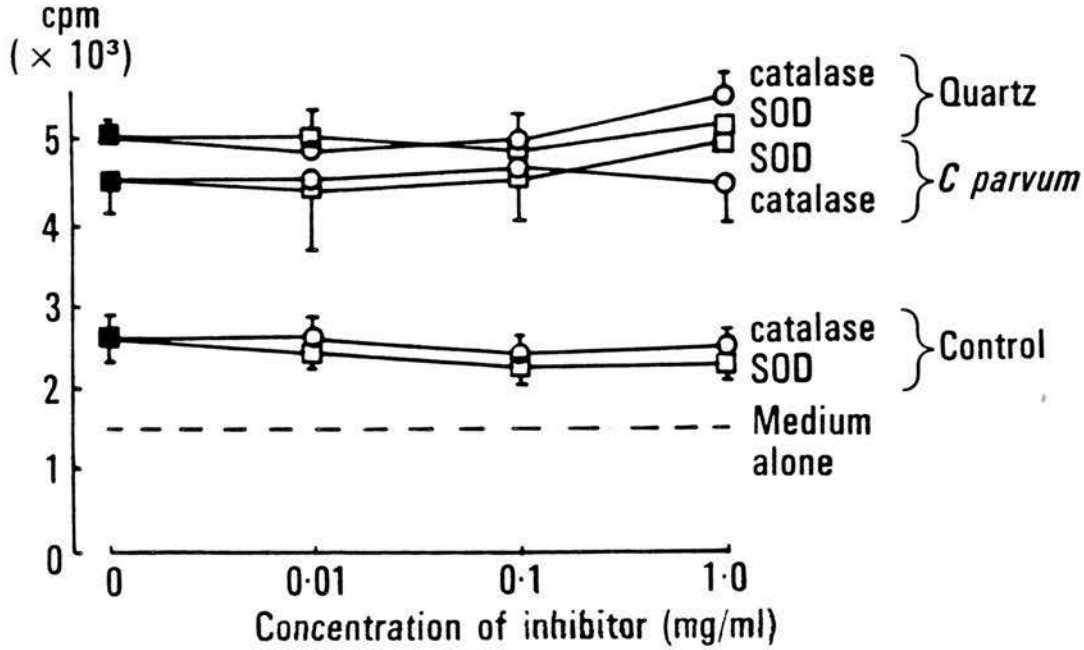


Fig 5 *Dose-response relationship of the effec the oxidant scavengers superoxide dismutase (SOD) and catalase on degradation of a fibron matrix by control cells cells elicited by Corynebacterium parvu and quartz. Results are means and standard err three experiments, with triplicate samples in ea no inhibitor.*

Degradation of a fibronectin matrix by control bronchoalveolar cells and those elicited by Corynebacterium parvum and quartz in the presence of the protease inhibitors α_1 protease inhibitor and α_2 macroglobulin (results (means (SEM)) derived from triplicate samples in three separate experiments)

Inhibitor	Final conc (μg/ml)	Control cells	C parvum elicited cells	Quartz elicited cells
None		2603 (299)	4587 (456)	5108 (304)
α_1 protease inhibitor	0·01	2380 (251)	2885 (569)**	3963 (374)**
	0·1	2428 (261)	2795 (379)**	3260 (335)***
	1·0	2333 (235)	2097 (230)***	2531 (274)***
α_2 macroglobulin	0·005	2787 (321)	4298 (418)	4648 (335)
	0·05	2456 (281)	4157 (571)	4198 (348)**
	0·5	2286 (288)	3528 (418)***	3534 (275)***

**Significant reduction compared with no inhibitor: $p < 0·01$.
***Significant reduction compared with no inhibitor: $p < 0·001$.

protease appeared to be related to an increased proportion of neutrophils in the bronchoalveolar lavage fluid. Recalculation of the data on the basis of the differential cell count suggested that the neutrophils in the two inflammatory populations were secreting similar amounts of neutral protease; this was estimated to be five times more than the amount secreted by the bronchoalveolar macrophages.

To determine the nature of the process of matrix degradation, we tested the effects of inhibitors of protease and of reactive oxygen intermediates in the assay. In vitro studies have suggested that reactive oxygen intermediates may be important in connective tissue damage,[30 31] but in our assay neither catalase nor superoxide dismutase reduced matrix proteolysis, thus indicating that neither hydrogen peroxide nor superoxide anion are involved in damage to the extracellular matrix. These results are in general agreement with the findings of several in vitro studies discounting reactive oxygen intermediates as a source of extracellular matrix degrading activity,[10 32 33] although in one study H_2O_2 was implicated.[32] To confirm that oxidant injury did not contribute to matrix proteolysis in the present study we tested exogenous hydrogen peroxide and superoxide anion and no proteolysis occurred (results not published). These results are in agreement with the findings of a previously published study in this laboratory, which indicated that reactive oxygen intermediates are not a major arbiter of tissue injury in mineral dust inflammation.[33]

To elucidate the mechanisms of proteolysis further we examined the inhibitor profile of the three cell populations, using protease inhibitors. The low level of proteolysis shown by control cells could not be inhibited, possibly owing to the presence of cysteine proteases[34] or to exclusion of soluble inhibitors from sites of close contact between leucocytes and the extracellular matrix.[10 35 36]

The *C parvum* population (>95% neutrophils) and the quartz population (50% neutrophils, 50% macrophages) showed similar inhibition profiles in response to α_2 macroglobulin and to α_1 protease inhibitor. Macrophage neutral proteases have been reported to be largely metalloproteases[37 38] and thus not capable of inhibition by α_1 protease inhibitor.[39] The inhibition profiles of the two inflammatory populations should therefore have been different in terms of their response to α_1 protease inhibitor if the macrophages in the quartz population were secreting metalloproteases. Since α_1 protease inhibitor inhibited the two inflammatory populations to the same extent, we tested inhibition of exogenous serine protease (porcine elastase) and metalloprotease (bacterial collagenase) in the fibronectin assay and confirmed that in our assay system serine proteases but not metalloproteases were inhibited by α_1 protease inhibitor (results not published). These results suggest that the proteolytic activity of the quartz population may be due to a serine protease, or to cysteine proteases as suggested above.

In summary, we have elicited two distinct populations of inflammatory cells in rat lungs, one containing largely neutrophils and the other composed equally of macrophages and neutrophils. The proteolytic activity of the two populations of inflammatory cells in vitro was substantially greater on a per cell basis than that of control bronchoalveolar cells. By carrying out inhibitor studies we have discounted the role of reactive oxygen intermediates in matrix degradation and have indicated that the proteolysis is mediated by serine protease activity.

Our results show that inflammatory leucocytes from the bronchoalveolar region of the lung have substantial connective tissue proteolytic activity; we have also shown that this activity can be inhibited by α_2 macroglobulin and α_1 protease inhibitor. These two inhibitors are found in the alveolar region of the lung, but the presence of active proteases in the bronchoalveolar lavage fluid of patients with chronic lung disease[7-9] suggests that the presence of large numbers of inflammatory cells in the lung parenchyma overloads this antiprotease screen. Protease activity

derived from inflammatory leucocytes may thus be a major arbiter of connective tissue damage during chronic inflammatory lung disease.

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Degradation of an Extracellular Matrix Component by Bronchoalveolar Leukocytes *In Vitro*: Modulation by Mineral Dust

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Introduction

Occupational exposure to harmful mineral dusts is frequently associated with development of chronic fibrotic lung disease e.g. silicosis, asbestosis or coalworkers' pneumoconiosis (Morgan and Seaton, 1984). Alveolitis is a characteristic feature of such diseases (Begin *et al.* 1986; Voisin *et al.* 1985) and although the aetiology of the fibrosis is not yet fully established, such an accumulation of inflammatory leukocytes is firmly implicated in the pathogenesis of the occupational lung diseases.

Proteolytic enzymes are released from inflammatory leukocytes at sites of inflammation and some "bystander injury" to the surrounding tissue is an inevitable consequence (Ryan and Majno, 1977). Where the inflammation is short-lived, acute injury is quickly resolved and normal tissue architecture is restored. Persistent lung inflammation, however, leads to severe tissue derangement resulting in fibrosis; the mechanisms involved in the pathogenesis of fibrotic lung disease have recently been reviewed by Hunninghake *et al.* (1984). In occupational lung disease there are two likely causes of persistent inflammation, not mutually exclusive, and these are:- (a) the inherent harmfulness of the dust and (b) persistent exposure. This study has evaluated the role of inherent harmfulness in causing persistent inflammation by assessing the inflammation developing in response to a single intratracheal dose of the fibrogenic mineral dust quartz, compared with TiO_2, a mineral which is virtually inert in experimental studies *in vivo* (Donaldson *et al.* 1987) and *in vitro* (Donaldson and Brown, 1988) and is not associated with pathology in occupationally-exposed groups. We compared these responses with those elicited by two other model inflammogens which reflect the normal scope of the lung to mount an inflammatory response without causing irreversible tissue damage. *C. parvum* was chosen to represent a normal bacterial challenge in the lung and since mineral dusts can activate complement, zymosan was also used as it is a very efficient activator of complement. We have investigated one of the aspects of persistent exposure by assessing the effect of a second dust challenge (*in vitro*) on the functional status of inflammatory bronchoalveolar leukocytes obtained following *in vivo* dosing with dust. We measured two key functional

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parameters of the bronchoalveolar leukocytes relevant to proteolytic injury and its consequences by (a) assessing the ability of bronchoalveolar leukocytes to degrade fibronectin, a connective tissue component of major importance in maintaining tissue integrity and (b) measuring plasminogen activator production. Plasminogen activator is a leukocyte protease that is frequently cited as being central to the inflammatory response (Robinson, 1988).

Materials and Methods

Animal injection and lavage

Rats used were syngeneic, SPF, males of the PVG strain.

Zymosan and both mineral dusts (DQ_{12} standard quartz; titanium dioxide (rutile) Tioxide Ltd., Stockton were injected directly into the trachea as 1mg in 0.5ml saline, *C. parvum* as 1.4mg in 0.2ml saline; animals were killed at selected time points thereafter. The lavage procedure was performed as previously described (Donaldson *et al.* 1988).

Fibronectin proteolysis

The ability of leukocytes or leukocyte supernatants to degrade fibronectin was assessed by measuring breakdown of a solid phase [^{125}I] fibronectin matrix as previously described (Brown and Donaldson, 1988). Proteolytic activity was measured as counts per minute of degraded [^{125}I] fibronectin released into the supernatant medium over a 4 hour period.

Plasminogen activator assay

Plasminogen activator activity was measured as previously described (Donaldson *et al.* 1987) by measuring plasminogen-dependent breakdown of [^{125}I] fibrin. Results are shown as counts per minute of [^{125}I] fibrin released into the supernatant medium in a 4 hour period.

Statistical analyses

Differences between means of treatment groups were tested using Student's 't' test.

Results and Discussion

Alveolitis is a characteristic feature of chronic lung disease associated with exposure to harmful mineral dusts but there is little information regarding the role of the inflammatory leukocytes in the pathogenesis of the disease process. We have addressed this question by investigating inflammation developing in the lung in response to a single dose of the fibrogenic dust, quartz, compared with other non-fibrogenic stimuli. We have shown here that the acute inflammatory response to quartz was less than that caused by a bacterial preparation (*C. parvum*); it was, however, greater than that produced by TiO_2 or the particulate activator of complement, zymosan, in terms of total numbers of leukocytes recruited (Figure 1) and the percentage of those cells that were neutrophils (Figure 2).

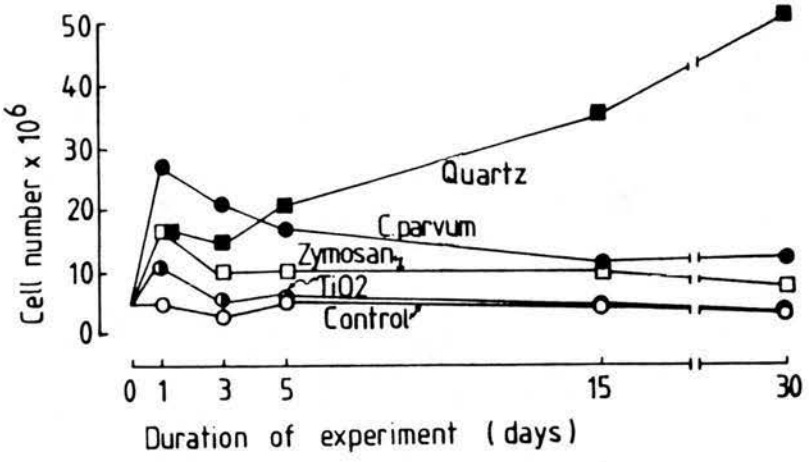


Fig. 1. Total number of leukocytes retrieved in bronchoalveolar lavage. Results represent the mean of 3 rats per treatment group.

The neutrophil is the classical leukocyte of acute inflammation and the neutrophil response to *C. parvum*, TiO_2 and zymosan was a typical acute one with the percentage of neutrophils falling off rapidly after a peak at 1 day and returning to around control levels by 5 days.

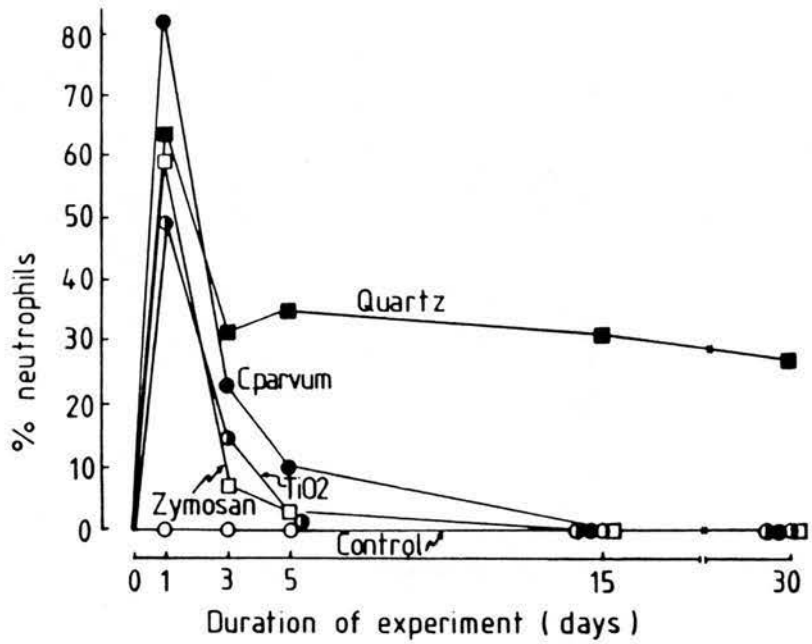


Fig. 2. Percentage of neutrophils in the bronchoalveolar lavage cell population. Results represent the mean of 3 rats per treatment group.

By 3 days, the difference between quartz and the other inflammogens was apparent – the percentage of neutrophils in the quartz lavage declined less than in the others and thereafter persisted at around 30%. This result is in agreement with the finding of approximately 6–8 fold more neutrophils in bronchoalveolar lavage in silicosis, asbestosis and coalworkers' pneumoconiosis compared with the normal population (Begin *et al.* 1986; Voisin *et al.* 1985 and of the neutrophil alveolitis found in rats inhaling quartz (Donaldson *et al.* 1988). The macrophage alveolitis which persisted up to 30 days in the *C. parvum* and zymosan animals was not present in the TiO_2 rats. This suggests that the leukocyte influx in TiO_2–exposed lungs (which was less than any other group) may occur largely in response to chemotactic stimuli released during phagocytosis of the TiO_2 particles by macrophages. Subsequent to phagocytosis, there is no further reaction between the macrophage and the TiO_2 particle and thus no means of prolonging the inflammation, so no pathological change results.

The proteolytic activity of the inflammatory leukocytes reflected the severity of the inflammation, peaking at 1 day with all of the inflammogens (except quartz which was maximal at 3 days) and declining rapidly thereafter in all but the quartz-elicited group (Figure 3).

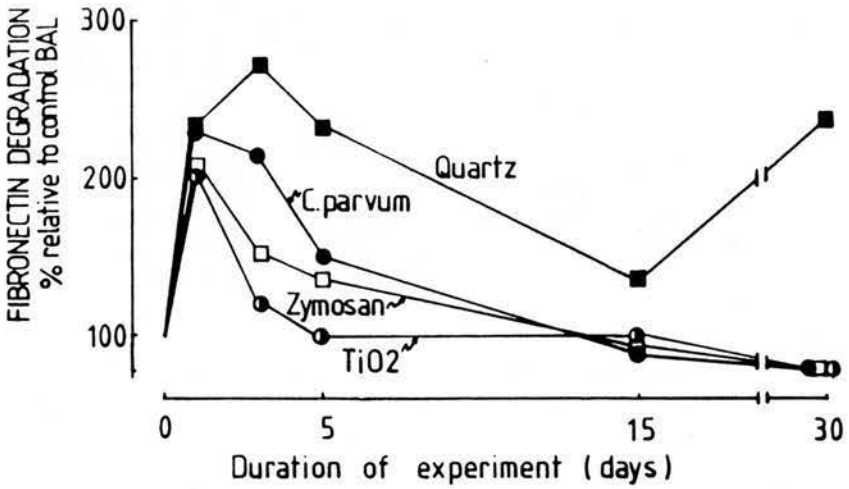


Fig. 3. Protease activity as the percentage of fibronectin degraded in relation to control leukocyte proteolytic activity.

The proteolytic activity of the quartz-elicited leukocytes remained at least double that of the other inflammatory leukocyte populations from 1 day onwards and although there was a slight drop in the percentage of neutrophils at day 30, total leukocytes in the bronchoalveolar lavage increased and the proteolytic activity of these leukocytes also increased. The increase in proteolytic activity at day 30, concomitant with a fall in the percentage of neutrophils in that population, suggests that the macrophages were involved in the increased proteolysis either directly by secreting more proteolytic enzymes or indirectly by triggering increased neutrophil-mediated proteolysis. Neutrophil elastase has previously been considered to be of major importance in leukocyte-mediated injury in the lung (Cohen and Rossi, 1983) but this enzyme is susceptible to α1-protease inhibitor, the major source of antiprotease activity in the alveoli. If, as we suggest, macrophage proteases were secreted at high levels during chronic inflammation in the quartz-treated rats, this might be more likely to result in tissue damage since macrophage proteases are not inhibited by α1-protease inhibitor (Fels and Cohn, 1986).

Though we confirmed that we could measure increased plasminogen activator activity by using bronchoalveolar leukocytes elicited by thioglycollate broth (results not published) we found no evidence of enhanced plasminogen activator activity, on a cell for cell basis, (Figure 4) in leukocytes elicited by dusts or bacteria in rat lungs. This, however, does not preclude a role for plasminogen activator in the inflammatory response since enhanced levels of this enzyme may occur in the alveoli due to the increased accumulation of leukocytes.

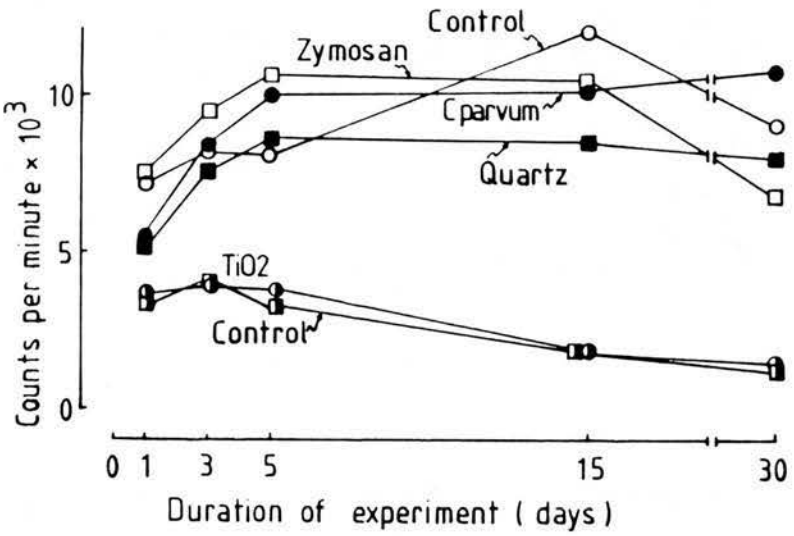


Fig. 4. Plasminogen activator secretion as counts per minute of degraded fibronectin released. TiO_2 was tested independently of the other samples and thus has a separate control.

In the key parameter we measured, proteolysis of fibronectin, the differential between controls and the different inflammatory leukocyte populations remained constant, despite addition *in vitro* of the triggers quartz, TiO_2, zymosan or PMA. In the case of control leukocytes, this suggests that in addition to the dust, one or more *in vivo* signals are required for leukocyte activation; mediators such as interleukin 1, interferon or tumour necrosis factor suggest themselves as likely priming agents. The dust-elicited leukocytes may have been maximally triggered *in vivo* and thus would be incapable of further stimulation *in vitro*. The assay of *in vitro* triggering was carried out within a four hour period which may have been too short for measurable stimulation to occur; experiments are currently in progress to measure *in vitro* stimulation over several days.

In summary, we have demonstrated marked differences in the inflammation engendered in rat lungs by a dust of known fibrogenic activity, quartz, compared with the inert dust, TiO_2. TiO_2 elicited a transient acute inflammatory response but alveolar inflammation in response to quartz persisted throughout the 30 days of the study and secretion of proteolytic enzymes by these leukocytes increased. Thus, tissue injury mediated by leukocyte proteases may be a major arbiter of the permanently deranged tissue structure and functional impairment found in the chronic mineral dust-related lung diseases. Work is in progress to characterize the cellular origin of the connective tissue degrading activity described in inflammatory leukocyte populations.

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In Vitro Studies of Leukocytes Lavaged from the Lungs of Rats Following the Inhalation of Mineral Dusts.

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Introduction

Much has been learned concerning the harmful effects of mineral dusts and other toxic materials by both *in vivo* and *in vitro* techniques. Both approaches have disadvantages, however. With *in vivo* studies exposure can be conducted under physiologically normal conditions but it is more difficult to unravel the complex sub-cellular and molecular events. *In vitro*, target cells can be examined in much greater detail but dose levels may have to be excessive to produce effects in an acceptable time scale and the absence of the whole of the body's defensive systems and factors such as recruitment of new populations limit this approach. An approach now being adopted more frequently where target cells are readily available by simple techniques such as pulmonary lavage is to undertake exposure to harmful substances *in vivo* followed by examination of target cells and their products *in vitro*. This paper reports studies in which rats were exposed to three coalmine dusts, two varieties of asbestos, quartz and titanium dioxide administered by inhalation. Subsequently, their lungs were lavaged and a series of studies undertaken with the cell populations obtained.

Materials and Methods

Syngeneic rats of the PVG strain were exposed to dust clouds of chrysotile or amosite asbestos, quartz, titanium dioxide or three coalmine dusts from collieries mining anthracite, high rank coal and low rank coal. Dose levels were either 10mg/m^3 or 50mg/m^3 of respirable dust although chrysotile asbestos was used only at the 10mg/m^3 dose. Dust exposure continued for up to 75 days followed by a recovery period of 64 days. Animals were killed at intervals between 1 day of dusting and the end of the study and their lungs subjected to lavage with 0.85% NaCl at 37°C. The procedure was repeated

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4 times with massage of the lungs between each wash to increase the yield of cells. The first 10ml of saline recovered from the lungs was used to assess levels of enzymes present while the cell yield from all washes was centrifuged and pooled. Total cell numbers were obtained using a Neubauer chamber and the percentage viability was assessed using Trypan blue exclusion. Differential counts were undertaken following May Grunwald–Giemsa staining and these preparations were also used to examine the proportion of cells containing dust particles. Lactate dehydrogenase was estimated in the first saline wash by the method of Wroblewski and Ladue, (1955). The levels of macrophage activation was studied using the spreading assay described by Donaldson *et al*, (1984) and the ability of lavaged cells to phagocytose additional material was examined by treating coverslip cultures with fluorescinated latex beads. The production of the reactive oxygen intermediates (ROI) by lavaged cells was estimated by the method of Johnstone, (1981) for superoxide anion and the method of Pick and Keisari, (1980) for hydrogen peroxide. The effect of dust exposure on the ability of leukocytes to chemotact towards serum, either activated with zymosan or treated with dust was examined using Blindwell chambers and Nuclepore filters and the ability of lavage fluids to cause chemotaxis was studied using the same techniques with indicator cell populations of control bronchoalveolar cells or a neutrophil–rich bronchoalveolar cell population obtained following the intratracheal injection of *Corynebacterium parvum*.

Results

Pulmonary lavage of dusted rats showed a variable response in respect of cell numbers depending on the dust type. At a dose level of 10mg/m^3 macrophage numbers did not increase significantly overall compared to controls, with any dust except quartz. With quartz macrophage numbers remained at normal levels for approximately 7 weeks but thereafter increased dramatically. For chrysotile, macrophage numbers were below control levels at 2 days, raised between 8 and 32 days of dusting before falling again by 75 days. At dose levels of 50mg/m^3 the increase in macrophage numbers following treatment with quartz began sooner, at approximately 4 weeks and rose to much higher levels. With quartz at this dose, over 100 x 10^6 macrophages could be lavaged from each rat after 10 weeks of exposure. At the 50mg/m^3 dose levels the three coalmine dusts all caused a significant increase in macrophage numbers by 75 days of exposure although the figures were approximately 5 times less than with quartz. Only amosite asbestos was used at the 50mg/m^3 dose level. This caused some increase in macrophage numbers by 32 days but less than found with the coalmine dust samples. Macrophage numbers were not

increased following treatment with titanium dioxide at 50mg/m^3.

An additional difference between the dusts in relation to macrophage numbers was found during the 64 day recovery period following dusting for either 32 or 75 days at both dose levels. With coalmine dusts, macrophage numbers, where raised, returned to control levels. With chrysotile at 10mg/m^3 and amosite at 50mg/m^3 macrophage numbers fell to below control levels during recovery after 32 days of dusting but remained slightly raised after 75 days of dusting. With quartz, macrophage numbers continued to increase, more than doubling during the recovery period at both dose levels. Examples of macrophage numbers lavaged from rat lungs following various dust treatments are illustrated in Figures 1 and 2. (see p. 343)

While several million pulmonary macrophages can be lavaged from the lungs of control rats, neutrophils are not found in these cell populations. The presence of neutrophils, therefore, represents a sensitive indication of pulmonary inflammation. At 10mg/m^3, all dust except titanium dioxide resulted in a significant increase in neutrophils by 32 days although once again the response to quartz was much greater than with coalmine dusts or asbestos. A similar pattern was found at 50mg/m^3 although with much higher neutrophil numbers reached and a significant increase detectable by 16 days or earlier. At 50mg/m^3 dose level the coalmine dust produced large numbers of neutrophils, although less than quartz and titanium dioxide produced a significant increase in neutrophil numbers between 52 and 75 days. Amosite asbestos although producing raised levels of neutrophils actually produced fewer than titanium dioxide at these later time points.

During the recovery period following treatment at 10mg/m^3, neutrophil numbers returned largely to control levels with all dusts except quartz. The number of lavageable neutrophils increased during this period with quartz although much less than the increase found with macrophages. A similar pattern was seen with quartz at the 50mg/m^3 dose level. At this dose, however, the number of lavageable neutrophils found during recovery had not returned to control levels by 75 days in animals treated with coalmine dusts, amosite or even titanium dioxide. Numbers of neutrophils lavaged from rat lungs in representative experiments are illustrated in Figures 3 and 4. (see p. 343)

The liberation of lactate dehydrogenase from cells is a very good indicator of cell damage and LDH levels in lavage fluid during the present study were found to parallel the neutrophil response very closely with most dusts. With a few dusts, however, a different pattern was seen. The low rank coalmine dust produced an early increase in LDH levels

but at both 10mg/m^3 and 50mg/m^3 this fell back between 52 and 75 days to levels close to the controls. Chrysotile at 10mg/m^3 produced the most rapid rise in LDH of all dusts with very significant increases by 8 days of dusting. Thereafter the chrysotile figures were the highest recorded until 52 days when they were overtaken by quartz. Amosite at 10mg/m^3 also produced higher LDH figures in the later stages of dusting than the neutrophil figures would have indicated. With amosite, however, there was little variation in LDH release between 10mg/m^3 and 50mg/m^3 of dust. During the recovery period at both dose levels, LDH release fell with the coalmine dusts towards control figures. With amosite at 10mg/m^3 a reduction in LDH release was found during the recovery period but at 50mg/m^3 and with chrysotile at 10mg/m^3 the LDH figures remained at their high levels. With quartz at both dose levels the release of LDH continued to increase during the recovery period. Levels of LDH present in pulmonary lavage fluids from representative experiments are illustrated in Figures 5 and 6. (see p. 344)

Cells in the lavaged populations always showed over 90% viability when newly isolated. Where the dust was of a type clearly visible by light microscopy (quartz and chrysotile are not) the proportion of macrophages containing dust was also over 90% by as little as 8 days of dusting. The proportion of neutrophils containing dust was much lower and seldom exceeded 50%. Over 90% of macrophages were also capable of further phagocytosis of latex spheres.

The macrophage spreading assay indicated increased levels of macrophage activation with all dust types. However, no consistent pattern of increase was found and levels did not differ between the 10 and 50mg doses. There was no overall difference in the production of ROI between control or dusted leukocytes with any of the dusts although all populations were stimulable by treatment with PMA *in vitro*.

Cells from animals dusted with quartz, coalmine dust, titanium dioxide or chrysotile all showed reduced ability to chemotact towards serum, whether normal, zymosan-treated or dust-treated (chemotaxis studies were not undertaken with amosite asbestos). This reduction was least marked with titanium dioxide and most marked with quartz and it increased with exposure time particularly for studies using the 10mg/m^3 dose level (Table 1). Spontaneous production of chemotaxins by the different leukocyte populations showed no pattern consistent with levels of cell recruitment found in the lungs. The only consistent evidence for any triggering of chemotaxin release was zymosan-stimulated release of chemotaxins for control bronchoalveolar cells. This stimulation was evident in all the lavaged cell populations regardless of dust treatment.

Discussion

A combination of *in vivo* and *in vitro* techniques has permitted an examination of the early inflammatory effects produced by the inhalation of a range of mineral dusts. As might have been predicted, quartz a known toxic dust caused a severe pulmonary inflammation which continued to increase after the end of dust exposure. Coalmine dusts of both high and low rank, while less active, also produced significant inflammation as indicated by the presence of neutrophils and LDH in lavage fluids but this tended to subside after the end of dusting. Even titanium dioxide, a supposedly innocuous dust, caused an influx of neutrophils with LDH release after 50 days of dusting at a high dose. Amosite asbestos caused significant levels of pulmonary inflammation but no higher than coalmine dusts at the same dose levels and this is in keeping with low levels of *in vitro* toxicity previously reported (Gormley *et al.*, 1985). The findings for chrysotile asbestos appear somewhat anomalous. *In vitro* studies show chrysotile to be a highly toxic dust and this is probably reflected in the high levels of LDH found in lavage fluid. At the same time neutrophil levels, while raised, were less than with some coalmine dusts and numbers of lavageable macrophages were only above control levels between 8 and 52 days. After the recovery period following 32 days exposure, macrophage levels were below control figures. A possible explanation of these findings is that macrophages containing asbestos fibres, which accumulate around the respiratory and terminal bronchioles, tend to become bound together in agglomerates which sometimes fuse to form foreign body giant cells. It may well be that macrophages containing asbestos fibres are much less easily dislodged by the lavage process than macrophages containing particulate dust so that the number of cells obtained underestimates the inflammatory response in the lung. Neutrophils may well become involved in the same cellular aggregates as macrophages with their numbers in lavage fluid correspondingly reduced.

While previous *in vitro* studies have demonstrated that at least some of the dust types considered here were able to kill macrophages within a few hours (Gormley *et al.*, 1980,1985) the present study found that >90% of lavaged macrophages were still viable at all time points while a similar proportion of cells contained visible dust. This indicates that under physiological conditions of exposure macrophage killing by dusts may occur but a great excess of the leukocytes are alive and have altered phenotypes from the resting population. The altered activity of these cells is likely to be expressed as increased release of cytokines and mediators of great relevance to disease production. Dusted macrophages were indeed shown to be more activated than controls and activation is

associated with increased levels of secretion. At the same time, the dusted cells showed reduced ability to chemotact which may reduce clearance rates and retain the cells in the lung tissue for long periods during which time these products can affect surrounding cells. This ability of macrophages and other inflammatory cells to orchestrate disease development in lung tissue is a key field of research which can best be undertaken using cell populations derived from *in vivo* exposure to harmful materials. We have already examined some aspects of leukocyte response that could be important in pulmonary disease (Brown *et al.*, 1988; Donaldson *et al.*, 1988[a,b]) but much remains to be done before we can fully understand the mechanisms by which inhaled mineral particles are able to initiate and control the disease process.

TABLE 1

CHEMOTAXIS OF BRONCHOALVEOLAR LEUKOCYTES FOLLOWING DUST EXPOSURE AT 10mg/m^3

DUST TYPES	SERUM TREATMENT	CONTROL	DUST-EXPOSED	CONTROL	DUST-EXPOSED
	-	13.5 (0.7)	8.5 (3.5)	17.0 (5.7)	14.0 (8.5)
TiO_2	Zymosan	80.0 (4.2)	55.5 (2.1)	73.5 (3.5)	66.0 (8.5)
	TiO_2	17.5 (4.9)	8.0 (2.8)	10.5 (0.7)	18.5 (2.1)
	-	26.5 (0.7)	11.0 (0.0)	54.5 (6.4)	14.0 (0.0)
Quartz	Zymosan	69.5 (3.5)	37.0 (2.8)	50.0 (17.0)	38.0 (2.8)
	Quartz	29.5 (3.5)	10.5 (2.1)	57.5 (6.4)	16.0 (1.4)
	-	26.0 (2.8)	9.5 (2.1)	15.5 (3.5)	4.0 (1.4)
H	Zymosan	89.0 (9.9)	58.0 (14.1)	60.0 (9.9)	38.5 (2.1)
	H	27.0 (7.1)	12.5 (4.9)	13.0 (0.0)	3.0 (0.0)
	-	35.0 (1.4)	21.0 (2.8)	39.5 (4.9)	14.5 (0.7)
A	Zymosan	67.0 (0.0)	48.0 (14.1)	70.0 (8.5)	35.5 (2.1)
	A	42.5 (9.2)	26.5 (3.5)	30.0 (1.4)	7.0 (0.0)
	-	16.5 (2.1)	9.5 (3.5)	4.0 (1.4)	6.0 (0.0)
L	Zymosan	69.5 (6.4)	34.0 (1.4)	38.5 (4.9)	8.5 (0.7)
	L	37.0 (0.0)	14.5 (2.1)	2.5 (0.7)	8.5 (0.7)
	-	23.5 (4.9)	6.5 (0.7)	30.0 (4.2)	9.0 (1.4)
Chrysotile	Zymosan	63.0 (4.2)	20.0 (1.4)	62.5 (0.7)	20.0 (4.2)
	Chrysotile	32.0 (1.4)	7.0 (0.0)	39.5 (4.9)	7.0 (0.0)

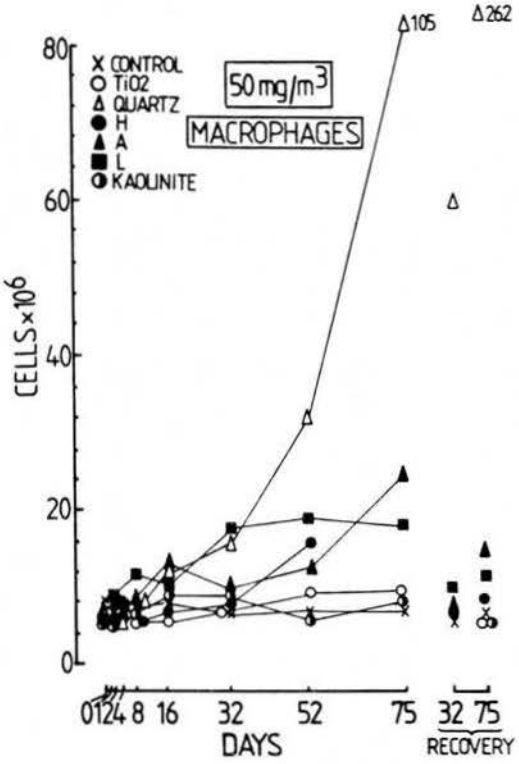


Figure 1

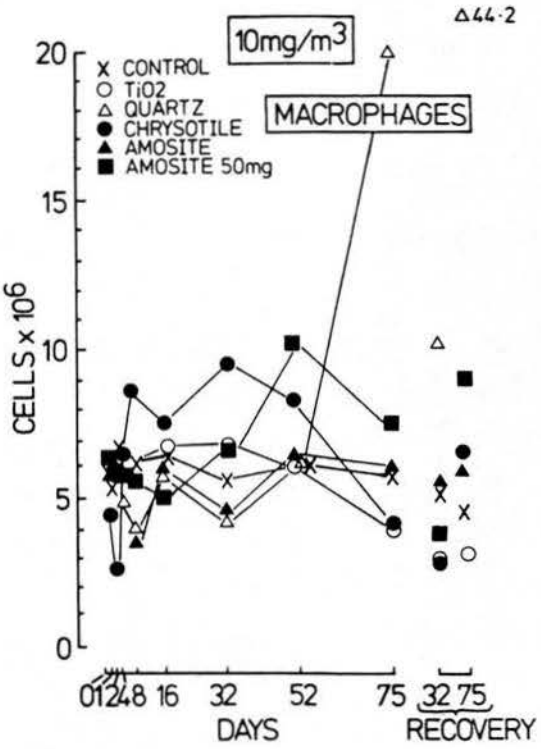


Figure 2

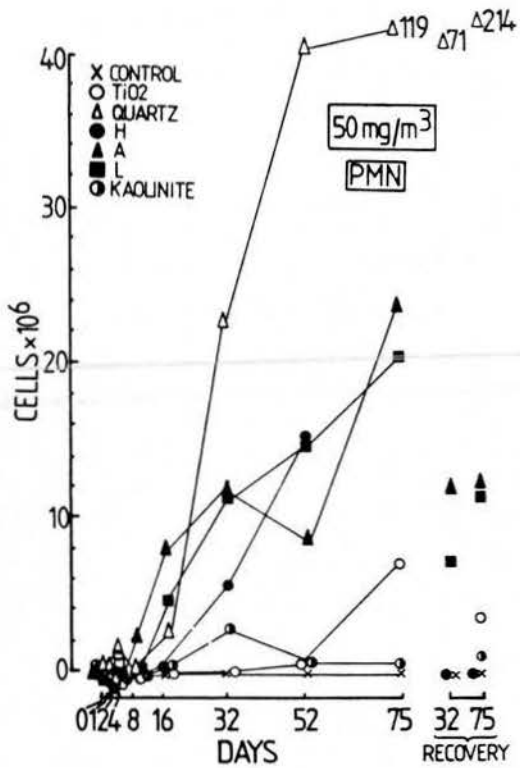


Figure 3

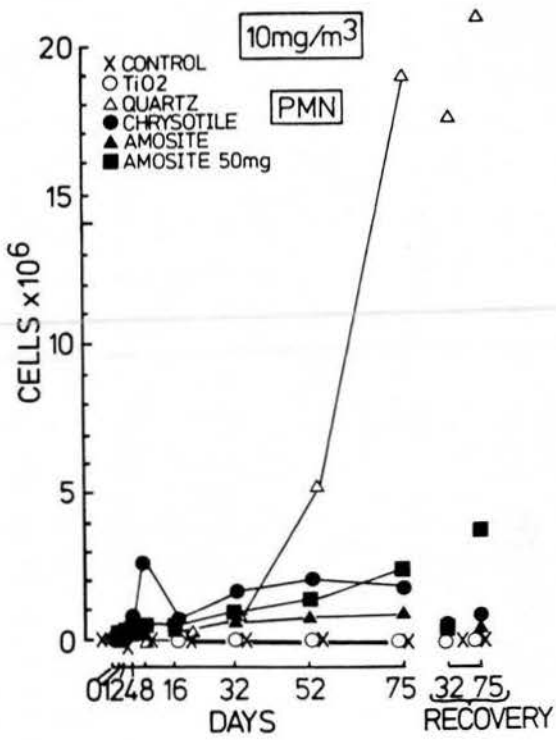


Figure 4

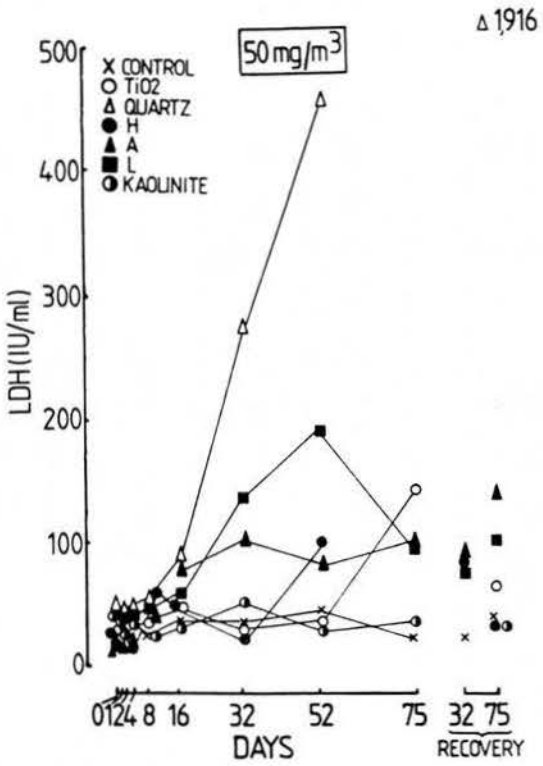


Figure 5

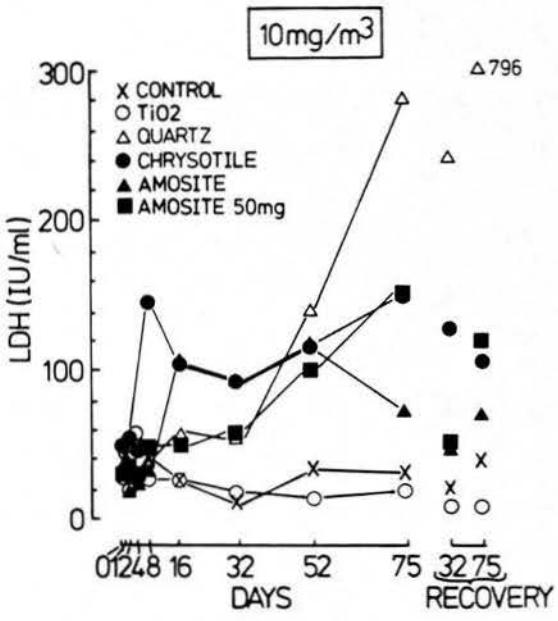


Figure 6

Figure Captions

Figures 1–6 illustrate representative figures for different components of pulmonary inflammation obtained by lavage. These were from animals treated by inhalation to a variety of mineral dusts at either 10mg/m^3 or 50mg/m^3 for periods of up to 75 days with some animals allowed to survive during a recovery period of 64 days. Figures 1 and 2 represent macrophage recovery from lungs. Figures 3 and 4 illustrate levels of PMN present. Figures 5 and 6 demonstrate the LDH content of lavage fluid.

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Inflammation generating potential of long and short fibre amosite asbestos samples

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ABSTRACT Previous studies have shown that long thin asbestos fibres are more pathogenic in in vivo and more active in in vitro assays than short fibre samples. In the present study a long fibre amosite asbestos sample and a short fibre sample prepared from it were tested for ability to cause inflammation in the peritoneal cavity of the mouse; a UICC sample intermediate in fibre size and an inert compact dust, TiO_2, were also tested. The ability of the dust samples to cause inflammation, as judged by macrophage and neutrophil recruitment, was ranked in the order long fibre > UICC > short fibre > TiO_2. Ability of amosite samples to cause inflammation was therefore related to the proportion of long fibres. The enhanced ability of long fibres to cause inflammation and cause macrophage activation is probably a key factor in the ability of long fibres to cause pulmonary fibrosis and may also be important in fibre carcinogenesis.

Inhalation of asbestos dust is associated with the development of interstitial pulmonary fibrosis and pulmonary neoplasia.[1] Experimental studies have shown, however, that not all asbestos samples are imbued with the same potential to cause lung disease. The shape of the fibres is one factor of major importance in this phenomenon and has been extensively studied (see review[2]) showing that long thin fibres are more pathogenic than short fibres. Studies from our own laboratory have recently shown that long amosite asbestos fibres, administered by inhalation, are substantially more pathogenic than a short fibre sample prepared from the long fibres and having essentially the same crystallinity and elemental composition.[3] The in vivo findings on the pathogenicity of long fires have been, in general, supported by in vitro studies showing that long fibres are most active in short term assays.[4]

Inflammatory responses in the lung parenchyma, while an important defence mechanism in normal circumstances, is considered to be an important arbiter of tissue damage,[5] leading to alveolar destruction or fibrosis if the inflammation persists.[6 7] The inflammatory potential of mineral dusts is therefore likely to be an important correlate of their pathogenic potential.[8]

Recent work from our laboratory has described an assay of the ability of mineral dust to cause inflammation in vivo[9] and differences in pathogenicity of long and short fibre amosite samples.[3]

The present study brings these together and seeks to examine the inflammation generating potential of the long and short fibre amosite samples to determine whether this correlates with the ability to cause damage to rat lungs as previously described.[2]

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Materials and methods

DUSTS

The titanium dioxide was the rutile form supplied by Tioxide Limited (Stockton-on-Tees).

The long fibre amosite comprised a batch of commercially available, milled South African amosite. This sample was generated as a cloud in an exposure chamber (for details see ref 3) and the airborne fibres were found to have a size distribution substantially longer than that of the standard UICC amosite which we have used previously.[10] The long fibre sample used in the present study was collected from the chamber air on to filters. Details of preparation of the short fibre amosite samples have also been given previously.[3] Briefly, a quantity of the bulk long fibre sample described above was ground in a ceramic ball mill and sedimented in water; comprehensive analysis of the final sample showed no loss of crystallinity and an elemental composition close to the parent long amosite sample. Details of preparation of the UICC amosite sample have been given previously.[11]

CHARACTERISATION OF THE AMOSITE ASBESTOS SAMPLES

Long and short fibre amosite were characterised by collecting airborne samples of the dust in exposure chambers on to membrane filters. Fibres $>0{\cdot}4$ μm in length and with an aspect ratio $>3{:}1$ were sized by scanning electron microscopy at a magnification of $\times 10\,000$. The UICC amosite sample was collected and sized as part of an earlier study.[10] Once again the dust was collected from a cloud in an exposure chamber on to membrane filters and sized by scanning electron microscopy, but in this case only fibres longer than $0{\cdot}6$ μm were included in the count.

ANIMALS

Syngenic, male C57B16 mice, 10–12 weeks of age at the time of injection, were used throughout.

INJECTION OF DUSTS

Dusts were suspended in sterile phosphate buffered saline (Gibco; Paisley) such that 0·5 ml contained 50, 500, or 2500 μg; in addition long and short amosite were injected at 5 μg. Groups of three mice were then injected intraperitoneally with 0·5 ml of suspended dust.

PERITONEAL LAVAGE

The leucocyte population of the peritoneal cavity was obtained by lavaging with three sequential injections of 2 ml of PBS containing 10 U/ml of heparin. A total volume of 5 ml was generally retrieved and the cells were kept on ice in plastic tubes.

CELL COUNTS

The total cell number retrieved from each mouse was obtained by dilution and counting in a Neubauer chamber. The proportion of each cell type was obtained from cytocentrifuge smears stained with Diff-Quik (Merz-dade, Dudingen, Switzerland). The percentage of each cell type and total numbers were used to calculate the total number of macrophages and neutrophils; other cell types were present at low levels ($<10\%$).

MACROPHAGE SPREADING ASSAY

The activational status of the macrophages was assessed by measuring the ability of the cells to spread on glass for one hour. The method used was that described previously[12]; 10^5 cells in 100 μl of RPMI 1640 + 10% fetal calf serum (Gibco; Paisley) were placed on 6 × 22 mm coverslips and incubated at 37°C for one hour. The maximum diameter of 200 Diff-Quik stained macrophages was assessed using a digitising board interfaced with a personal computer and a light microscope.[12]

STATISTICS

Data were examined by analysis of variance and differences in the means of treatment groups were examined for statistical significance using a t test appropriate for populations with different variances.[13]

Results

DUST SAMPLES

The titanium dioxide sample was an isometric dust of $2{\cdot}5$ μm median volume diameter. The amosite samples used differed in their size distribution. As shown in figs 1 and 2, the short and long fibre samples had similar diameters but the length distributions differed considerably. Thus the percentage of fibres >10 μm was 0·1–0·2% in the short fibre sample but 10–12% in the long fibre sample. The UICC sample, which was fibre sized according to slightly different rules (see methods) comprised 2–3% fibres >10 μm[10] and so lay midway between the long and short in terms of length distribution; the UICC sample was similar in diameter to the long and short fibre sample.[10]

GENERATION OF INFLAMMATION IN THE MOUSE PERITONEAL CAVITY

Control

In preliminary studies we found that the peritoneal cavity of control mice injected with saline alone yielded $6{\cdot}76 \times 10^6 \pm 0{\cdot}86$ macrophages and $0{\cdot}06 \times 10^6 \pm 0{\cdot}05$ neutrophils two days after injec-

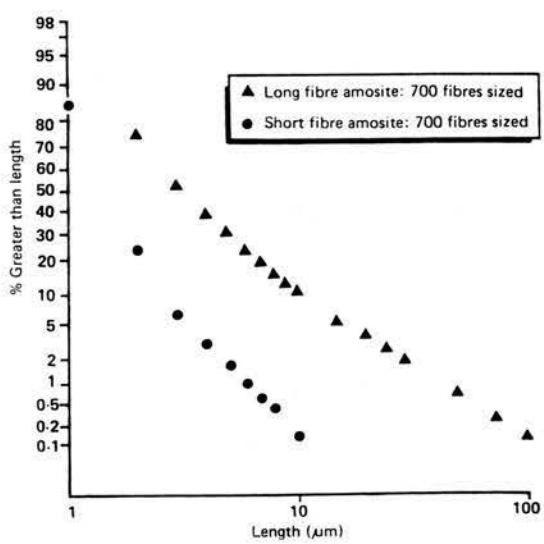


Fig 1 *Length distribution of long and short fibre amosite samples. (SEM sizing: 10 000 × magnification. Fibres longer than 0·4 μm diameter less than 3 μm and aspect ratio greater than 3 to 1.)*

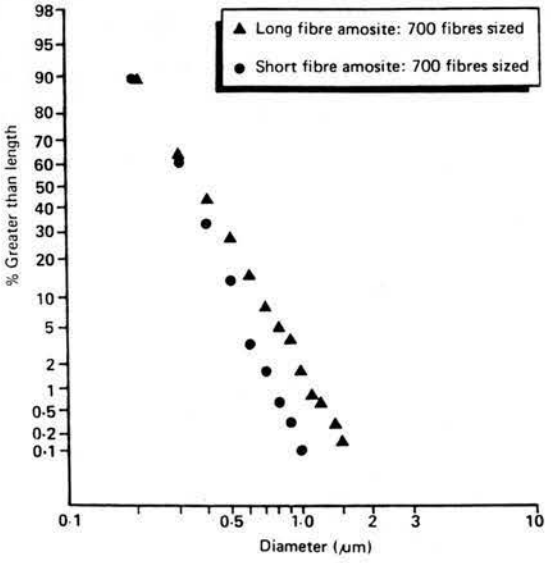


Fig 2 *Diameter distribution of long and short fibre amosite samples. (SEM sizing as in fig 1.)*

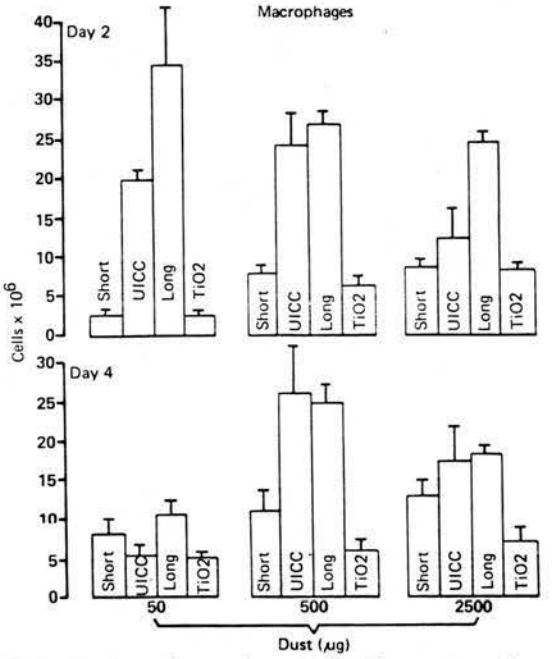


Fig 3 *Numbers of macrophages lavaged from peritoneal cavity of mice injected with short amosite, long amostie, UICC amosite, or titanium dioxide at indicated doses two or four days previously.*

tion. These figures are similar to those found after injections of titanium dioxide which is normally considered to be an innocuous dust. For this reason titanium dioxide was used as a negative control in these studies and the inflammatory response of the three amosite samples was compared with this.

Inflammatory cell recruitment in response to dust

The inflammatory cell recruitment caused by the different dust samples is shown graphically in figs 3 and 4; the statistical significance of comparisons between dusts are shown in tables 1 and 2.

The pattern of response found consistently with virtually all doses and times was that TiO_2 caused least response followed by short amosite whose activity was greater than TiO_2 but still was not substantial even at the higher dose. The UICC amosite was more active than short, and long fibre amosite caused, by far, the greatest degree of inflammation.

Amosite samples compared with TiO_2

The tables show that, on day 4, in 13/18 of the comparisons there were no differences between TiO_2 and any amosite sample in terms of macrophage response. All, except one, of the exceptions occurred with the middle (500 μg) dose. On day 2 however, particularly with UICC v TiO_2 and long v TiO_2 the asbestos samples caused significantly more macrophage recruitment (significant differences in 12/18 of comparisons). The neutrophil response tended to be more sensitive with significant increases in numbers with short and UICC compared with TiO_2 in 8/12 comparisons; long, however, showed the most consistent, highly significant, increases in recruitment over TiO_2, being $p > 0{\cdot}001$ in 6/6 comparisons.

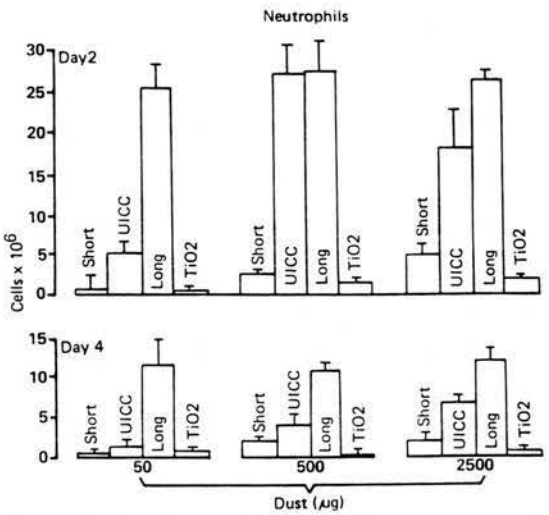


Fig 4 *Number of neutrophils lavaged from peritoneal cavity of mice injected with short, long, or UICC amosite or titanium dioxide at indicated doses two or four days previously.*

Table 1 *Results of comparisons between amosite asbestos samples and TiO_2 in ability to cause macrophage recruitment. Significant differences relate to increases in the response with dust (a) compared with dust (b)*

Dust (a)	(b)	Macrophages					
		50 μg		500 μg		2500 μg	
		2 Days	4 Days	2 Days	4 Days	2 Days	4 Days
Short	v TiO_2	NSD	NSD	NSD	<0·05	NSD	<0·05
UICC	v TiO_2	NSD	NSD	<0·001	NSD	<0·001	NSD
Long	v TiO_2	<0·001	NSD	<0·001	<0·001	<0·001	NSD
UICC	v short	<0·001	NSD	<0·01	<0·01	NSD	NSD
Long	v UICC	<0·01	NSD	NSD	NSD	<0·05	NSD
Long	v short	<0·001	NSD	<0·001	<0·01	<0·001	NSD

Comparisons between amosite samples

At four days short and UICC did not, in general, significantly differ in causing inflammation (no significant differences in 5/6 comparisons), although the pattern of UICC > short was consistent; at two days, however, UICC was consistently significantly more active in causing both macrophage and neutrophil recruitment than short (significant differences in 5/6 comparisons). A similar pattern was evident with long v UICC with more activity of long over UICC on day 2 than on day 4. The greatest difference was evident between long and short amosite where long was consistently highly significantly more active than short (significant differences in 10/12 comparisons).

Inflammatory potential of low dose long and short amosite asbestos

The doses used in the present study (50, 500, 2500 μg) were those we have used previously.[9] Since the inflammatory response was still evident at 50 μg with long amosite, an extra experiment was carried out using 5 μg. The results of this, shown in table 3, show that even at 5 μg there is still a pronounced degree of inflammation with long amosite; short amosite remained virtually inactive.

Macrophage spreading assay

The macrophage spreading assay was undertaken only with cells from animals injected with the long and short amosite samples and killed four days later. While cells from animals treated with short amosite were similar to controls, the long fibre dust had produced pronounced macrophage activation ($p < 0·001$) (table 4).

Discussion

This study examined the ability of long and short amosite asbestos samples to cause inflammation in the mouse peritoneal cavity; UICC amosite was included as an amosite sample whose length distribution was intermediate between the short and the long. All three asbestos samples caused significantly more inflammation than a titanium dioxide sample which we have previously found to be low in activity when administered by intraperitoneal injection,[9] inhalation (K Donaldson *et al*, in preparation), and by intratracheal instillation (unpublished observations). The short fibre sample was closest to titanium dioxide in activity and long fibre amosite produced the greatest amount of inflammation relative to the titanium dioxide sample. There were also significant differences between the three amosite samples in their inflammation generating potential with long > UICC > short. At low dose (50 μg) long amosite caused macrophage activation, as assessed by spreading, whereas short amosite did not.

A length of about 10 μm has been found to be a key

Table 2 *Results of statistical comparisons between amosite asbestos samples and TiO_2. Significant differences relate to increase in the response with dust (a) compared with dust (b)*

Dust (a)	(b)	Neutrophils					
		50 μg		500 μg		2500 μg	
		2 Days	4 Days	2 Days	4 Days	2 Days	4 Days
Short	v TiO_2	NSD	NSD	<0·05	<0·01	<0·001	<0·01
UICC	v TiO_2	<0·05	NSD	<0·001	NSD	<0·001	<0·05
Long	v TiO_2	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001
UICC	v short	<0·05	NSD	<0·001	NSD	<0·001	NSD
Long	v UICC	<0·001	<0·05	NSD	NSD	<0·05	NSD
Long	v short	<0·001	<0·01	<0·001	<0·01	<0·001	<0·01

Table 3 *Numbers of macrophages and neutrophils recruited to the peritoneal cavity two days after intraperitoneal injection of 5 μg of long or short fibre amosite. Significant difference ($p < 0.001$) between treatments*

	Cells × 10⁶	
Treatment	*Macrophages*	*Neutrophils*
Long amosite	23·25 (3·56)*	13·28 (3·71)
Short amosite	4·65 (0·56)	0·20 (0·02)

* ± (SEM).

one in terms of the biological reactivity of fibrous dusts since fibres of this size and above are of maximal pathogenicity whereas below this length the activity begins to decrease[14 15]; similar results are found in in vitro assays.[4] In the three amosite populations used here the figure for percentage of fibres longer than 10 μm was: short 0·1–0·2%, UICC 2–3%, long 10–12%. The ranking of the amosite samples in terms of their ability to cause inflammation therefore correlated with proportion of long fibres in the samples.

The short fibre amosite sample produced the lowest levels of inflammation of the three amosite samples with long amosite producing the highest. The differences between short amosite and UICC amosite, and UICC amosite and long amosite, were significant for the influx of both macrophages and neutrophils two days after injection of 50 μg of dust. At higher doses, the difference between UICC amosite and long fibre amosite was less pronounced. This may be because at these doses even the UICC sample contained sufficient long fibres to produce a cell response close to the maximum for the mouse peritoneal cavity, with the additional long fibres in the long fibre sample, for this reason, having little effect. At four days after injection the macrophage response at 50 μg and the neutrophils response at all doses declined considerably, similar to that found with other dusts we have examined. The reduction in macrophage influx at 50 μg probably indicates that sufficient macrophages had been recruited by two days to engulf or surround all the injected fibres and to begin granuloma formation by four days with consequent reduction of the accessibility of these macrophages to the lavage techniques used. At the two higher doses, levels of macrophage recruitment remained at similar levels between two and four days. This may imply that sufficient free dust had remained over this period, or that there was sufficient tissue damage, to keep recruitment high even though "fixing" of cells by early granuloma formation would have been taking place.

Table 4 *Macrophage spreading on glass after injection of 50 μg of dust four days previously. Significant differences ($p < 0.001$) between long amosite and the other two treatments*

Treatment	*Macrophage spreading (μm)*
Saline control	10·90 (1·93)*
Long amosite	17·38 (7·08)
Short amosite	10·93 (5·01)

* ± (SD).

The results described here are complementary to those published[3] using the same long and short amosite samples. In that study the long fibre sample was much more fibrogenic than the short and caused more long tumours. We have shown here that the increased ability to produce injury and inflammation in vivo, shown by the long fibre sample, is consistent with the increased pathogenic potential of the same sample.[3] We are aware of only one previous study relating asbestos fibre length to inflammatory potential[16]; this showed similar results to those described here with long fibres being much more active. In that study, however, chrysotile from two different sources was used; unlike the present study which used a long fibre sample and a short fibre sample prepared from it so excluding the potential for differences in asbestos composition in the two samples.We have also shown the effect across a range of doses and shown that, even at 5 μg per mouse, the long fibre sample is intensely active in causing inflammation while even at 2500 μg the short fibre sample is low in activity.

Evidence of a link between inflammation in the alveolar region of the lung and development of pulmonary fibrosis has come from many studies in different fibrotic lung disease.[6] In the case of mineral dust related fibrosis there is compelling evidence that fibrogenic dusts cause pulmonary inflammation in occupationally exposed men,[17 18] and experimentally exposed animals.[8 19] Inflammatory leucocytes have the potential to cause damage to the fragile lung parenchyma through release of oxidants and proteases,[5] which may lead to a repair response and fibrosis if damage is sufficiently great. Macrophages also possess the ability to stimulate fibroblast growth and this is increased in activated macrophages[20] such as those found in inflammatory sites and in macrophages from lungs exposed to toxic dust.[16] Inflammation may also play a part in tumorigenesis in the case of fibrous dusts such as asbestos. Here the genotoxic effects of asbestos[21] combined with a generalised "promoting" effect during the proliferative phase of inflammation could favour neoplastic change.[22]

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2 Soter NA, Wasserman SI, Austen KF. Cold urticaria: release into the circulation of histamine and eosino-phil chemotactic factor of anaphylaxis during cold challenge. *N Engl J Med* 1976;**294**:687-90.
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Injurious Effects of Mineral Dust-elicited Bronchoalveolar Leukocytes on Epithelial Cells *in vitro*: The Role of Extracellular Matrix Components

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Introduction

Deposition of mineral dust associated with the development of pneumoconiosis causes a range of changes in the lung parenchyma including alveolar inflammation and, in the longer term, septal fibrosis and epithelial abnormalities (Begin *et al.* 1986; Gibbs *et al.* 1984). We have been studying the role of inflammatory leukocytes in the development of pathology in dust-exposed lung and have previously reported on the ability of inflammatory bronchoalveolar leukocytes from rat lung, exposed by intratracheal installation to quartz, to cause proteolytic injury to cells of an alveolar epithelial cell line *in vitro* (Donaldson *et al.* 1988[c]). In the present paper we extend these studies by examining inflammatory bronchoalveolar leukocytes lavaged from chrysotile asbestos-exposed lung, in terms of ability to cause epithelial injury *in vitro*. We further report on the ability of bronchoalveolar leukocytes from lung exposed by the more appropriate inhalation route to coalmine dust, with the regard to causing epithelial injury and proteolytic degradation of fibronectin. In order to study the role of the extracellular matrix in epithelial injury *in vitro*, we have examined the ability of exogenous protease and mineral dust-elicited inflammatory bronchoalveolar leukocytes, to injure epithelial cells cultured on surfaces coated with purified extracellular matrix components.

Materials and Methods

Models of mineral dust exposure

Mineral dusts used in the study were quartz (DQ_{12} standard), chrysotile asbestos (UICC sample A) and titanium dioxide (TiO_2: rutile form; Tioxide Ltd., Stockton on Tees). For intratracheal instillation, dusts were suspended at 2mg/ml in sterile saline and 0.5ml (1mg) instilled into the lungs of anaesthetised rats. For inhalation exposure using coalmine dust, the dusts were collected from the air of a colliery mining medium rank

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coal (26% ash) containing 0.7% quartz. Dust was generated in purpose-built exposure chambers as a monodispersed airborne cloud at 50mg/m^3 - full details of the generation and monitoring procedures are given elsewhere (Donaldson *et al.* (1988[a]). At selected time points after instillation of dust or 45 days after airborne exposure to coalmine dust, rats were killed by barbital overdose and their lungs lavaged with 4 x 8ml volumes of warm saline with massaging to increase the yield of leukocytes (Donaldson *et al.* (1988[a]).

Assay of bronchoalveolar leukocyte-mediated injury to alveolar epithelial cells

This assay has been described in detail elsewhere (Donaldson *et al.* (1988[c]). Briefly cells of the A549 alveolar epithelial cell line were prelabelled with ^{51}Cr overnight and washed. The labelled cells were co-cultured with inflammatory bronchoalveolar leukocytes at varying effector:target ratios and leukocyte-mediated detachment injury and lytic injury was assessed.

Assay of bronchoalveolar leukocyte-mediated proteolysis of ^{125}I fibronectin

This assay is described in detail elsewhere (Brown and Donaldson, 1988[b]). Briefly ^{125}I-labelled fibronectin were prepared and adhered, as a matrix, to the bottom of microtitre plate wells. The bronchoalveolar leukocytes under test were added to the microtitre plate wells and their ability to degrade ^{125}I fibronectin was assessed.

Results

Detachment injury caused to alveolar epithelial cells by co-culture with dust-elicited bronchaolveolar leukocytes

Figure 1 shows the epithelial-injuring activity of bronchoalveolar leukocytes from the lungs of rats exposed, by intratracheal instillation, to chrysotile asbestos or titanium dioxide. It shows clear detachment injury with both chrysotile asbestos and TiO_2-elicited leukocytes at day 1 with chrysotile leukocytes producing more detachment activity at the higher effector:target ratios. The ability of the TiO_2-elicited bronchoalveolar leukocytes to injure epithelial cells was virtually over by 3 days whilst the chrysotile asbestos bronchoalveolar leukocytes were still able to cause epithelial injury at 3 days although this

had returned almost to normal control levels by 7 days. The percentage of neutrophils in each of the populations tested was:- TiO_2 - 1 day 48%, 3 days 15%, 7 days 0%; chrysotile - 1 day 40%, 3 days 12%, 7 days 4%; the remainder of the cells were macrophages with <5% lymphocytes.

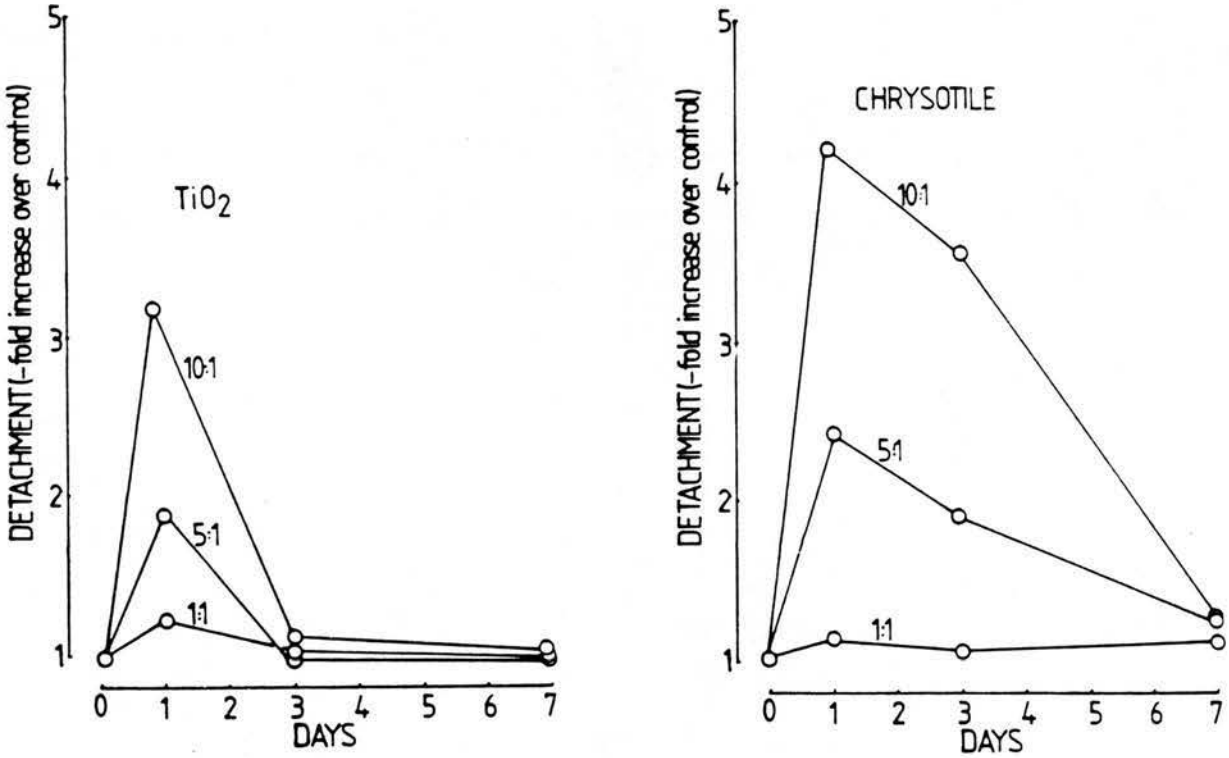


Fig. 1. Left. Detachment injury caused to alveolar epithelial cells by TiO_2- (left hand panel) or chrysotile asbestos (right hand panel) elicited bronchoalveolar leukocytes at varying effector:target ratios; rats received 1mg of dust intratracheally on day 0

Exposure to coalmine dust (50mg/m^3) by inhalation elicited a bronchoalveolar leukocyte population with ability to cause detachment injury to alveolar epithelial cells *in vitro* (Figure 2); concomitant with the increased detachment caused by these inflammatory leukocytes there was increased proteolysis of ^{125}I fibronectin. The coalmine dust-elicited population contained 56% neutrophils.

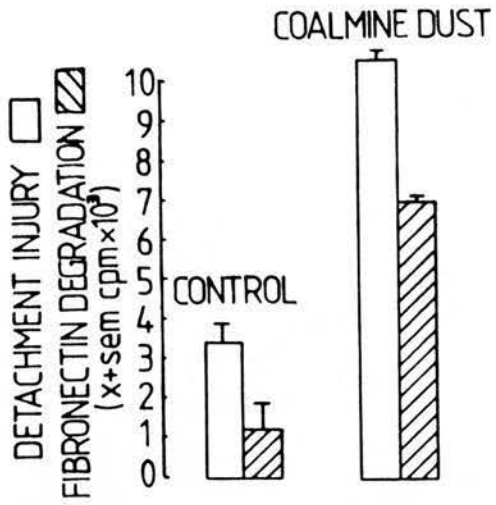


Fig. 2. Epithelial cell detachment injury and breakdown of fibronectin caused by bronchoalveolar leukocytes from control rats or rats inhaling coalmine dust for 44 days.

Role of extracellular matrix in detachment injury

Culture of the target epithelial cells on microtitre plates coated with purified extracellular matrix components profoundly altered their ability to be detached on culture with exogenous elastase (Figure 3). It is clear that this microbial elastase preparation causes rapid detachment of cells grown on plastic alone even at very low doses but growing cells on fibronectin or Type IV collagen matrix renders the alveolar epithelial cells resistant to detachment injury even at very high levels of this exogenous enzyme. Figure 4 shows that, in the presence of quartz-elicited bronchoalveolar leukocytes, culture on extracellular matrices also causes protection against detachment injury. Clear differences exist in the ability of different components to produce protection, with Type IV collagen promoting greatest attachment and resistance to inflammatory cell damage and laminin and fibronectin being less protective.

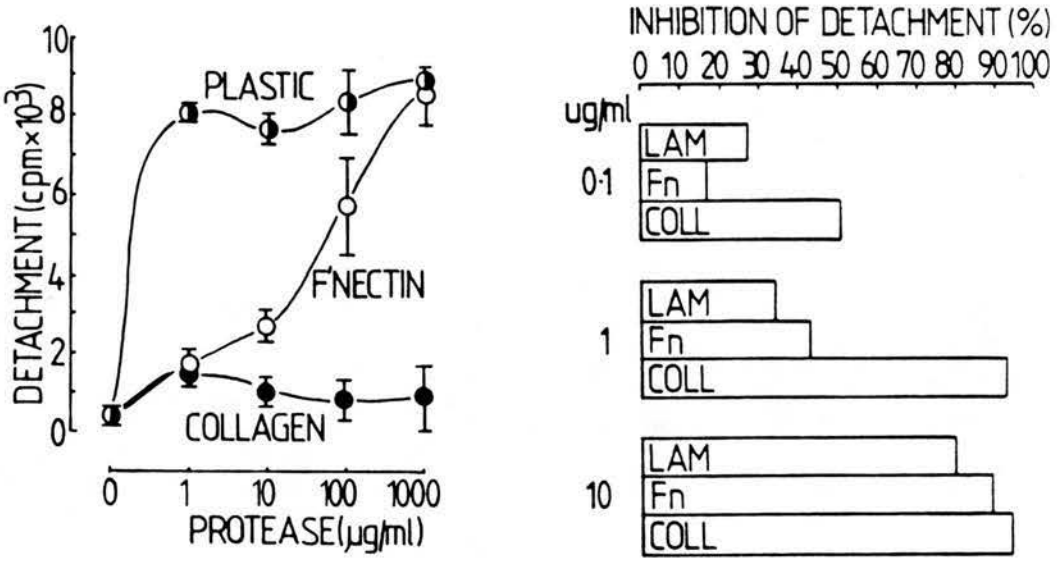


Fig. 3. Epithelial cell detachment injury caused by exogenous elastase to alveolar epithelial cells cultured on plastic, fibronectin or collagen-coated plates.

Fig. 4. Epithelial cell detachment injury caused by quartz-elicited bronchoalveolar leukocytes to alveolar epithelial cells cultured on laminin, fibronectin or collagen-coated plates. Results shown as inhibition of detachment compared to cells cultured on plastic.

Discussion

We have been examining the relationship between inflammatory leukocyte recruitment into the lungs following the deposition of pathogenic mineral dust, and subsequent development of pathological change. This paper examines, in particular, the ability of inflammatory leukocytes to injure alveolar epithelial cells *in vitro* and the role of the extracellular matrix has been studied in this phenomenon. Injury to epithelial cells could lead to alveolar epithelial hyperplasia and, possibly through failure to attain re-epithelialisation, also contribute to alveolar fibrosis (Witschi et al. 1980). Our previous results have shown that bronchoalveolar leukocytes from quartz-exposed lung have the ability to injure alveolar epithelial cells by causing detachment through a largely protease-mediated mechanism (Donaldson et al. 1988[c]) and that these leukocytes can also cause degradation of extracellular matrix components (Brown and Donaldson, 1988[b]). In the present paper we have extended these findings and have demonstrated that inflammatory leukocytes from

lungs exposed to chrysotile asbestos or TiO_2 by instillation both have, in the short-term, the ability to injure epithelial cells. The single intratracheal instillation of asbestos elicited leukocytes with epithelial-injuring activity which was present at both 1 and 3 days and still above control levels by day 7. With intratracheal TiO_2, a dust not associated with pneumoconiosis and having little inflammatory potential (Donaldson et al. 1988[b]), epithelial-detaching activity was present at 1 day but had returned to the same level as control bronchoalveolar leukocytes by 3 days. Delivered by intratracheal instillation, chrysotile asbestos is substantially less active than quartz in causing leukocyte recruitment to the rat lung and a similar pattern is found with inhalation exposure (Donaldson et al. 1988[b]).

We report here also that bronchoalveolar cells from rats exposed to coalmine dust by inhalation show increased epithelial-injuring activity with concomitant increased fibronectin degrading activity; details of the time relatedness of this response are described elsewhere (Brown and Donaldson, 1988[a]). It therefore seems likely that bronchoalveolar leukocytes from rats exposed to any mineral dust which cause inflammation on inhalation exposure will have the potential to cause epithelial injury and extracellular matrix damage. Our experience has shown that such dusts include the classical pneumoconiosis-generating dusts quartz, asbestos (Donaldson et al. 1988[b]) and coalmine dust (Donaldson et al. 1988[a]). TiO_2, which does not cause pneumoconiosis, does not cause inflammation when inhaled except after very long exposure at very high dose (Donaldson *et al.* 1988[a]). We suggest therefore that some of the epithelial abnormalities and hyperplasia commonly present in association with pneumoconiotic fibrosis may be due to injurious effects on the epithelial cells caused by inflammatory bronchoalveolar leukocytes.

The present study has also demonstrated an important modulating role of extracellular matrix elements in detachment injury caused to the alveolar epithelial cells by inflammatory bronchoalveolar leukocytes. Epithelial cells appear to have affinity for extracellular matrix which may be receptor-mediated particularly in the case of Type IV collagen which was particularly protective against proteolytic injury. Leukocyte proteases may be excluded from interacting with the areas of contact between the cell and the matrix which could be important targets for the proteases during detachment *in vitro*. Epithelial cells lay down their own basement membrane containing extracellular matrix elements but in the time scale of this assay it is unlikely that they have had time to do so. These results suggest that, if a proper basement membrane could be produced by the target cells, their susceptibility to detachment injury could be much less. However, studies on whole basement membrane and mixtures of purified extracellular matrix

components are necessary to clarify this response. The results suggest that epithelial detachment injury *in vivo* might not be so severe as suggested by detachment experiments carried out with the target cells grown on plastic and highlights the importance of the culture conditions in assessing epithelial cell response to leukocyte-mediated injury. During inflammation *in vivo*, however, activated leukocytes are trafficking through the alveolar epithelium during which the basement membrane and other extracellular matrix associated with the epithelial barrier may be more susceptible to injury. An assay system which would allow migration of the effector leukocytes through a barrier of epithelial cells would provide a better model.

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Bronchoalveolar Leukocyte Response in Experimental Silicosis: Modulation by a Soluble Aluminum Compound

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Bronchoalveolar Leukocyte Response in Experimental Silicosis: Modulation by a Soluble Aluminum Compound. BROWN, G. M., DONALDSON, K., AND BROWN, D. M. (1989). *Toxicol. Appl. Pharmacol.* **101**, 95–105. The biological properties of quartz have been related to its surface reactivity. We have addressed the role of particle surface reactivity in mediating the biological activity of quartz in mixed dusts, by treating the quartz with aluminum lactate. Intratracheal instillation of untreated quartz in rats caused a rapid, sustained alveolitis and bronchoalveolar leukocytes obtained from these animals had enhanced activity in degrading fibronectin, but reduced ability to mount a respiratory burst. Quartz pretreated with aluminum elicited a markedly reduced inflammatory response; the reduced activity of the treated quartz was also reflected in the attenuated change in the key functional parameters, oxidant production and proteolysis of fibronectin. Late intratracheal dosing with aluminum after the quartz-induced alveolitis was well established reduced the inflammatory response and abrogated the effect of quartz on the respiratory burst, but did not alter fibronectin degradation by the leukocytes. Aluminum did not affect the inflammatory response to *Corynebacterium parvum* and thus the effect was on the quartz particles and not on the inflammatory leukocytes. These findings have implications for the likely pulmonary responses to mixed dusts containing quartz and aluminum silicate clays. © 1989 Academic Press, Inc.

Occupational exposure to silica as either the pure form of the mineral or in a mixed dust is associated with the development of fibrotic lesions in the lungs (Morgan and Seaton, 1984). Despite the known causal relationship between silica exposure and lung damage, the quartz content of a mixed dust is not always correlated with the incidence of disease in men exposed to that dust (Davis *et al.*, 1975). It has been suggested that this might be due to the presence of minerals in a mixed dust which could have an ameliorating effect on quartz toxicity (Bremner, 1939) and this theory is supported by epidemiological evidence (Walton *et al.*, 1977).

The biological activity of silica has been related to its surface properties (Kriegseis *et al.*, 1987) which may be modified by compounds occurring in conjunction with quartz in mixed dusts. Such compounds include iron, which reduces the fibrotic potential of silica (Gross *et al.*, 1960; Reichel *et al.*, 1977), and aluminum, which reduces both the inflammogenicity and the fibrogenicity of silica (Begin *et al.*, 1986; Le Bouffant *et al.*, 1977a). Aluminum is adsorbed onto the surface of silica particles (Denny *et al.*, 1939), and thus alters various properties of the silica, rendering it less refractive by electron diffraction (Le Bouffant *et al.*, 1977b), increasing its zeta potential (Nolan *et al.*, 1981), and increasing flocculation by reducing the solubility of the particles (Denny *et al.*, 1939). In addition, the quartz component of native silica dust can be surface-modified by techniques which remove contaminants from the surface of the quartz particles and render them more toxic to cells *in vitro* (Kreigseis *et al.*, 1987). The

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importance of the surface reactivity of quartz has also been demonstrated by showing that freshly fractured quartz has greater biological activity—presumably because new, uncontaminated surfaces are revealed (Heffernan, 1932; Fubini *et al.*, 1987). Clay minerals such as kaolin, mica, illite, or smectite are potential sources of aluminum in mixed dusts and a pilot study by Le Bouffant *et al.* (1977b) has demonstrated reduced biological activity of quartz when administered in conjunction with illite. Further experimental evidence *in vivo* in support of the "antidotal rocks" theory of Bremner (1939) has demonstrated that treatment with various aluminum or iron compounds in parallel with quartz dosing markedly reduced the pathogenicity of the quartz in rats (Gross *et al.*, 1958; Le Bouffant *et al.*, 1977a,b), guinea pigs (Gross *et al.*, 1958), mice (King *et al.*, 1953), and rabbits (Dworski, 1955). However, these studies reflected only the final outcome of the disease process by measuring the extent of the pathological change in the lungs. In addition, assessment of the protective effects of the aluminum and iron was complicated by the fact that they were particulate in nature and could elicit an adverse reaction in the lung in their own right (Reichel *et al.*, 1977; Shaver, 1948; Jordan, 1961).

More recently, experimental studies on the biological activity of quartz *in vivo* have used soluble aluminum to modulate the effect of quartz in eliciting an inflammatory response and subsequent pathological change in the lungs of sheep (Begin *et al.*, 1986). These studies have shown that pretreatment of quartz with soluble aluminum reduces the inflammatory response as measured by bronchoalveolar lavage; tissue damage was also markedly reduced.

Tissue injury caused by toxic products of inflammatory leukocytes is deemed to be central to subsequent pathological change in chronic inflammatory lung disease (Hunninghake *et al.*, 1979) and is also implicated in the fibrotic change associated with silica exposure (Voisin *et al.*, 1985). In this study we have investigated the mechanisms of silica pathogenicity by assessing the inflammogenic and fibrogenic potential of native silica and silica modified by pretreatment with aluminum lactate. The inflammatory response to these materials in rat lungs was assessed by measuring the total number of leukocytes and the percentage of polymorphonuclear leukocytes in the bronchoalveolar lavage; we assessed changes in the functional status of the leukocytes, relevant to tissue damage, by measuring degradation of a fibronectin substrate and production of hydrogen peroxide *in vitro* and have related such changes to the pathology seen at autopsy. To investigate the mode of action of aluminum in reducing quartz toxicity, we used a two-pronged approach—first we investigated the ability of aluminum to alter the biological activity of quartz *in vivo* by administering the aluminum after quartz-induced alveolitis was established and second, we addressed the question of whether the aluminum was acting on the quartz particle or on the cells of the inflammatory response by measuring the ability of aluminum *in vivo* to reduce the inflammation elicited by a heat-killed pathogen, *Corynebacterium parvum*.

The results are discussed in relation to the mode of action of silica in causing inflammation and pathological change in the lung.

MATERIALS AND METHODS

Reagents. Phorbol myristate acetate (PMA), human plasma fibronectin, bovine serum albumin, catalase, superoxide dismutase, cytochrome *c*, and hydrogen peroxide were obtained from Sigma Chemical Co., Poole, Dorset. Ham's F10 medium was purchased from Gibco BRL, Paisley, Renfrewshire. Iodine-125 (^{125}I) was obtained from Amersham International, Aylesbury, Buckinghamshire. The silica used was the DQ12 quartz standard sample.

Animals. SPF, syngeneic, female PVG rats were obtained from the breeding unit of the Institute of Occupational Medicine, Edinburgh. The animals were housed in RB3 cages with raised stainless steel tops (North Kent Plastics, Kent) and were maintained at 23–24°C, with a 12-hr photoperiod. Food was rat and mouse mainte-

nance diet No. 1, supplied by Special Diet Services, Witham, Essex; water was provided *ad libitum*.

Aluminum lactate treatment of quartz. Quartz was coated with aluminum according to the method of Begin *et al.* (1986). Briefly, quartz was mixed by rotation in a solution of 1% aluminum lactate in water for 3 hr at room temperature and was then washed twice with saline before being resuspended in saline for intratracheal injection.

Experimental protocol. 1. Intratracheal injections. These were performed under deep ether anesthesia within a period of 7 min for each animal. The trachea was exposed by dissection, and a small incision was made to allow access of a blunt catheter. The cannula was inserted as far as the first bifurcation of the bronchi and the dust, in a volume of 0.5 ml sterile PBS was injected as a single bolus. Thereafter, the cannula was withdrawn, and the wound was closed using two 11-mm michel clips and was then dusted with Cicatrin antibiotic powder (Wellcome Foundation, London).

2. Time response. Animals were treated by intratracheal instillation of 1 mg DQ12 quartz or 1 mg DQ12 coated with aluminum lactate (quartz/aluminum lactate); control animals were untreated; previous work with this rat model has shown that intratracheal dosing with saline causes minimal leukocyte recruitment which lasts only 24 hr (unpublished results). One, four, or twelve weeks after injection three animals per group were killed and the lungs resected and lavaged to assess the inflammatory response; to assess tissue injury the lungs were subsequently fixed by inflation with 10% formol saline, processed for histology, and stained with hemotoxylin and eosin.

3. Delayed aluminum treatment. Animals were dosed by intratracheal instillation of 1 mg DQ12 as above. One month after quartz dosing the animals were treated by intratracheal instillation, with 0.5 ml saline containing 50 mg aluminum lactate; 1 week or 1 month thereafter, the lungs were resected and treated as above. Appropriate controls, either untreated or dosed with quartz alone, were killed in parallel with those treated with aluminum lactate and quartz.

4. *C. parvum*-induced inflammation. *C. parvum* (1.4 mg) was administered alone or in conjunction with 50 μg aluminum lactate in 0.5 ml saline; leukocyte recruitment and tissue injury were assessed 16 hr later.

Bronchoalveolar lavage. Animals were killed by phenobarbitone overdose administered by the intraperitoneal route. The thoracic cavity was opened and the lungs cannulated with a blunt 16-gauge needle. After resection, the lungs were lavaged with four sequential 6-ml washes of saline at 37°C, with massage prior to each lavage. The washes were pooled and placed immediately on ice. Total viable cells were estimated by trypan blue exclusion and cytospin preparations were made and stained with May–Grunwald–Giemsa for differential counts.

Assay of leukocyte proteolysis. Leukocyte proteolysis was estimated by measuring degradation of a ^{125}I-labeled fibronectin matrix, details of which have been published elsewhere (Brown and Donaldson, 1988). Briefly, fibronectin was iodinated by the chloramine-T method and adhered in microtiter wells. Cell suspension (200 ml) at 5×10^5 cells/ml in Ham's F10 medium + 2% BSA was pipetted into triplicate wells and incubated for 4 hr at 37°C. Supernatant (150 μl) was then harvested from each well and counted by gamma counter, increasing counts denoting increased proteolysis of the fibronectin matrix.

Hydrogen peroxide and superoxide assays. Hydrogen peroxide and superoxide were measured as detailed previously (Donaldson *et al.*, 1988). Increasing content of hydrogen peroxide or superoxide, in the presence or absence of PMA, was manifest as a color change and was measured spectrophotometrically.

Histology. Lungs were fixed in 10% formol saline and were then routinely processed for histology, prior to staining with hematoxylin and eosin.

Statistical analysis. Results were analyzed by paired comparisons using Student's *t* test.

RESULTS

Leukocytes in bronchoalveolar lavage. Intratracheal instillation of quartz into rat lungs evoked a marked and persistent inflammatory response (Fig. 1); 1 week after dosing with quartz the total number of leukocytes in the bronchoalveolar lavage (26 ± 1.9 [$\bar{x} \pm$ SE] $\times 10^6$/rat) had increased sevenfold compared with controls (3.7 ± 0.4); >90% of these leukocytes were viable. The increase in cell numbers peaked at 4 weeks (47.0 ± 6.3) but was sustained up to 12 weeks after quartz dosing (32.9 ± 3.6). In animals dosed with quartz/aluminum lactate there was a small increase in the number of leukocytes lavaged (8.6 ± 2.0) 1 week after treatment but the difference was not statistically significant and thereafter, the cell numbers returned to control levels. Polymorphonuclear leukocytes formed a large proportion of the accumulating bronchoalveolar leukocytes in the quartz-dosed animals (45–55%; Fig. 2) and were also present in substantial numbers in the quartz/aluminum lactate-dosed animals (10–20%) but there were no neutrophils in the bronchoalveolar lavage cell population of the control animals. Lymphocytes formed <2% of the total leukocytes and were not altered in the treated animals.

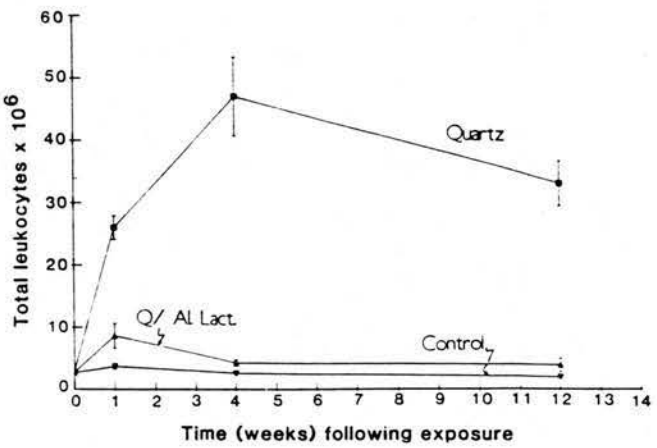


FIG. 1. Effect of pretreating quartz with aluminum lactate on the recruitment of leukocytes to the alveolar region. Q/Al. Lact., quartz pretreated with aluminum lactate. Results are the means (SE) of three rats per group in two separate experiments.

Proteolytic activity of bronchoalveolar leukocytes. Bronchoalveolar leukocytes from quartz-dosed animals had greater ability to degrade fibronectin than control leukocytes (significant at 1 and 4 weeks, $p < 0.001$, $p < 0.005$, respectively) or those obtained from rats dosed with quartz/aluminum lactate (significant at 4 weeks, $p < 0.05$; Fig. 3). The latter were also more active than control leukocytes (significant at 4 weeks, $p < 0.005$). The proteolytic activity of both the quartz-dosed and the quartz/aluminum lactate-dosed animals was maximal 1 week after treatment and declined linearly thereafter. Plotting the proteolytic activity of the individual leukocyte populations as a function of the total number of leukocytes in the lavage fluid revealed a marked difference in the putative

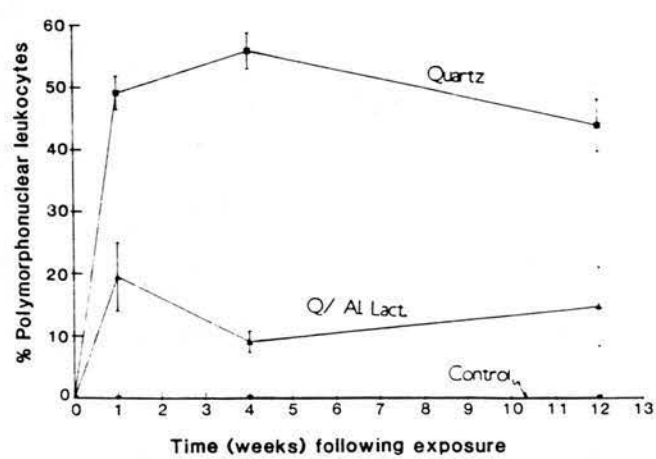


FIG. 2. Percentage of neutrophils in bronchoalveolar lavage leukocyte population from control and quartz-dosed rats or those dosed with quartz pretreated with aluminum lactate. Q/Al. Lact., quartz pretreated with aluminum lactate. Results are the means (SE) of three rats per treatment group in two separate experiments.

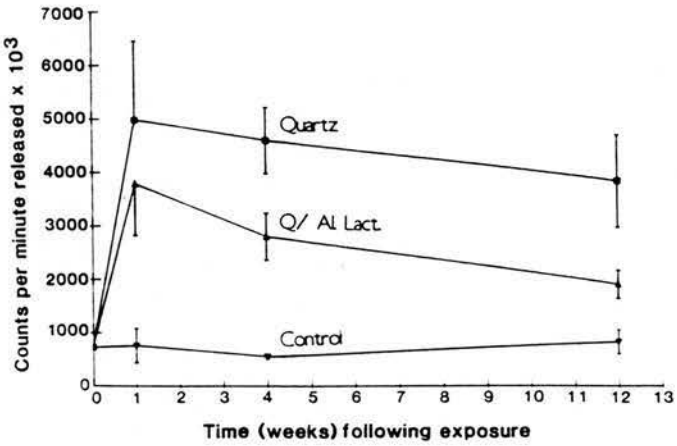


FIG. 3. Proteolytic activity assessed as ability to degrade a ^{125}I-labeled fibronectin matrix. Q/Al. Lact., quartz pretreated with aluminum lactate. Results are the means (SE) of triplicate wells for three rats per treatment group in two separate experiments and are expressed as counts per minute of degraded substrate released.

lung proteolytic burden between treatment groups (Fig. 4).

Oxidant activity. Quartz showed a marked ability to reduce the oxidant burst of bronchoalveolar leukocytes in response to triggering with PMA, the quartz-elicited leukocytes showing consistently less hydrogen peroxide production than controls (significant at 4 and 12 weeks, $p < 0.001$; Fig. 5). The untriggered oxidant burst was also reduced by quartz treatment (results not shown). Hydrogen peroxide production by quartz/aluminum lactate-elicited bronchoalveolar leukocytes was also less than controls at 4 and 12 weeks, but the difference was not statistically significant. Superoxide production mirrored the hydrogen peroxide results (data not included).

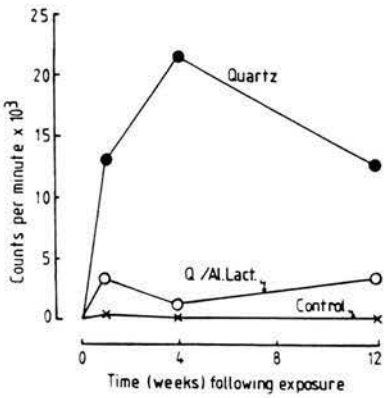


FIG. 4. Proteolytic activity per cell expressed as a function of the total number of leukocytes in the bronchoalveolar lavage to give an indication of the potential overall proteolytic burden in the alveolar region. Results are the means (SE) of three rats per treatment group in two separate experiments.

Delayed treatment with aluminum lactate. Animals dosed with aluminum lactate 1 month after quartz dosing showed no significant reduction in alveolitis by 1 week after aluminum treatment in terms of the total number of leukocytes in bronchoalveolar lavage (Fig. 6) or the percentage of neutrophils in lavaged leukocytes (Fig. 7). However, 1 month after aluminum treatment, total cells in lavage (15.3 ± 2.2) were significantly less than those in lavage of rats treated with quartz alone (28.0 ± 7.8, $p < 0.0005$; Fig. 6); the total number of leukocytes in both treatment groups was significantly greater than that in controls (2.2 ± 0.4, $p < 0.0005$).

Although the cellular response to quartz was suppressed 1 month after aluminum treatment, the functional status of the inflammatory leukocytes remained unchanged

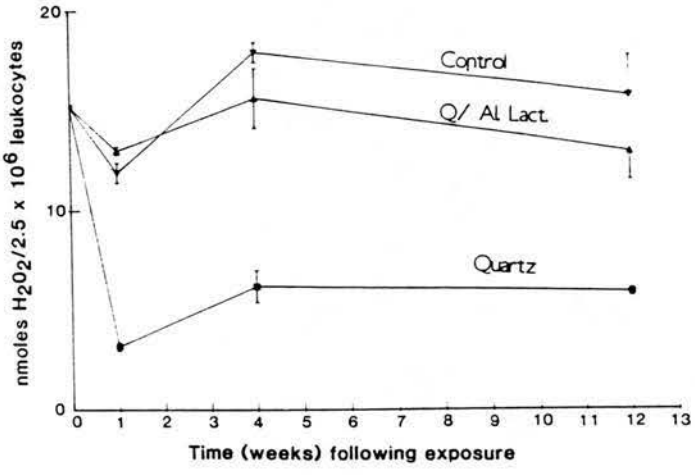


FIG. 5. The effect of quartz or quartz pretreated with aluminum lactate on the oxidant activity of bronchoalveolar lavage leukocytes. Results are the means (SE) of three rats per treatment group in two separate experiments and are expressed as nanomoles H_2O_2 per 2.5×10 leukocytes.

either 1 week or 1 month after aluminum treatment. Both fibronectin degradation and hydrogen peroxide production by bronchoalveolar leukocytes from rats dosed with quartz followed by aluminum lactate were similar to the activity of leukocytes obtained from rats dosed only with quartz (Table 1).

Effect of aluminum lactate treatment on the inflammatory response to C. parvum. Lungs of rats dosed concomitantly with *C. parvum* and aluminum lactate were equally effective in mounting an inflammatory response as those dosed with *C. parvum* alone. Aluminum lactate had no effect on total leukocytes, percentage of neutrophils in the bronchoalveolar lavage, or on the proteolytic activity of the bronchoalveolar leukocytes (Table 2).

Histological examination. One week after quartz or quartz/aluminum lactate treatment there was little evidence of tissue damage but occasional macrophage accumulations were

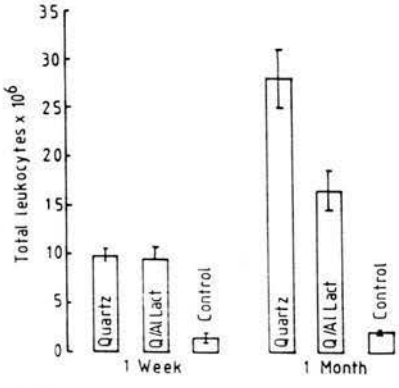


FIG. 6. Effect of late treatment with aluminum lactate on total number of leukocytes in the bronchoalveolar lavage: rats were exposed to quartz for 1 month and then aluminum lactate for a further 1 week or 1 month. Results are the means (SE) of three rats per treatment group.

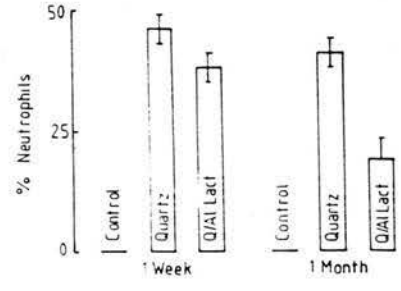


FIG. 7. Effect of late treatment with aluminum lactate on percentage of neutrophils in bronchoalveolar lavage leukocytes from rats exposed to quartz for 1 month and then aluminum lactate for a further week or month. Results are the means (SE) of three rats per treatment group.

TABLE 1

EFFECT OF DELAYED TREATMENT WITH ALUMINUM LACTATE ON THE PROTEOLYTIC ACTIVITY AND HYDROGEN PEROXIDE PRODUCTION BY QUARTZ-ELICITED BRONCHOALVEOLAR LEUKOCYTES[a]

	Proteolytic activity[b]		H_2O_2[c]	
	1 Week	1 Month	1 Week	1 Month
Control[d]	431 (34)	531 (169)	14.9 (4.6)	18.8 (3.9)
Quartz alone[e]	2072 (59)	2510 (591)	7.6 (2.0)	6.2 (0.5)
Quartz/aluminum lactate[f]	2595 (76)	2316 (613)	10.5 (2.6)	8.9 (2.0)

[a] Results represent the means (SE) for triplicate samples and two experiments at each time point.
[b] Proteolytic activity is expressed as counts per minute of degraded substrate released into the culture medium.
[c] Hydrogen peroxide is expressed as nanomoles released per 2.5×10^5 leukocytes.
[d] Controls, leukocytes lavaged from untreated rats.
[e] Quartz alone, leukocytes from rats lavaged after a single intratracheal dose of quartz.
[f] Quartz/aluminum lactate, 1 month after a single intratracheal dose of 1 mg quartz, a single 50-μg dose of aluminum lactate was administered. Leukocytes were retrieved after a further 1 week or 1 month.

present at bifurcations of alveolar ducts. By 1 month after quartz treatment, areas of alveolar lipoproteinosis were seen in conjunction with septal hyperplasia; these areas were minimal in animals treated with quartz/aluminum lactate. After 3 months, the tissue injury had progressed in both treatment groups with the greatest damage apparent in the quartz-treated animals (Fig. 8). Rats treated with quartz/aluminum lactate showed marked areas of tissue injury but these were less severe than in animals dosed with quartz alone and comprised only about 5% of the entire tissue, compared with around 80% in the quartz-treated animals. Late dosing with aluminum had no effect on the tissue response to quartz.

DISCUSSION

Although the action of aluminum in ameliorating quartz-induced lung injury has been

TABLE 2

EFFECT OF ALUMINUM LACTATE ON THE INFLAMMATORY RESPONSE ELICITED BY INTRATRACHEALLY INSTILLED *C. parvum*

	Days following intratracheal injection[a]					
	1		3		15	
	Cp	Cp/Al[b]	Cp	Cp/Al	Cp	Cp/Al
Total cells ($\times 10^6$)	21.9 (2.6)	21.2 (2.1)	13.4 (3.0)	22.6 (0.6)	11.1 (1.8)	13.0 (2.9)
% Neutrophils	76 (3.5)	82 (0.3)	34 (4.1)	37 (7.6)	0.3 (0.3)	0.3 (0.3)
Proteolysis[c]	5.0	4.8	3.5	3.2	1.3	1.3

[a] Results represent the means (SE) for triplicate samples at each time point, except where the mean proteolytic activity is expressed as a ratio of background counts.
[b] Cp, *C. parvum;* Cp/Al, *C. parvum* administered concomitantly with aluminum lactate.
[c] Fibronectin proteolysis (released counts as a ratio of medium control).


b
a

well documented in animals and man, there has been no systematic study of the mechanisms whereby this effect might occur. Accordingly, we have assessed the role of aluminum in modulating quartz-induced alveolitis and we have measured two key aspects of bronchoalveolar leukocyte function that are important in the lung injury associated with development of fibrosis; these are proteolysis of extracellular matrix and oxidant production. We reported previously that bronchoalveolar leukocytes from rats dosed with silica have enhanced ability to damage lung connective tissue components (Brown and Donaldson, 1988). We now demonstrate that pretreating the quartz with aluminum lactate reduces its inflammogenic properties and decreases the proteolytic activity of the inflammatory bronchoalveolar leukocytes, on a "per-cell" basis, by about half; the protective effect was sustained throughout the 12-week period of study. The increased proteolytic activity per leukocyte is related to the percentage of neutrophils in the population but preliminary results from this laboratory suggest that inflammatory alveolar macrophages, while less active than exudate neutrophils, do have enhanced proteolytic activity compared with control alveolar macrophages and so both cell types may contribute to the increase (Brown *et al.*, 1989). To assess the potential for proteolytic injury in the lung, we calculated the putative overall leukocyte protease burden by multiplying the proteolytic activity per leukocyte by the number of cells in the bronchoalveolar lavage. This revealed that the potential protease burden in lungs of rats dosed with native quartz was at least sixfold greater than in those animals dosed with quartz/aluminum lactate.

In contrast to the proteolysis experiments where inflammatory bronchoalveolar leukocytes had enhanced activity, intratracheal dosing with quartz caused a marked reduction in oxidant production, a result which is consistent with previous reports of impaired leukocyte function (Dauber *et al.*, 1982), and reduced respiratory burst in quartz-dosed animals, (Donaldson *et al.*, 1988), and may explain the impaired clearance of bacteria in mice treated with silica (Goldstein *et al.*, 1969). The reduction in hydrogen peroxide production was less for leukocytes elicited by aluminum lactate. These results indicate that aluminum acts to modulate quartz-induced leukocyte function and is able to enhance or suppress leukocyte function.

To further investigate the effect of aluminum in altering quartz reactivity, established quartz-induced alveolitis was subsequently treated *in vivo* with aluminum lactate. The progression of the alveolitis was markedly attenuated by such treatment, thus, the late aluminum treatment reported by Begin *et al.* (1986) to be effective in reducing alveolitis in quartz-dosed sheep is also effective in quartz-treated rats. Since aluminum lactate treatment failed to abrogate the inflammatory response to *C. parvum*, we conclude that the effect of the aluminum was by some interaction with the quartz particles and not with the inflammatory leukocytes.

The tissue response seen at pathology agrees with the progressive nature of the bronchoalveolar lavage results in response to quartz challenge, increasing tissue injury culminating in marked alveolar lipoproteinosis and hyperplasia of the alveolar septa at 3 months. As with the alveolitis, the tissue response to quartz pretreated with aluminum lactate was also progressive but was markedly attenuated compared with quartz alone. These results are in agreement with Begin *et al.* (1987a), who also demonstrated attenu-

FIG. 8. Tissue response 3 months after intratracheal inoculation of (a) 1 mg quartz or (b) 1 mg quartz pretreated with aluminum lactate (×400 magnification). Areas of hyperplasia (arrows) were more extensive in lungs treated with quartz alone than in those treated with quartz/aluminum lactate. Lipoproteinosis (indicated by X (b)) was also seen extensively in quartz-treated lungs but is not apparent in (a).

ated cellular and tissue responses to aluminum-coated quartz in sheep. In the present study, dosing with aluminum after the quartz response was established produced divergent responses—the alveolitis was markedly reduced but there was no effect on the extent of tissue injury; Begin *et al.* (1987b) reported similar results in exposed sheep.

Aluminum administered to the lungs at some time after the quartz treatment may gain access to the quartz particles when they are released from dead cells or may be endocytosed to react intracellularly. Our results show that the aluminum reduces the progression of alveolitis associated with silica exposure but does not modulate the function of the inflammatory leukocytes in the two parameters we have measured, proteolysis of fibronectin and oxidant production. Thus, the reaction between quartz and aluminum in the lung, whether it occurs intra- or extracellularly, may be incomplete. Alternatively, if the amount of quartz per cell is important in causing toxicity and ultimately, pathogenicity, then only those particles which had been in sufficient numbers within a leukocyte to kill it would be released to react with aluminum in the extracellular environment, subsequently losing their toxic potential on reaction with the aluminum. Those cells containing fewer active quartz particles might then remain functionally activated within the lung, causing a lower level of alveolitis than previously, but still secreting toxic products which would damage the lung tissue. Toxicity of quartz to alveolar leukocytes is thus unlikely to be the sole mechanism of quartz-induced alveolitis. Our results suggest that the harmful effects of silica are exerted not only by direct toxicity to alveolar leukocytes but also by persistence of active quartz particles sequestered within leukocytes causing release of phlogistic agents into the alveolar region and thus leading to the sustained alveolitis which we observed to be much greater with native quartz compared with aluminum-coated quartz.

This study demonstrates that the biological activity of quartz is diminished in the presence of aluminum. In mixed dusts, the pathogenic effect of quartz may be similarly modulated by prior interaction of the quartz with other minerals in the dust such as iron or aluminum silicate clays. The interaction between quartz and aluminum can occur subsequent to a substantial quartz-induced alveolitis but still significantly reduces the activity of the quartz. Thus, in situations such as coal mining, where there can be significant temporal variation in the mineralogical content of mixed dust, the pathogenic effect due to inhalation of a high quartz content dust might be attenuated by subsequent inhalation of a dust containing ameliorating minerals.

These results may have a bearing on assessing the potential harmfulness of any mixed dust but further research is required to understand the nature of the interactions between quartz and other mineral components present in mixed dusts. This includes interaction at the cellular level between components of mixed dusts and quartz, further studies on the role of these components in modifying quartz-mediated lung inflammation, and physical studies of the quartz surface before and after interaction with ameliorating components of mixed dust.

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Inflammatory responses in lungs of rats inhaling coalmine dust: enhanced proteolysis of fibronectin by bronchoalveolar leukocytes

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ABSTRACT Chronic exposure to coalmine dust is associated with the accumulation of inflammatory leukocytes in the bronchoalveolar region of the lung and, in the long term, with fibrosis and emphysema of the lung parenchyma. Degradation of connective tissue by inflammatory leukocytes has been implicated in the parenchymal damage that precedes the development of fibrotic or emphysematous lesions in the lung. The ability of inflammatory leukocytes obtained by bronchoalveolar lavage from rats inhaling coalmine dust to degrade fibronectin in vitro was assessed. The animals were exposed to an airborne mass concentration of dust similar to the maximum permissible level in United Kingdom collieries. The bronchoalveolar lavage cell population showed changes with duration of dust exposure; there were increases in the total number of leukocytes and in the percentage of polymorphonuclear leukocytes, and the macrophage component of the lavage became increasingly activated, as assessed by the ability of these cells to spread on glass. In addition, degradation of a radiolabelled fibronectin matrix by the coalmine dust exposed bronchoalveolar leukocytes increased with duration of dust exposure. Thus exposure to airborne coalmine dust causes an influx of inflammatory leukocytes to the alveolar region. These cells have enhanced ability to degrade fibronectin and this may be important in subsequent disease development.

Coalworkers' pneumoconiosis is a disease directly attributable to the inhalation of coalmine dust and is characterised by the development in the lungs of fibrotic lesions and emphysema.[1] Patients with coalworkers' pneumoconiosis have increased numbers of inflammatory leukocytes in the bronchoalveolar region[2] and this alveolitis, as in other lung diseases,[3] is likely to be central to the remodelling of lung tissue which leads to the lesions described above. The accumulation of inflammatory leukocytes in the bronchoalveolar region is also one of the earliest manifestations of disease in animals exposed experimentally to coalmine dust,[4] asbestos,[5 6] and silica.[6 7] During inflammation, inflammatory macrophages and neutrophils secrete an array of molecules[8]; included among these are the neutral proteases, active at the normal pH of lung tissue, which are secreted in increased amounts by inflammatory leukocytes[9] and which may damage connective tissue in vivo[10] and in vitro.[11] Degradation of connective tissue may be central to the pathogenesis of chronic inflammatory lung disease by enhancing and prolonging the inflammatory and repair responses[12–15] at sites of inflammation in the lung parenchyma. This type of connective tissue damage may occur where neutral protease activity is sufficient to overload the antiprotease screen.[16] This is strongly suggested by the presence of active neutral protease in bronchoalveolar lavage of patients with chronic inflammatory lung disease,[17 18] including coalworkers' penumoconiosis[19] and, experimentally, in the bronchoalveolar lavage of animals exposed to silica,[20 21] a powerful inflammation generating agent.

In the present study we have investigated the development of pulmonary inflammation in rats exposed to airborne coalmine dust for up to 52 days; rats exposed for 32 days and then allowed to breathe room air for a further 64 days were also studied. Animals were exposed to 10 mg/m^3 airborne coalmine dust; this is of the same order of magnitude as the maximum permissible level in British collieries which is 7 mg/m^3, measured in the return roadway and about four to six times the level of dust to which miners might

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expect to be exposed on a daily basis for their working lives. The leukocyte population of the bronchoalveolar space was obtained by bronchoalveolar lavage and the following parameters assessed: total number, differential count, the ability of the population to degrade fibronectin in vitro and the ability of the macrophages to spread on glass as a measure of macrophage activation. Comparisons were made with control rats maintained in room air throughout.

Materials and methods

REAGENTS

Alpha-1-protease inhibitor (α-1-PI) from human plasma, alpha-2-macroglobulin (α-2M) from human plasma, bovine serum albumin (BSA), human plasma fibronectin, phorbol myristate acetate (PMA), and zymosan were obtained from Sigma Chemical Co Ltd, Poole, Dorset. Ham's F10 medium, fetal calf serum (FCS), and Dulbecco's phosphate buffered saline (PBS) were purchased from Gibco BRL, Paisley, Renfrewshire. ^{125}-Iodine (^{125}I) was obtained from Amersham International, Aylesbury, Buckinghamshire. Microtitre plates were purchased from Sterilin UK Limited, Feltham, Middlesex, and Nembutal from Ceva Limited, Watford. Sodium EDTA was obtained from BDH Limited, Glasgow.

COALMINE DUST

Dust was collected from the air of a British colliery using dry fabric filters. The dust was irradiated to kill microbial spores (100 KRad) before being generated as a cloud using a Timbrell dust generator. The dust was then passed through a cyclone to produce a respirable fraction that was dispersed as a cloud in a 1 m^3 exposure chamber[22] at an airborne mass concentration of 10 mg/m^3. Airborne samples collected on to filters showed that the mean target concentration of 10 mg/m^3 was achieved throughout the exposure. The mineralogical composition of the dust, as assessed by *x* ray diffraction and infra red analysis, was total ash 53·2, quartz 6·7, kaolin 18·1, and mica 0. The dust was low rank with a mass median aerodynamic diameter of 2–3 μm, measured by an eight stage cascade impactor.

INHALATION EXPOSURE OF RATS

Male, syngeneic, PVG rats were obtained from the laboratory animal breeding unit, Institute of Occupational Medicine. Rats were placed in the inhalation chamber and exposed to dust for seven hours a day, five days a week. On days 8, 16, or 32, groups of four rats were removed from the chamber, killed, and the bronchoalveolar leukocyte population obtained by lavage. At each time point, two control rats of identical age, maintained in room air, were also lavaged. One group of four rats was removed from the chamber on day 32 and maintained in room air for a further 64 days before being lavaged; these will be referred to as "recovery" rats.

BRONCHOALVEOLAR LAVAGE

Animals were killed by overdose with intraperitoneal pentobarbitone sodium (Nembutal); the thoracic cavity was dissected open to expose the lungs and the trachea cannulated with a blunt 16 g needle. The lungs were then resected and lavaged with four sequential washes of 8 ml saline at 37°C, with gentle massage of the lungs on the second wash to increase the yield of cells; the four washes were pooled and placed immediately on ice.

CELL COUNTS

The pooled bronchoalveolar lavage fluid was centrifuged and the cell pellet resuspended in phosphate buffered saline. The total cell number was measured using a Neubauer chamber and differential cell counts were performed using May Grunwald Giemsa stained cytocentrifuge preparations.

SPREADING ASSAY

Cells were resuspended to a concentration of 1 × 10^6/ml in Ham's F10 medium containing 10% heat inactivated fetal calf serum; 100 μl aliquots were placed on 6 × 22 mm glass coverslips and incubated at 37°C for one hour. The coverslip preparations were then washed, fixed, and stained with May Grunwald Giemsa. The maximum diameter of 200 macrophages (any adherent neutrophils were excluded from sizing) was measured using a graphic instruments image analyser, interfaced to an optical microscope.[23]

NEUTRAL PROTEASE ASSAY

Neutral protease activity was assessed as previously described by measuring the degradation of radioiodine labelled fibronectin adhered as a solid phase matrix[20]; intact cells were cultured on the matrix for four hours at 37°C; solubilised ^{125}I correlated with increased enzyme concentration. Cells from animals exposed for eight days to coalmine dust were similarly tested for neutral protease activity in the presence of the protease inhibitors, α-1-PI at concentrations of 0·01, 0·1, and 1·0 mg/ml or α-2M at concentrations of 0·005, 0·05, or 0·5 mg/ml and also in the presence of fetal calf serum (1%, 5%, or 10%) or ethylene diamine tetra-acetic acid (EDTA) (0·01, 0·1, and 1·0 mg/ml). Triggering experiments with eight day dust elicited cells were carried out in the presence of PMA (0·1, 1·0, or 10 μg/ml) or zymosan (1, 10, or 100 μg/ml).

STATISTICAL ANALYSIS

Results were analysed by paired comparisons using Student's *t* test.

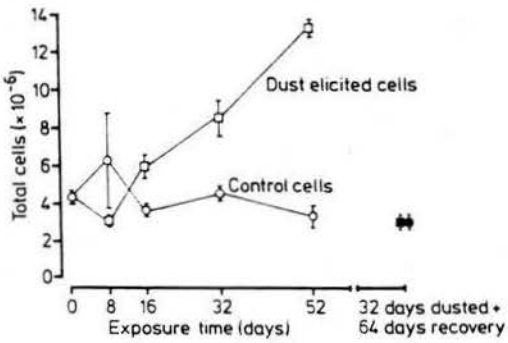


Fig 1 *Total leukocytes in bronchoalveolar lavage fluid with duration of exposure to dust. Dust elicited leukocytes were significantly more active than controls after 52 days exposure to dust ($p < 0.001$). Results are mean ± SE of four dust exposed or two control rats at each time point.*

Results

TOTAL CELLS IN BRONCHOALVEOLAR LAVAGE

The total number of cells retrieved by bronchoalveolar lavage increased with duration of exposure to dust (fig 1); after 52 days of exposure to dust the total cell number in bronchoalveolar lavage (13·2 ± 0·9) (mean ± SE; × 10^6) was significantly greater than bronchoalveolar lavage cells from control animals (4·3 ± 0·8) ($p < 0.001$). Animals exposed to coalmine dust for 32 days followed by recovery in room air for a further 64 days had fewer cells in the bronchoalveolar lavage (3·2 ± 0·2) than animals killed immediately after 32 days exposure to dust (8·4 ± 1·9) ($p < 0.05$), but the difference was not statistically significant.

PERCENTAGE OF NEUTROPHILS IN BRONCHOALVEOLAR LAVAGE

No neutrophils were observed in bronchoalveolar lavage of control animals but were present after eight and 16 days of exposure to dust (2·8 and 3·5% respectively, fig 2). The percentage of neutrophils was greater at the later time points; 32 days: 18%, 52 days; 15·8%. The percentage of neutrophils remained raised (17·8%) in recovery rats exposed to dust for 32 days and maintained in room air for a further 64 days.

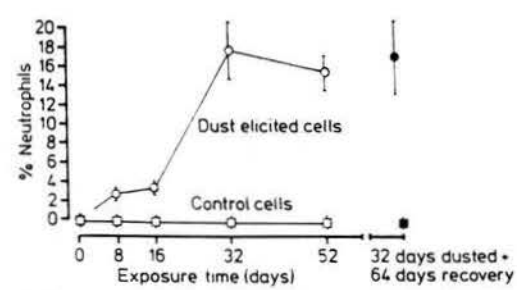


Fig 2 *Percentage of neutrophils in broncholaveolar lavage with duration of exposure to dust. Results are mean ± SE of four dust exposed or two control rats at each time point.*

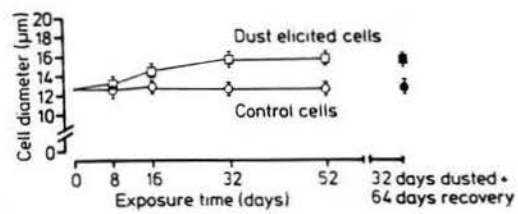


Fig 3 *Macrophage spreading with duration of exposure to dust. Spreading was significantly greater than controls at all but eight day time point ($p < 0.001$). Results are expressed as cell diameter in μ and are mean ± SE of 200 macrophages counted for ech of four dust exposed or two control rats at each time point.*

MACROPHAGE ACTIVATION

The diameter of alveolar macrophages obtained from coalmine dust exposed animals allowed to spread on glass for one hour increased in a time dependent manner from 13·1 ± 0·7 μm on day 8 to 15·6 ± 0·2 μm on day 52 (fig 3). Spreading was significantly greater than the controls (12·7 ± 0·2 μm) at all but the eight day time point ($p < 0.001$) and remained raised when the 32 day dust exposed animals were allowed to recover for 64 days in room air (15·4 ± 0·4 μm).

NEUTRAL PROTEASE ACTIVITY

The neutral protease activity of coalmine dust elicited leukocytes was significantly greater than that of control cells at all time points ($p < 0.001$) and remained raised on recovery (fig 4). By comparison, there was little increase in the percentage of neutro-

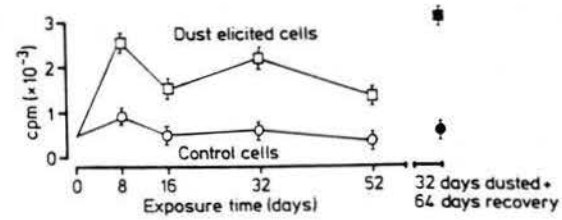


Fig 4 *Neutral protease activity of bronchoalveolar leukocytes with duration of exposure to dust. Neutral protease activity of dust elicited cells was significantly greater than the controls at all time points ($p < 0.001$). Results are expressed as counts per minute (cpm) degraded substrate/ 1×10^5 cells and are mean ± SE of three wells for each of four dust exposed or two control rats at each time point.*

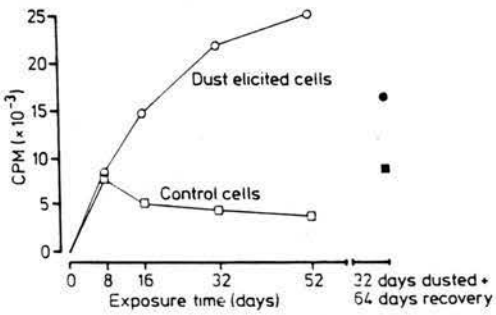


Fig 5 *Neutral protease activity as a function of total number of leukocytes in bronchoalveolar lavage. Total neutral protease activity pertaining to whole population of leukocytes from dust exposed animals increased with duration of exposure to dust and was significantly greater than controls at all time points ($p < 0.001$). Results are expressed as mean neutral protease activity, multiplied by total cell number at each time pont. Closed symbols represent recovery animals.*

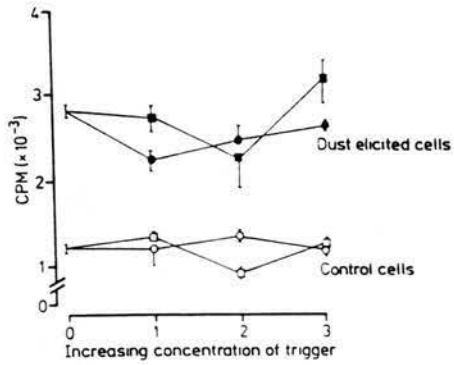


Fig 7 *Triggering of neutral protease activity of eight day dust elicited leukocytes and controls. Zymosan at 1, 10, and 100 μg/ml, phorbol myristate acetate at 0·1, 1·0, and 10 μg/ml. There was no significant enhancement of neutral protease activity by either trigger.*

phils in the lavage population until day 32. We therefore assumed that both macrophages and neutrophils were contributing to the neutral protease burden and accordingly calculated neutral protease activity as a function of the total number of cells present in lavage (fig 5). The putative total neutral protease activity of the whole population of bronchoalveolar lavage cells increased with duration of exposure to dust and was sixfold greater than that of control cells at day 52.

The neutral protease activity of BAL cells from eight day coalmine dust exposed animals (fig 6) was inhibitable in a dose dependent manner by α-1-PI (significantly reduced at 0·01, 0·1, and 1·0 mg/ml $p < 0.005$), α-2M (significant at 0·5 mg/ml, $p < 0.005$), serum (significant at 1%, 5%, and 10%, $p < 0.005$) and by EDTA (significant at 1·0 mg/ml $p < 0.001$). There was no increase in the neutral protease activity of control or bronchoalveolar leukocytes from eight day dust exposed animals on triggering in vitro with PMA or zymosan (fig 7).

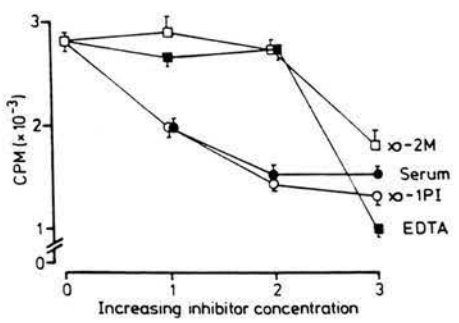


Fig 6 *Inhibition of neutral protease activity of eight day dust elicited leukocytes by α1-PI and EDTA at 0·01, 0·1 and 1 mg/ml, α2-M at 0·005, 0·05 and 0·5 mg/ml, serum at 1%, 5%, and 10%. Results are expressed as counts per minute (cpm) of degraded substrate/1 × 10^5 cells and are mean ± SE of three wells for each inhibitor concentration. α1-PI and serum significant reduction at all inhibitor concentrations, $p < 0.001$; α2-M-significant reduction at 0·5 mg/ml, $p < 0.005$; EDTA significant reduction at 1·0 mg/ml, $p < 0.005$.*

Discussion

The aim of this study was to evaluate the pulmonary inflammatory response to inhaled coalmine dust with particular regard to the proteolysis of fibronectin by inflammatory leukocytes from the bronchoalveolar space. We have shown that chronic inhalation exposure to low levels of coalmine dust leads to the gradual accumulation of inflammatory cells—neutrophils and activated macrophages—in the alveolar region. These results are contrary to those of Bingham *et al*, who reported no increase in the total number of cells in the bronchoalveolar lavage of rates exposed by inhalation to similar levels of coalmine dust.[24] In that study, however, the dusts were obtained by crushing seam coal. This procedure produces a dust that may differ fundamentally from that found in the air of coalmines since it (*a*) is not produced by coal cutting equipment but is ground and (*b*) does not include material from the roof and floor that is cut concomitantly with the coal seam. The latter factor may be particularly important, since this is likely to contribute substantially to the non-coal minerals including quartz, which are considered to be important factors in determining the toxicity of any coalmine dust sample.[25,26] In addition, the samples used by

Bingham *et al* were particularly fine dusts compared with the dust used in the present study and would be less likely to deposit in the lower airways.[24] Studies from this laboratory have shown that the inflammatory response to the inhalation of coalmine dust is dose dependent[27] and the finding that alveolar inflammation did result in the study by Bingham *et al* when the dusts were administered by intratracheal instillation,[26] gives added weight to the suggestion that the lack of response to inhaled dust was due to insufficient dust depositing in the alveolar region.

The increased number of leukocytes in the bronchoalveolar lavage of coalmine dust exposed rats in the present study is consistent with the increases observed in lavage of patients with the toxic dust related lung diseases coalworkers' pneumoconiosis, silicosis, and asbestosis.[2 28] Similar increases in total leukocyte numbers in bronchoalveolar lavage fluid have also been shown in experimental studies with silica, where exposure was by intratracheal instillation, in rabbits,[29] rats,[20 30] mice,[31] and guinea pigs,[32] or by inhalation in rats.[6 7]

Inflammatory leukocytes elicited in rat lungs[20] and in the mouse peritoneal cavity[33] have increased proteolytic activity in vitro and bronchoalveolar lavage of cigarette smokers[34] and patients with coalworkers pneumoconiosis[35] yields cells with enhanced secretion of elastase-like activity. In the present study bronchoalveolar lavage leukocytes from animals exposed by inhalation to coalmine dust had greater proteolytic activity than control cells; we confirmed that the measured substrate degradation was due to proteolysis, as reported previously,[20] by inhibition with serum, α1-PI, EDTA, and α2-M. This study has focused on proteases as these are considered to be key inflammatory cell products in causing injury and remodelling of the lung, both in emphysematous[16] and fibrotic[17 18] lung disease.

The proteolysis assay used in this study has shown that coalmine dust elicited bronchoalveolar leukocytes can damage at least one component of the extracellular matrix, fibronectin; previous studies from this laboratory have shown that inflammatory leukocytes can also degrade other connective tissue components such as collagen and laminin.[11]

We have thus shown that proteolytic activity in coalmine dust inflamed lung can be increased in two ways: (1) there are increased numbers of inflammatory leukocytes in the alveolar region and (2) these cells are up to fivefold more active in degrading fibronectin than the resident alveolar macrophages. The connective tissue damage associated with such increased proteolytic activity may be central to the pathogenesis of the disease process by enhancing inflammation[15 36] and by altering cell to cell and cell to matrix interactions which are important in maintaining normal tissue architecture.[13 14]

The finding that the inflammatory response was sustained during 64 days of recovery after 32 days of exposure to airborne dust may have relevance to the progression of progressive massive fibrosis seen in some coalmine workers even when removed from further occupational exposure to dust[1] and lends support to one report that pulmonary inflammation persists in retired coalminers as measured by clearance of Tc99m DTPA.[37] The persistence of an alveolitis, with neutrophils and activated macrophages, long after cessation of exposure to the coalmine dust is strong circumstantial evidence for the importance of leukocytes in the development of the lesions of coalworkers' pneumoconiosis; such a role for leukocytes in fibrosis due to dusts and other aetologic agents has frequently been suggested.[8 38 39]

Unstimulated control rat macrophages had low levels of fibronectin degrading activity compared with the dust exposed cells. It has been reported previously that triggering with PMA over a similar period to that used in the present study caused enhanced levels of neutral protease release.[40] Phagocytosis of latex beads[41] and colchicine treatment[42] were also shown to enhance neutral protease secretion by mouse peritoneal macrophages, although this was measured over several days. We assessed the roles of phagocytosis and leukocyte activation in inducing enhanced proteolysis of fibronectin by measuring the proteolytic activity of eight day dust elicited cells (97% macrophages) in the presence of a particulate (zymosan) or non-particulate (PMA) trigger. The results were contradictory to those reported previously,[39–41] with no evidence of stimulation; this may be due to differences in the cell types, duration of the experiment, or the proteolysis assays used. In the present study the failure of either trigger to stimulate increased proteolytic activity by the control leukocytes in vitro may reflect a requirement for activation in vivo, by the inflammatory milieu, to prime the leukocytes for increased proteolytic activity on contact with triggers in vitro. Since the proteolytic activity of the coalmine dust elicited leukocytes remained constant throughout the exposure period, it appears that they were maximally triggered in vivo after eight days of exposure to dust and therefore triggering in vitro would not enhance this response.

In conclusion, this study has shown that exposure to 10 mg/m^3 airborne concentration of respirable coalmine dust collected from a British colliery caused inflammation in the lungs of rats. The inflammation was characterised by the presence of an increasing proportion of neutrophils in the bronchoalveolar lavage and the presence of large numbers of activated alveolar macrophages. Proteolysis of fibronectin by the inflammatory leukocytes was increased compared with control cell proteolysis throughout the dusting period and persisted for 64 days of recovery during

which time the rats breathed room air. Alveolitis, as defined by the presence of neutrophils and activated macrophages in bronchoalveolar lavage and an increased ability to degrade fibronectin, also persisted in the recovery animals, although the total number of leukocytes in the bronchoalveolar region was no greater than in control animals. In a previous study in this laboratory rats were exposed to coalmine dust for one year followed by a four month recovery period.[43] In that study both the total number of leukocytes and the percentage of polymorphonuclear leukocytes in the lavage cell population remained raised. The continued presence of inflammatory leukocytes with enhanced ability to degrade extracellular matrix components such as fibronectin could be one important factor in long term pathological change associated with exposure to coalmine dust.

We thank Dr J M G Davis and Dr R E Bolton who were in the inception and development of this project and Mr A D Jones and his staff who were responsible for generating and monitoring the dust exposure system. We also thank Mrs J Slight, Dr M D Robertson, and Mr D M Brown who were concerned in the lavage procedure and in enumerating the bronchoalveolar leukocyte populations. We acknowledge the continuing interest in this project shown by Professor D M Weir.

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Correspondence and editorials

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Thorax 1990;45:281–282

Production of interleukin-1 like activity by neutrophils derived from rat lung

Yukinori Kusaka, Kenneth Donaldson

Abstract

Interleukin-1 like activity was produced by neutrophils obtained by bronchoalveolar lavage from experimentally inflamed rat lung. Activity was released spontaneously from neutrophils at high levels but it was enhanced by stimulation with endotoxin in vitro.

The role of the alveolar macrophage in immune and inflammatory responses in the lung has been well documented.[1] During inflammatory responses in the lung, however, other leucocyte types are recruited to the alveolar region, including polymorphonuclear neutrophils.[2] These have been implicated in the pathological changes that follow inflammation in the lung in the adult respiratory distress syndrome, idiopathic pulmonary fibrosis, and lung disease related to mineral dusts. Some inflammatory lung diseases, including idiopathic pulmonary fibrosis and extrinsic allergic alveolitis, have an important immunological component, whereas diseases such as pneumoconiosis have a less obvious immunological element. In all these diseases, however, neutrophils are found in increased numbers in bronchoalveolar lavage fluid. Hitherto the neutrophil has not been considered to have a major influence on the immune responses in the lung, but we report neutrophil derived interleukin-1 like activity in cells obtained from inflamed rat lungs.

Methods

RATS

We used 12–15 week old female specific pathogen free, inbred PVG rats from the Institute of Occupational Medicine's own breeding unit.

INDUCTION OF INFLAMMATION IN THE LUNG

Pulmonary inflammation was induced by transtracheal instillation of 1 mg of quartz (DQ_{12} standard) or 1·4 mg of a heat killed preparation of *Corynebacterium parvum* (Wellcome, Beckenham). One to seven days later the lungs were lavaged and bronchoalveolar cells obtained.[3] The lungs of control rats were lavaged to obtain normal bronchoalveolar cells (>95% alveolar macrophages).

SEPARATION OF CELLS

Whole inflammatory bronchoalveolar cell preparations were separated into neutrophil rich populations by centrifugation through Sepracell medium (Sepracell, Oklahoma).

MEASUREMENT OF INTERLEUKIN-1

Interleukin-1 like activity was determined in dilutions of supernatant from overnight cultures of the whole or separated cell populations in the presence or absence of lipopolysaccharide (*Escherichia coli*, serotype 0111: B4, 100 ng/ml; Sigma, Poole). Cells were cultured at 37°C in RPMI 1640 with 10% fetal calf serum (Gibco, Paisley). Dilutions of supernatant were incubated with C3H mouse thymocytes at 6×10^5 cells/well in microtitre plates. Phytohaemagglutinin was added to a final concentration of 4 μg/ml, a concentration previously determined to be suboptimal, and the plates were cultured for 72 hours. Thymocyte proliferation was determined by the incorporation of tritiated thymidine added during the final 16 hours of culture. Controls included wells without supernatant and wells with a supernatant collected from C57B16 mouse peritoneal macrophages cultured with 10 μg/ml lipopolysaccharide recombinant interleukin-1α.

STATISTICAL ANALYSIS

Differences between treatment groups were analysed by Student's *t* test.

Results

INTERLEUKIN-1 LIKE ACTIVITY

In all experiments the background level of thymocyte proliferation produced counts of 500–1000 cpm and the two positive controls counts ranging from 3500 to 12 500 cpm. The neutrophil rich populations obtained from rats treated with *C parvum* and from rats instilled with quartz produced substantial quantities of interleukin-1 like activity; in both cases the proportion of neutrophil approached 80% (fig 1). Interleukin-1 like activity was also produced by alveolar macrophages. Although substantial, this could not account for the increased interleukin-1 like activity produced by the neutrophil enriched populations (fig 1). In one experiment the neutrophils from rats treated with *C parvum* were enriched in Sepracell separation medium, which increased their proportion from 76% to 100%. This caused the mean interleukin-1 like activity to increase from 2094 (SD 107) to 2604 (198) cpm.

Incubation of control macrophages or an 83% pure neutrophil population with lipopolysaccharide (100 ng/ml) produced a substantial stimulation of interleukin-1 like activity (fig 2).

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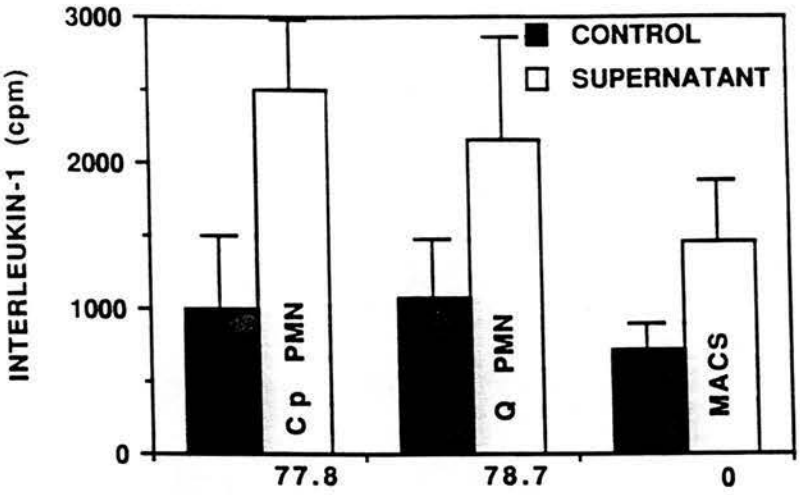


Figure 1 Presence of interleukin-1 like activity in culture supernatants of polymorphonuclear neutrophils obtained from Corynebacterium parvum stimulated lung (Cp PMN) and quartz stimulated lung (Q PMN); supernatants from alveolar macrophages (MACS) are included for comparison. Significant ($p < 0.001$) increases occurred with leucocyte populations compared with thymocyte controls (black columns) for each sequence of experiments. Data are derived from triplicate wells in four (Cp PMN), six (Q PMN) or three (MACS) separate experiments. Numbers underneath the open bars indicate the percentage of neutrophils in the culture.

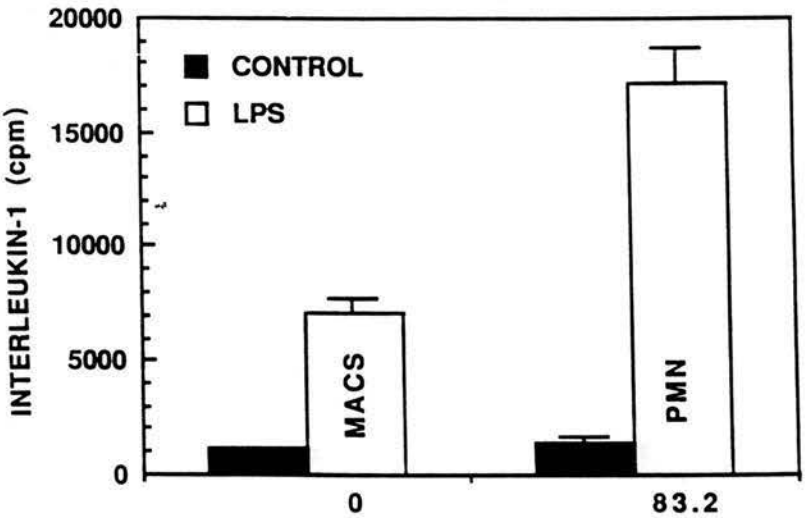


Figure 2 Presence of interleukin-1 like activity in supernatants obtained from alveolar macrophages (MACS) and Corynebacterium parvum (PMN), cells being unstimulated or stimulated by incubation with lipopolysaccharide (LPS) (100 ng/ml) for 24 hours. There were significant increases ($p < 0.001$) from treatment with lipopolysaccharide. Means and SEM of results from triplicate wells are given. Numbers under the open bars indicate the percentage of neutrophils.

EXCLUSION OF THE CONTRIBUTION OF OTHER CYTOKINES IN THE THYMOCYTE ASSAY

As other cytokines could be present in the supernatants and could enhance thymocyte responses we sought to examine whether interleukin-2 or tumour necrosis factor was present. Using the interleukin-2 sensitive cell line CTTL-2, we found no interleukin-2 activity in any supernatant. Recombinant tumour necrosis factor had negligible activity in the thymocyte assay.

Discussion

Interleukin-1 derived from neutrophils is potentially important in the alveolitis caused by quartz (silica).[4] We believe that neutrophil derived cytokines could be important in inflammatory lung disease and so report the production of interleukin-1 like activity by neutrophils from rat lung inflamed by quartz and heat killed bacteria.

Neutrophils derived from the inflamed lung are capable of producing large amounts of interleukin-1 like activity as assessed in the thymocyte enhancement assay. We excluded interleukin-2 and tumour necrosis factor as likely contaminating activities in the thymocyte assay. Neutrophils are found in the alveolar region and in the alveolar spaces in a range of diseases in which an immunological component is suspected. The release of interleukin-1 like activity by neutrophils could modulate the function of lymphocytes and other cells in the lung parenchyma and in the lung lymph nodes during inflammation and potentially cause local and systemic modulation of the immune response.

Neutrophils occur in the lungs of humans and animals exposed to non-antigenic mineral dusts such as asbestos and quartz.[4] They might be important in the local and systemic immune changes reported in dust exposed workers and experimental animals, which could be important in the disease process. Interleukin-1 is a fibroblast growth factor as well as having multiple effects on other cells, including endothelial cells and leucocytes.[5] If the interleukin-1 like activity is derived from neutrophils, this has important potential consequences for understanding pathological changes occurring in a range of inflammatory lung diseases.

Interleukin-1 can be produced and released by neutrophils from the rabbit peritoneal cavity[6] and from both bovine[7] and human[8] peripheral blood. This is to our knowledge the first report of interleukin-1 like activity in neutrophils derived from experimentally inflamed lung.

This work was funded by the Commission of the European Communities and the Colt Foundation, and YK had a personal grant from the Colt Foundation.

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HEMOTACTIC RESPONSES OF LEUKOCYTES ROM THE BRONCHOALVEOLAR SPACE OF RATS XPOSED TO AIRBORNE QUARTZ, COALMINE USTS OR TITANIUM DIOXIDE

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ITRODUCTION

udies on humans and in laboratory animals have revealed at bronchoalveolar deposition of dusts commonly associated ith pneumoconiosis, results in recruitment of leukocytes the lung parenchyma.[1,2] In view of the important role of e leukocytes in mediating both injury and mesenchymal ll proliferation, the resulting alveolitis is considered to be important factor in determining the progress of disease.[3,4] uring inflammation leukocytes are known to marginate and en migrate from the capillaries to the interstitium and veolar space under the influence of chemotactic factors enerated in this region. As part of a study on leukocyte cruitment into the lungs of rats exposed, by inhalation, to neumoconiosis-producing dusts,[5] we examined the emotactic activity of bronchoalveolar leukocytes lavaged om these animals. We report here on the chemotactic ac- vity of bronchoalveolar leukocytes from the lungs of rats posed to 10 mg/m^3 or 50 mg/m^3 airborne mass concen- ation of; (a) the pathogenic particulate quartz, which causes licosis; (b) dusts collected from the air of coalmines min- g anthracite, high rank coking coal and low rank bituminous al; (c) as a negative control, titanium dioxide, a fine par- culate of respirable size which is not associated with neumoconiosis.

ATERIALS AND METHODS

ats

ngeneic, PVG rats, SPF maintained and fifteen weeks of e at commencement of exposure, were used.

inerals

ne dusts used in the study were (a) titanium dioxide (Rutile), tained from Tioxide Limited, Stockton-on-Tees; (b) the artz standard DQ_{12}; (c) coalmine dusts collected from the r of British collieries mining anthracite, high rank (cok- g) coal and low rank (bituminous) coal. Airborne coalmine st samples were collected on dry Bondina socks mounted the return roadway of a single face at each of the three llieries; full details of this procedure are given sewhere.[5] Details of the mineralogical composition of the mples used are shown in Table I.

Inhalation Exposure

Groups of 48 rats were exposed to airborne dust for 5 days per week, seven hours per day in exposure chambers described by Beckett.[6] The dusts were dispensed using either Wright or the dust dispensers. The concentration of dust in the chambers was monitored as the mass concentration of respirable dust defined by the BMRC Johannesburg sampling criterion[7] using a Casella MRE 113A dust sampler. Full details of the exposure system are described in full elsewhere.[5]

Bronchoalveolar Lavage

At 8, 32 and 75 days into exposure, groups of four rats, and two control rats maintained in room air, were removed from the chamber and subjected to bronchoalveolar lavage. The method is described in detail elsewhere[5] but involved removal of the lungs, exsanguination, followed by lavage of the bronchoalveolar space with 4 × 8 ml volumes of saline at 37°C. The bronchoalveolar leukocytes, so obtained, were concentrated by centrifugation, counted and the proportions of the different leukocyte types assessed by differential counting of May-Grunwald Giemsa stained cytospin preparations.

Assay of Bronchoalveolar Leukocyte Chemotaxis

Chemotaxis was assessed using Blindwell chambers. Three hundred microlitres of 10% zymosan-activated serum (ZAS) (high in the chemotactic complement component C5a), were placed in the lower compartment and a filter (Nuclepore, Pleasanton, California) placed on top. The top compartment was screwed down and 6 × 10^5 alveolar macrophages in 400 µl of RPMI medium (Gibco, Paisley) were placed in the upper compartment. The filters used were 5 µm pore size and incubation was for 3.5 hours at 37°C in 5% CO_2 to allow migration of cells through the filter towards the chemotactic material in the lower compartment. At the end of the incubation period the filter was removed from the chamber, washed, stained and allowed to dry before being mounted on a slide in plastic mountant. Two chambers were set up for each condition and the number of migrated cells in 5 high power fields (x1000) were assessed for each filter.

Table I
Mineralogical Composition of Dusts Used in the Study

DUST	CLASSIFICATION	% ASH	% IN DUST KAOLIN	MICA	QUARTZ
Coalmine dust A	Anthracite	10.6	0.8	4.3	1.4
Coalmine dust H	High rank	13.2	0.6	0.5	1.0
Coalmine dust L	Low rank	53.2	18.1	0.0	6.7
Quartz	DQ_{12} Standard	NOT APPLICABLE - PURE QUARTZ			
Titanium dioxide (TiO_2)	Rutile	NOT APPLICABLE - PURE TiO_2			

tistical Analysis of Results

ılts were obtained from four experimental and two con-rats at each time point. Data were analysed by analysis ariance using the Genstat computer package and com-sons made using a 't' test.[5]

SULTS

:motaxis versus Chemokinesis in kocyte Migration

ensure that chemotaxis was the dominant activity being sured in each sample, and not chemokinesis, we used odified "checkerboard" method:—(mean ± standard ation migrated cells/high power field); spontaneous ration 0.0 ± 0.0; chemokinesis (measured as migration 5% ZAS in both the upper and lower compartments) ± 6.6; chemotaxis (measured with 5% ZAS in the lower partment) 46.4 ± 4.8. These results confirm that the ority of the migration was in fact chemotaxis and migra- in the Blindwell Chambers will henceforth be referred s chemotaxis.

ect of Dust inhalation on Chemotaxis of nchoalveolar Leukocytes

ure 1 shows typical data obtained for chemotaxis ex-ments with leukocytes from rats exposed, by inhalation, 0 mg/m³ of the five dusts. This data clearly shows that lation exposure to TiO_2 had very little effect whereas osure to quartz and the coalmine dust was associated with arked reduction in the ability of the bronchoalveolar kocytes to chemotact.

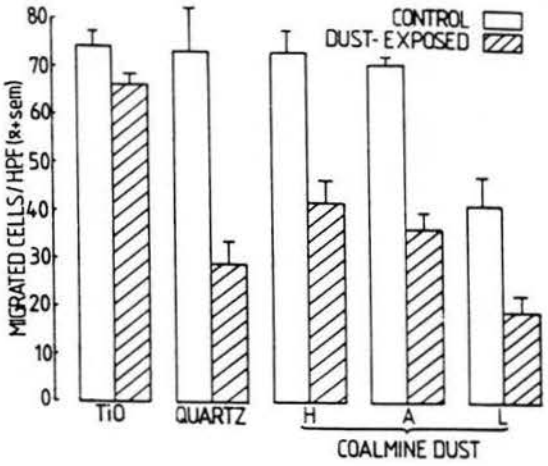
ure 2 shows the data, from all experiments at 10 mg/m³ orne mass concentration, expressed as percentage inhibi- of chemotaxis compared to the controls on that day, to e clearly highlight the effect of dust exposure. It is clear , although inhibition of 30% was present with TiO_2 at ys, thereafter the inhibitory effect of TiO_2 did not ex-l 17%. In the case of quartz, however, this was as great 9.4% by day 75. All three coalmine dusts tended to show adual rise in the impairment of chemotaxis shown by the nchoalveolar leukocytes as time of exposure progressed, ching 50–70% inhibition by day 75.

Figure 3 documents the effects of increasing airborne mass concentration of coalmine dust, on the inhibition of chemotatic activity. The increase from 10–50 mg/m³ airborne mass concentration was associated with a marked increase in the impairment of chemotaxis, observable in the coalmine dust-exposed bronchoalveolar leukocytes, reaching 70–90% at 50 mg/m³.

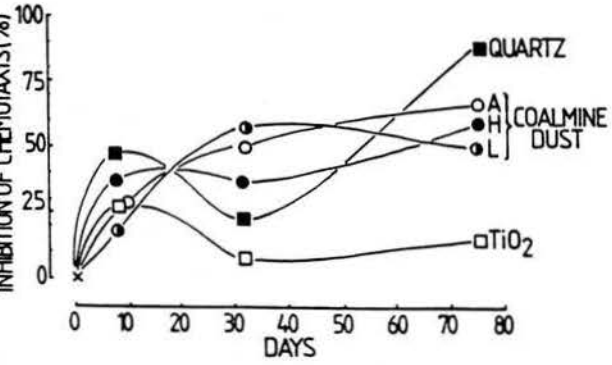
Attempts to Elucidate the Mechanism of Dust-Related Impairment of Leukocyte Chemotaxis

Limited experiments were carried out to try and elucidate the mechanism whereby dust deposition in the lungs of rats, as described above, caused loss of ability to chemotact. (a) Effect of ingested dust on macrophage chemotaxis. Control rat alveolar macrophages were allowed to adhere to filters and then incubated with quartz or TiO_2 for 1 hour to allow phagocytosis. A chemotaxis gradient was then set up by placing the filters in a chamber with ZAS in the bottom compartment. We then allowed chemotaxis to proceed:—all data given as migrated cells/high power field mean ± standard deviation; untreated macrophages, with no phagocytic burden 54.4 ± 11.3, TiO_2-exposed 51.8 ± 6.2, quartz-exposed 59.8 ± 6.0. Clearly merely having a phagocytic burden inside the macrophages was not sufficiently detrimental to cause impairment of chemotaxis. (b) Effect of incubation for 4 hours on chemotaxis. Allowing dust-exposed macrophages with impaired chemotaxis (obtained after 75 days of exposure to coalmine dust L) to incubate for 4 hours in medium to allow recycling of chemotaxin receptors had no effect on the impaired ability of the cells to chemotact:—control alveolar macrophages, freshly derived 55.0 (7.0)—incubated for 4 hours 48.2 (11.0); dust-exposed bronchoalveolar leukocytes, freshly derived 12.6 (3.6)—incubated for 4 hours 9.1 (2.4). (c) Relationship between % neutrophils in the lavage and % inhibition of chemotaxis. Since neutrophils were present to substantial proportion in some samples of bronchoalveolar leukocytes we assessed whether the presence of neutrophils was related to impairment of chemotaxis. There was no clear relationship between the proportion of neutrophils present in any bronchoalveolar leukocyte sample and impairment of chemotaxis—10–60% inhibition was caused with $< 10\%$ neutrophils while increasing the percentage of neutrophils

between 10 and 50%, only caused a maximum further 20% ıibition.



gure 1. Chemotactic response of bronchoalveolar from rats exposed to the indicated dusts at 10 mg/m³. Data derived as mean + sd of pooled results obtained for days 8, 32 and 75 (6–12 rats per group). Significant differences dust-exposed v control for all except TiO_2.



gure 2. Mean percentage inhibition of chemotaxis shown by bronchoalveolar leukocytes from dust-exposed compared to control rats exposed to 10 mg/m³ of the indicated dusts. Mean percentage inhibition obtained as:

$$00 - \frac{\text{mean migration}}{\text{mean migration}} \text{ of } \frac{\text{dusted bronchoalveolar leukocytes}}{\text{control bronchoalveolar leukocytes}} \times 100$$

Raw data obtained from 2 control and 4 dust-exposed rats.

ISCUSSION

iO_2 is a fine particulate used extensively in industry and not associated with pneumoconiosis in exposed populaons.[8] It causes minimal response in rats when given by inalation or intraperitoneal injection.[2,9] Coalmine dust and uartz both cause pneumoconiosis and 3 coalmine dusts of

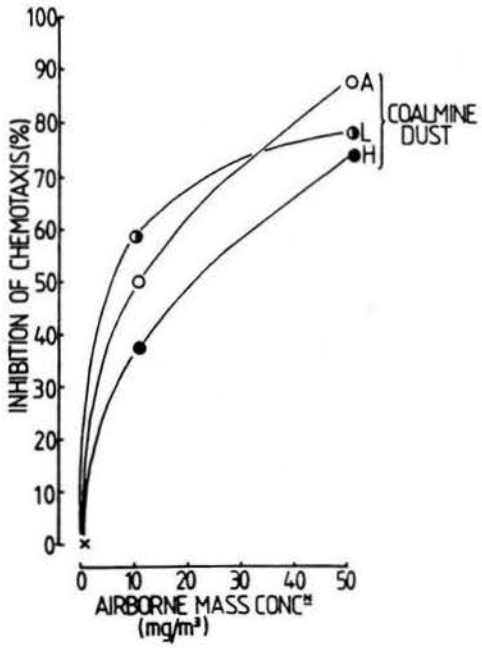


Figure 3. Airborne mass concentration dependence of the chemotaxis inhibition present in bronchoalveolar leukocytes from rats exposed for 32 days to the indicated dusts; no 50 mg/m³ data available for quartz or TiO_2. Data derived as described in the legend to Figure 2.

different mineralogical composition, including quartz content, were included in order to test whether such differences would contribute to differences in leukocyte recruitment. These studies are reported in detail elsewhere[5] but revealed alveolitis in rats exposed to quartz and all 3 coalmine dusts and failure of TiO_2 to elicit any substantial leukocyte response except at high dose following a long period of exposure.

The studies on the chemotactic activity of bronchoalveolar leukocytes reported here show impairment of chemotaxis in line with the ability of the dust to cause inflammation, i.e., (a) titanium dioxide which caused minimal inflammation caused least impairment of leukocyte chemotaxis; (b) quartz, caused large scale inflammation and the bronchoalveolar leukocytes of the alveolitis had impaired chemotactic activity; (c) coalmine dusts were intermediate in response between TiO_2 and quartz in ability to cause inflammation and impair chemotactic responses. There were no well defined differences between the three coalmine dusts with different mineralogical composition, in terms of their ability to impair chemotaxis.

The results described here do show that chronic deposition of titanium dioxide, a dust not associated with pneumoconiosis did cause a measure of loss of impairment of chemotactic activity. In the cases where quartz and coalmine dusts caused impairment of chemotaxis there was

lear dose dependency in terms of the airborne mass concentration to which the rats were exposed. The data described ere was obtained as counts of all migrated leukocytes which ıcluded both macrophages and neutrophils in inflammatory opulations. However the decreased number of migrated eukocytes present in dust-exposed populations could not be xplained on the basis of the neutrophils present, either as ifferent migration characteristics compared to macrophages r effects of neutrophils on macrophage ability to migrate. his was evident since (a) profound inhibition was present ven with low percentages of neutrophils;[5] (b) in a limited umber of cases differential count of the migrated cells were arried out (data not included) revealing, in some cases, imilar proportions of macrophages and neutrophils in the ıigrated cells to those in the cells as lavaged; in some cases ıe proportion of neutrophils was decreased but this was ever sufficient to explain the overall reduction in migraion shown by the inflammatory population and impairment f macrophage chemotaxis must have been present. From his it is clear that macrophages from dust-exposed lung have mpaired chemotactic activity and that neutrophils from dustxposed lung have less chemotactic activity than control lveolar macrophages, at least under the conditions of the ssay. The net effect of this is that the ability of the leukocytes o clear dust from dust-inflammed alveoli is likely to be everely impaired.

Ve have shown that the biological mechanisms underlying he loss of ability to chemotact do not include mere difficulty encountered by dust-loaded cells in trying to pass through he pores of the filter towards the source of chemotaxin. Since he leukocytes lavaged from the bronchoalveolar space have xudated in response to a chemotactic stimulus, it seemed ossible that chemotaxin receptors might already be occupied. However, experiments allowing chemotaxin receptors to regenerate, by incubation for 4 hours, produced no effect and impairment was maintained. Other studies from our Institute have suggested that neutrophils could cause some nhibition of the chemotactic activity of macrophages.[10] However plotting % inhibition against % neutrophils in the avage failed to show any clear relationship between numbers f neutrophils and loss of chemotaxis.[5]

Ve believe that leukocytes from dust-exposed lung have imaired expression of chemotaxin receptors or inhibition of he cytoskeletal proteins involved in cell movement, or their nergy supply. Myrvik[11] reported inhibition of migration of abbit alveolar macrophages which had phagocytosed sbestos *in vitro*; whilst the impairment of chemotaxis could ot be attributed to toxicity in this study, unfortunately inert ontrol dusts were not included. Following exposure *in vivo* nd bronchoalveolar lavage, Warheit et al.[12] reported imairment of chemotaxis with asbestos whilst Dauber et al.,[13] nd Martin et al.,[14] both described impairment of leukocyte hemotaxis following inhalation exposure to silica. The preent response is the first, to our knowledge, showing that oalmine dusts also cause profound impairment of bronhoalveolar macrophage chemotaxis. It was noteable that inibition of chemotaxis was present following chronic inhalaion exposure at an airborne mass concentration of 10 mg/m^3 approximating to the maximum allowable level in British collieries (7 mg/m^3).

The fact that impairment of migration in bronchoalveolar leukocytes was much less with the inert dust TiO_2 than with the two pneumoconiosis-producing dusts suggests that this phenomenon may be important in contributing to lung damage and pathological change in pneumoconiosis. This could be brought about by the pneumoconiosis-producing dusts being allowed to persist in the bronchoalveolar region within alveolar macrophages chronically stimulated by the ingested pathogenic dust. Such stimulated macrophages, refractory to the normal chemotactic gradients which govern their movements, could persist in the alveolar region, releasing injurious agents such as proteases and oxidants and growth factors such as interleukin 1 and tumour necrosis factor, leading to fibrosis.

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VITRO INJURY TO ELEMENTS OF THE ALVEOLAR SEPTUM ISED BY LEUKOCYTES FROM THE BRONCHOALVEOLAR GION OF RATS EXPOSED TO SILICA

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RODUCTION

tz exposure is associated with lung fibrosis (silicosis) Type II alveolar epithelial cell hyperplasia is also com-y present.[1] Bronchoalveolar lavage studies using rats our own laboratories,[2] and in humans,[3] have onstrated that there is leukocyte recruitment into the following inhalation exposure to silica. Studies on other tic lung diseases have stressed the importance of the ocytes of the alveolitis in the progression of disease via se of important mediators.[4] We have therefore set out xamine the ability of bronchoalveolar leukocytes from exposed to silica by a single intratracheal instillation, ause injury to the extracellular matrix and cellular ents of the alveolar septum *in vitro*. Leukocytes from exposed to two other inflammogenic particulates—a heat d bacterial preparation and a yeast cell wall preparation nosan)—were similarly assessed, for comparison with tz.

TERIALS AND METHODS

nal Model of Silicosis

geneic PVG rats, SPF bred, were exposed by in-acheal instillation to 1 mg of DQ_{12} standard quartz. As rols, the heat killed bacterial preparation *Cor-acterium parvum* was also injected as was the yeast cell preparation zymosan; both of these particulates are vn to cause inflammation. Bronchoalveolar leukocytes e obtained by lavage as described in detail elsewhere[5] rious time points after injection. In this model quartz sure causes fibrosis, Type II epithelial cell hyperplasia alveolar lipoproteinosis beyond 1 month exposure which evident in histological sections of exposed lung.

ay of Leukocyte-Mediated Type II eolar Epithelial Cell Injury

assay is described in detail elsewhere[6] and involves lling of Type II alveolar cell line (A549) with ^{51}Cr. nchoalveolar leukocytes are then added to the labelled in microtitre wells and co-cultured for 4 hours; the abili-f the leukocytes to cause lysis or detachment of the helial cells is assessed.

Assay of Leukocyte-Mediated Proteolysis of Fibronectin

Leukocyte-mediated proteolysis of fibronectin was assessed using a solid phase assay of ^{125}I-labelled fibronectin in microtitre plate wells. This assay has been described in detail elsewhere[7] and measures protease-mediated injury. The leukocyte-mediated proteolyic activity shown here against fibronectin is also active against ^{125}I-labelled collagen and laminin. Leukocytes are cultured on the solid phase of ^{125}I-labelled fibronectin and allowed to degrade the matrix for 4 hours; products of proteolysis of fibronectin are measured as free counts in the supernatant.

Leukocyte Separation

Whole inflammatory bronchoalveolar leukocyte populations from quartz-exposed rats were separated by centrifugation through Sepra-Cell medium into macrophage and neutrophil-enriched fractions.

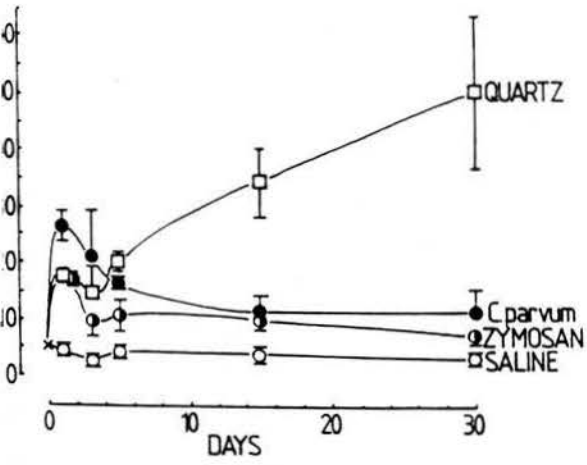
Statistical Analyses

Results were analysed by analysis of variance and differences in treatments compared for significance using a 't' test.

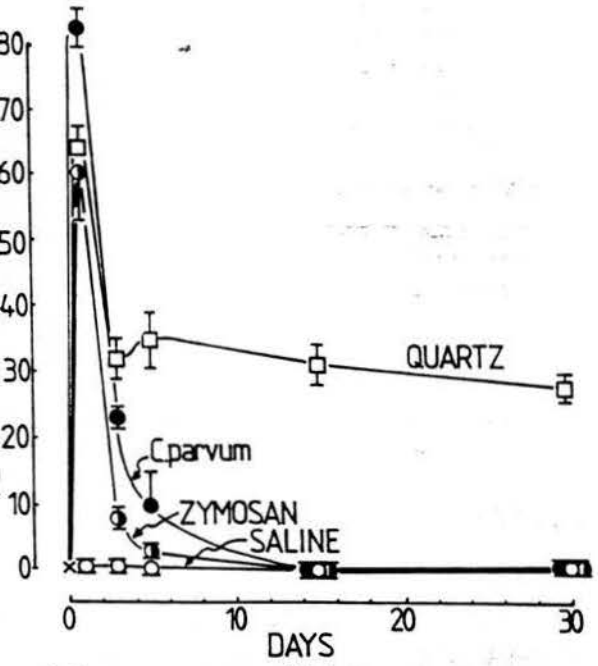
RESULTS

Inflammation Caused by a Single Injection of Silica, *C. parvum* or zymosan

Figures 1 and 2 show the total number of bronchoalveolar leukocytes and percentage neutrophils lavaged from rats injected intratracheally with quartz, *C. parvum* or zymosan. All three particles caused initial burst of inflammation characterized by recruitment of large numbers of leukocytes containing high proportions of neutrophils. In the case of *C. parvum* and zymosan this initial alveolitis was followed by a return to the normal situation where no neutrophils were present although the numbers of macrophages remained raised indicating a mild macrophage alveolitis. In the case of quartz, however, an intense macrophage/neutrophil alveolitis persisted until at least one month. Previous studies have shown that this alveolitis persists for up to three months.[8]



ure 1. Total leukocytes in bronchoalveolar lavage up to 30 days after instillation of saline, quartz, *C. parvum* or zymosan into the lungs of rats. Data is mean ± standard deviation from 3 rats. Significant ($P<0.01$–0.001) increases with all particulates compared to saline.



ure 2. Percentage neutrophils in bronchoalveolar lavage up to 30 days after instillation of saline, quartz *C. parvum* or zymosan. Data derived as in legend to Figure 1. Significant ($P<0.01$–0.001) increases in percentage neutrophils, compared to saline, for quartz at all time points and for *C. parvum* and zymosan at 1, 3 and 5 days.

tivity of Bronchoalveolar Leukocytes Breaking Down Fibronectin

shown in Figure 3 the bronchoalveolar leukocytes ob-ed from the lungs following injection of different particles showed varying abilities to break down fibronectin. During the acute inflammatory phase the leukocytes from lung exposed to all three particulates were capable of breaking down fibronectin. However, only quartz was capable of eliciting a sustained high level of proteolysis, in keeping with the persistence of the inflammation in quartz-exposed lung. It was notable that the ability to break down fibronectin correlated strongly with the presence of neutrophils.

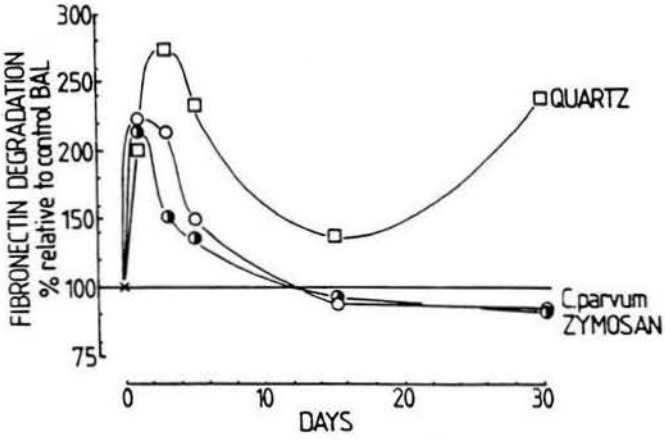


Figure 3. Proteolytic activity against fibronectin shown by bronchoalveolar leukocytes from rats injected with the indicated particulates. Data expressed as a percentage of the activity shown by control bronchoalveolar leukocytes.

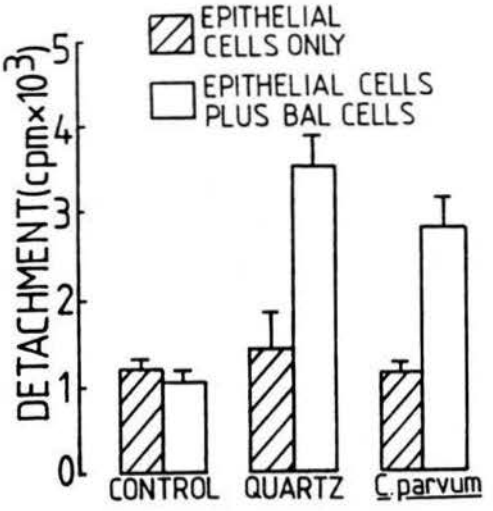
Injury to Alveolar Epithelial Cells Caused by Bronchoalveolar Leukocytes

Bronchoalveolar leukocyte populations elicited with quartz or C. parvum were tested for their ability to cause injury to cells of an alveolar epithelial cell line *in vitro*. Both *C. parvum*-elicited bronchoalveolar lavage cells obtained after one day (70–90% PMN) and 5-day quartz leukocytes (50% macrophages/50% neutrophils) were capable of causing the target cells to detach from the sub-stratum (Figure 4). There was no lytic injury to the target cells and the detachment injury could be completely inhibited by protease inhibitors such as alpha 1-protease inhibitor.[6]

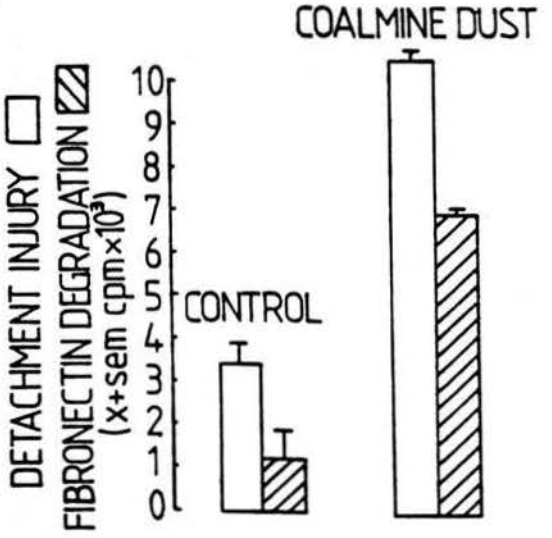
We have also examined the ability of leukocytes from the lungs of rats chronically inhaling coalmine dust to mediate injury. This showed that rats exposed, by inhalation, for 48 days to coalmine dust collected from the air of a British colliery[5] also caused epithelial injury and degradation of fibronectin (Figure 5).

Cellular Origin of Epithelial Cell Detaching Injury in Quartz-Elicited Bronchoalveolar Leukocyte Populations

As shown above, high proportions of neutrophils seem to accompany fibronectin-degrading and epithelial-injuring activity in the inflammatory leukocyte populations which we have examined. To determine whether the macrophages could also be producing proteolyic activity against fibro-



ıre 4. Detachment injury caused to alveolar epithelial cells *in vitro* by co-culture with control, quartz or *C. parvum* elicited bronchoalveolar leukocytes. All data given as mean + SEM of triplicate cells in 3 separate experiments. Significantly increased detachment caused by quartz and *C. parvum* treatment ($p<0.001$).



ıre 5. Detachment injury and fibronectin degradation caused by control bronchoalveolar leukocytes and bronchoalveolar leukocytes from rats inhaling coalmine dust for 45 days. Significant increases ($P<0.001$) in both parameters with coalmine dust exposed bronchoalveolar leukocytes compared to controls.

tin, and causing detachment injury, the 5 day quartz bron- alveolar leukocytes were separated into macrophage- ched and neutrophil-enriched populations. These were ı tested for their ability to cause epithelial cell detach- nt injury. Figure 6 demonstrates that separation of the ed population into the enriched populations resulted in very high levels of epithelial injury being caused by the neutrophil-enriched fraction. However, despite the macrophage-enriched fraction containing only 5% PMNs, this population caused 5-fold more detachment injury than control alveolar macrophages.

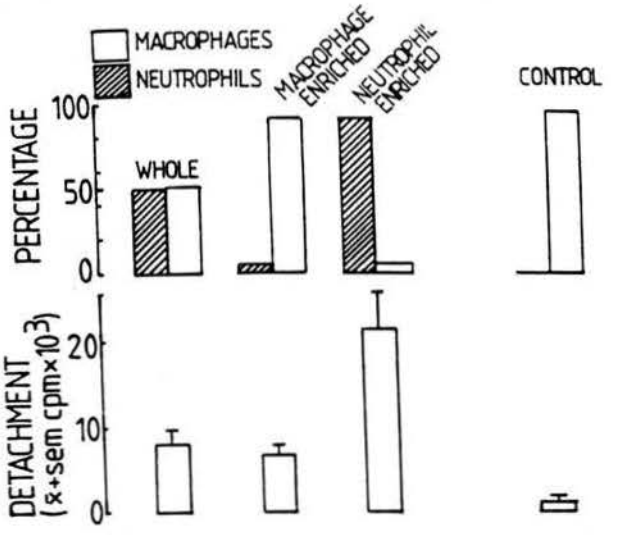


Figure 6. Cellular composition (upper panel) of, and detachment injury (lower panel) caused by, whole quartz-elicited bronchoalveolar leukocytes and both macrophage-enriched and neutrophil-enriched fractions obtained from it. Proportions of neutrophils and macrophages shown as mean percentage. Detachment injury shown as mean + SEM of cpm in detached cells.

DISCUSSION

This study has shown that a single injection of silica into the rat lung causes a long-term alveolitis. The alveolitis is characterized by a 3–12 fold increase in bronchoalveolar leukocytes comprising 30–40% neutrophils. Intratracheal instillation of a heat-killed bacterial preparation (*C. parvum*) or yeast cell walls (zymosan) also caused large scale burst of inflammation immediately following injection but these resolved quickly, returning to near normal levels by 15 days. Thus the initial severity of the alveolitis is not the main factor determining the persistence of silicotic inflammation in the intratracheal model.

The exact events which engender persistent inflammation with silica are speculative but cytoxicity of quartz towards alveolar macrophages might be central. The consequence of silica-induced alveolitis is likely to be fibrosis since the ability of inflammatory leukocytes to mediate further damage and pathological change in the lung is well established for a range of aetiologic agents.[4] In an attempt to understand which leukocyte-derived injurious factors might be important in the development of quartz-related pathology we examined the ability of the leukocytes from quartz-exposed lung to break down fibronectin. During the acute inflammation engendered

C. parvum and zymosan there were high levels of proteolytic activity present; the levels of protease however returned to normal within 15 days. An examination of the proteolytic activity of quartz-elicited leukocytes showed that this proteolytic activity, capable of breaking down fibronectin and other connective tissue elements[7] and so generating chemotaxin[10] and causing epithelial injury and basement membrane damage,[6] was 4, present persistently, and in increased quantities, for up to 1 month following quartz instillation; previous studies suggest that this inflammation and hence the increased protease burden persist for up to 3 months and possibly longer. The total proteolyic burden of the lung is not reflected adequately as the increase, on a per cell basis, in quartz-elicited bronchoalveolar leukocytes since the total number of leukocytes is also increased. If the increase in cell numbers is taken into consideration (a 16-fold increase on day 30) this produces a greater than 30-fold increase in the total protease burden of the lung following silica exposure at 30 days. Although the present study has utilized intratracheal instillation we have found that inhalation exposure to pneumoconiotic dust (coalmine dust containing quartz) has caused an alveolitis producing greatly enhanced lung burdens of fibronectin-degrading activity.[11]

The ability of inflammatory bronchoalveolar lavage leukocytes to injure epithelial cells correlates with proteolyic activity[6] and so we examined this aspect of injury produced by quartz bronchoalveolar leukocytes. The quartz bronchoalveolar leukocytes caused detachment injury which appeared to be mediated by both macrophages and neutrophils as shown by separation studies where the different leukocyte types were obtained in enriched form. It is therefore possible to conclude that the bronchoalveolar macrophages from quartz-exposed lung are activated with regard to proteolyic activity. The time course studies with *C. parvum* revealed that the oldest macrophage alveolitis present beyond 15 days but that this population was not activated with regard to protease production. The fact that the cell numbers were increased compared to controls argues for the fact that this did indeed represent an inflammatory population albeit one which was not characterized by increases in neutrophils. It is possible therefore that only inflammatory macrophages from mixed populations, where neutrophils are also present, show increased proteolytic activity. A likely explanation for this is that the alveolar macrophages from such populations have internalized neutrophil elastase as has been previously reported.[12] It was noteable that the neutrophil-enriched fraction had twice the proportion of neutrophils found in the whole population but produced a 3-fold increase in detaching activity. This suggests that either the separation procedure caused activation of neutrophils or that macrophages suppressed neutrophil proteolytic activity in the mixed population.

This study has shown that a single deposition of 1 mg of quartz in the rat lung causes a prolonged and intense alveolitis characterized by increased proteolytic activity of bronchoalveolar leukocytes, capable of causing injury to the epithelial and matrix elements of the alveolar septum. The results strongly suggest that leukocytes from rats exposed by inhalation to pneumoconiosis-producing dust also have these properties and that both macrophages and neutrophils express this injurious proteolytic activity, although in the case of macrophages this may be due to sequestered neutrophil elastase.

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Persistent biological reactivity of quartz in the lung: raised protease burden compared with a non-pathogenic mineral dust and microbial particles

Geraldine M Brown, David M Brown, Joan Slight, Kenneth Donaldson

Abstract

This study assessed the potential harmfulness of particles in the lung by measuring their ability to elicit and maintain an inflammatory response and to damage lung tissue. It compared the inflammogenicity of two non-durable, biological particulates (*Corynebacterium parvum* and zymosan) with a pathogenic mineral dust (quartz) and a non-pathogenic dust (titanium dioxide) by dosing rats via the intratracheal route and measuring the consequent alveolitis. The magnitude and duration of the inflammatory response were assessed by measuring the total number of leucocytes and the percentage of neutrophils obtained by bronchoalveolar lavage. Two key functional parameters of the lavaged leucocytes—ability to degrade fibronectin and production of plasminogen activator—were also measured. A marked inflammatory response had occurred by one day after instillation, characterised by increases in total leucocyte numbers and percentage of neutrophils in the bronchoalveolar lavages, with all four test materials. In all but the quartz exposed animals, the inflammation subsided rapidly thereafter, approaching control levels by 15 days after injection; in the quartz exposed animals the alveolitis persisted for up to 30 days. All of the inflammogens generated chemotaxins in rat serum in vitro and so, by analogy, might also be expected to generate chemotactic activity in alveolar lining fluid which could contribute to the generation of an inflammatory response. The cellular inflammatory response was accompanied by a concomitant increase in the proteolytic activity of the bronchoalveolar lavage leucocytes but production of plasminogen activator remained unchanged. In vitro exposure to the inflammogens had no effect on the proteolytic activity against fibronectin or on the plasminogen activator activity of bronchoalveolar leucocytes.

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Among the fibrotic lung diseases of known aetiology are those associated with chronic exposure to harmful mineral dusts—for example, silica (silicosis), asbestos (asbestosis), and coalmine dust (coalworkers' pneumoconiosis).[1] Exposure to such dusts is associated with the accumulation of inflammatory leucocytes in the alveolar region in exposed workers[2,3] and in animal models of lung disease.[4,5] The inflammatory response may be mediated, in part, by the action of leucocyte derived plasminogen activator in converting plasminogen to plasmin, a protease with potent inflammogenic properties.[6] Recruitment of leucocytes in response to deposition of particles in the lung may also occur through direct activation of complement by the particles in the alveolar lining fluid.[7] Toxic products and growth factors are released in increased quantities by inflammatory leucocytes at sites of inflammation in the lungs and so "bystander injury" to the surrounding host tissue and consequent overgrowth of mesenchymal cells are likely to result. Leucocyte proteases are considered to play a major part in this type of injury[8] and have been found in increased amounts in the bronchoalveolar lavage fluid of patients with fibrotic lung disease.[9,10] Proteolysis of connective tissue components by leucocyte proteases is likely to be central to the pathogenesis of chronic inflammatory lung disease as suggested by studies demonstrating connective tissue proteolysis by inflammatory leucocytes in vitro[11,12] and by leucocyte proteases in vitro[13] and in vivo.[14] The degree of tissue injury is associated with the number of inflammatory leucocytes in the alveolar region[6] and thus the severity of tissue damage is likely to be related to both the magnitude and duration of the alveolitis and to the cellular profile of the bronchoalveolar leucocytes.

In this study, we have considered key aspects of the

inflammatory response to particle deposition in the lung. We compared the inflammation engendered by two non-durable, biological particulates, *Corynebacterium parvum* (a heat killed bacterial preparation) and zymosan (an extract of yeast cell walls) with that evoked by the inert dust, titanium dioxide (TiO_2), or by a pathogenic dust, quartz; these are non-biodegradable particles. Titanium dioxide is a particulate that is widely used in the food and paint industries but has not been associated with pathology in occupationally exposed groups; it is commonly used as an "inert" control dust in experimental studies in vitro[15 16] and in vivo.[5 17] By contrast, occupational exposure to quartz is known to be associated with development of the fibrotic lung disease, silicosis, and quartz has been shown to have potent cytotoxic effects in vitro[18] and marked inflammogenic potential.[5]

The magnitude and duration of the alveolitis produced by these inflammogens was assessed using bronchoalveolar lavages; two key parameters of leucocyte protease activity, relevant to the inflammatory response, were then measured: (*a*) ability to degrade fibronectin, a connective tissue component of importance in maintaining tissue integrity in the lung and (*b*) production of plasminogen activator, a protease that converts plasminogen to plasmin and that may thus be central to the development of the inflammatory response. Also, to assess the contribution that dust might make directly to lung inflammation, we measured the inherent ability of the dust to activate complement. To more closely mimic the situation in vivo, with continuing dust exposure, we further exposed leucocytes to the inflammogens in vitro after previous in vivo exposure. The protease activity of these "twice challenged" leucocytes was assessed to see if a second challenge with inflammogen enhanced that activity.

The harmful effects of the in vivo treatments were assessed microscopically on fixed and stained sections of the lung tissue.

Materials and methods

ANIMALS

The rats used were syngeneic, SPF males of the PVG strain, obtained from the breeding unit of the Institute of Occupational Medicine.

REAGENTS

Iodine^{125} was obtained from Amersham Limited (Aylesbury, Buckinghamshire). Human plasma fibronectin, fibrinogen, and bovine serum albumin were purchased from Sigma Ltd, Poole, Dorset. Plasminogen was obtained from Kabi (Flow Laboratories, Rickmansworth, Herts). Hams F10 medium and phosphate buffered saline (PBS) were purchased from Gibco BRL (Paisley, Renfrewshire).

PARTICULATES

The quartz sample used was the DQ12 standard; TiO_2 (rutile) was obtained from Tioxide Ltd, Stockton-on-Tees. *Corynebacterium parvum*, a heat killed bacterial preparation was purchased from Wellcome Laboratories Ltd, Beckenham, Kent; and zymosan, which is a preparation of yeast cell walls, was obtained from Sigma, Poole, Dorset.

IN VIVO DUST EXPOSURE AND LAVAGE

Rats were injected intratracheally with 1 mg of quartz, TiO_2, or zymosan in 0·5 ml PBS, or 1·4 mg *C parvum* as a 0·2 ml suspension. One, three, five, 15, or 30 days thereafter, groups of three treated rats and three untreated control rats were killed and the lungs lavaged with warm saline to retrieve the bronchoalveolar leucocytes as previously described.[5] Total and differential counts were performed on the harvested leucocytes.

FIBRONECTIN PROTEOLYSIS

The ability of bronchoalveolar leucocytes, or their supernatants, to degrade fibronectin was assessed by measuring breakdown of a solid phase ^{125}I fibronectin matrix as previously described.[12] Proteolytic activity was measured as counts per min of degraded ^{125}I fibronectin released into the supernatant medium over a four hour period. To overcome inter-experiment variation due to decreasing counts of ^{125}I in the fibronectin coated plates with time, results are presented as a percentage of background proteolysis.

PLASMINOGEN ACTIVATOR ASSAY

Production of plasminogen activator was measured as previously described[19] by measuring plasminogen dependent breakdown of ^{125}I fibrin. Fibrinolysis was measured as counts per min of ^{125}I fibrin released into the supernatant medium in a 24 hour period.

IN VITRO TREATMENT WITH PARTICULATE OR SOLUBLE TRIGGERS

Bronchoalveolar leucocytes from particulate exposed or control rats were assayed in the fibronectin proteolysis and plasminogen activator assays in the presence of zymosan, TiO_2, or quartz at 0·01, 0·1, or 1·0 mg/ml, or phorbol myristate acetate (PMA) at 0·1, 1·0, or 10 μg/ml, to assess the effect of such treatment in modulating the activity of the leucocytes.

CHEMOTAXIS

Particulates were incubated at 1 mg/ml in rat serum for one hour at 37°C followed by heat inactivation at 56°C for one hour; the particles were then removed by centrifugation at 3000 rpm for 10 minutes. Generation of chemotactic activity in the serum was assessed by measuring its ability to induce chemotaxis of control rat alveolar macrophages in Blindwell chambers as previously described.[20]

STATISTICAL ANALYSIS

There was minimum day to day variation in total numbers of leucocytes or percentage of neutrophils in bronchoalveolar lavage of control animals, so comparisons of these two parameters between treatment groups and controls at one day after injection were made with pooled control data (12 animals). Differences were tested by Student's *t* test and the least significant difference reported.

HISTOLOGY

Tissue samples were prepared for histology at the 30 day time point. Lungs were fixed subsequent to lavage by inflating them with 10% formol saline before routine processing for histology. Whole lung sections were cut by microtome and stained with haemotoxylin and eosin to visualise the tissue.

Results

TOTAL LEUCOCYTES IN BRONCHOALVEOLAR LAVAGES

All the inflammogens provoked a transient peak of leucocyte recruitment one day after injection (fig 1); this was greatest with *C parvum* and least with TiO_2. The difference between the pooled data from all controls and each treatment group was compared at one day and was highly significant ($p < 0{\cdot}001$). The magnitude of the response to quartz was similar to that produced by zymosan at one day, but after a slight decrease in bronchoalveolar lavage leucocytes at three days, numbers of leucocytes in lavages from quartz dosed rats escalated and were 10-fold greater than controls at 30 days.

PERCENTAGE OF NEUTROPHILS IN BRONCHOALVEOLAR LAVAGES

The percentage of neutrophils in the cell populations from lavages reflected a typical acute inflammatory response (fig 2). A pronounced influx of neutrophils occurred at one day with each particulate and this was highly significant compared with the pooled control data ($p < 0{\cdot}005$). In all but the quartz dosed animals, the numbers of neutrophils returned rapidly to background levels; the percentage of neutrophils in quartz elicited leucocytes persisted at around 30% for the 30 days of the experiment (significantly greater than controls, $p < 0{\cdot}001$).

FIBRONECTIN DEGRADING ACTIVITY OF LEUCOCYTES IN BRONCHOALVEOLAR LAVAGES

The proteolytic activity of the leucocytes in lavages reflected the cellular response, peaking at one day with all of the inflammogens ($p < 0{\cdot}001$) (fig 3) and, with the exception of those from the quartz exposed group, returning rapidly to normal thereafter. Proteolysis of fibronectin by the quartz elicited leucocytes remained at least fivefold greater than that by control leucocytes for up to 30 days. The proteolytic activity of the inflammatory leucocytes, on a per cell basis, is an insufficient descriptor of the

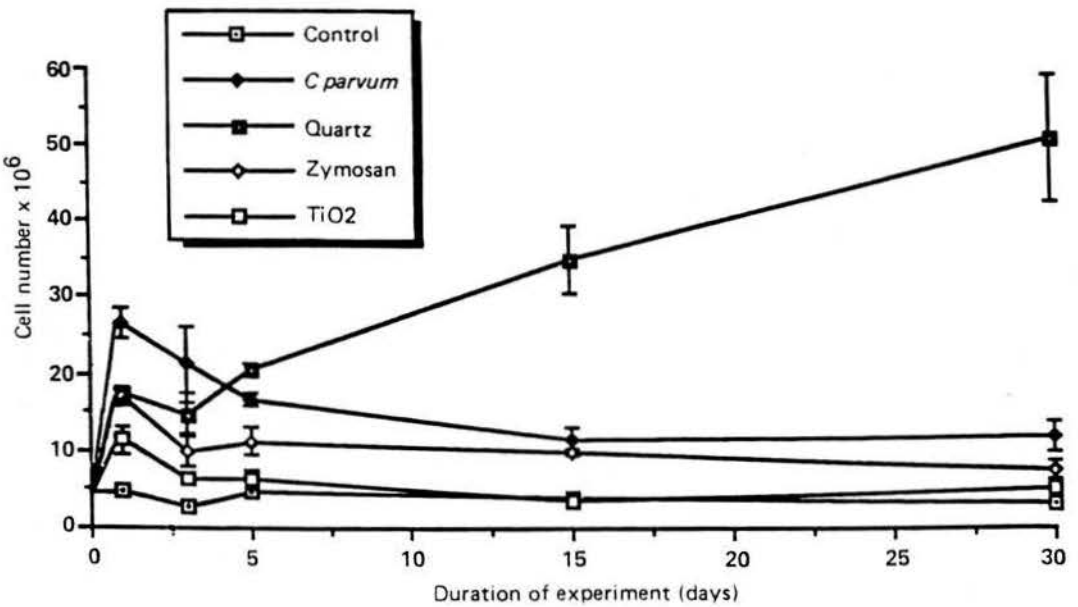


Figure 1 Total cells in lavage fluid with time following deposition of a single bolus of the inflammogen on day 0. Results are mean (SE) from three rats per sample at each time point.

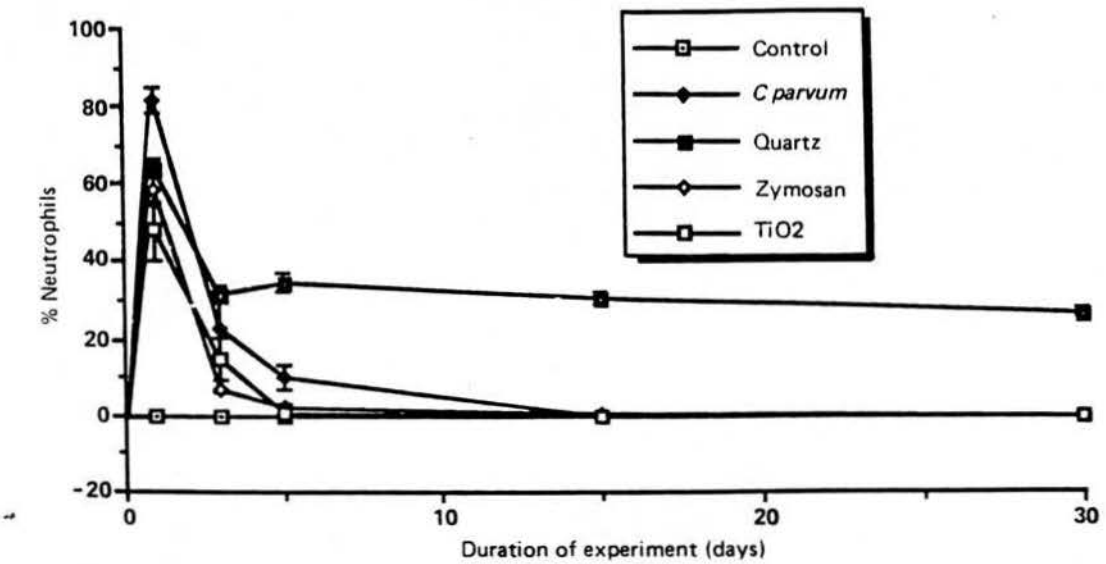


Figure 2 Percentage of neutrophils in lavage fluid with time following deposition of a single bolus of the inflammogen on day 0. Results are mean (SE) from three rats per sample at each time point.

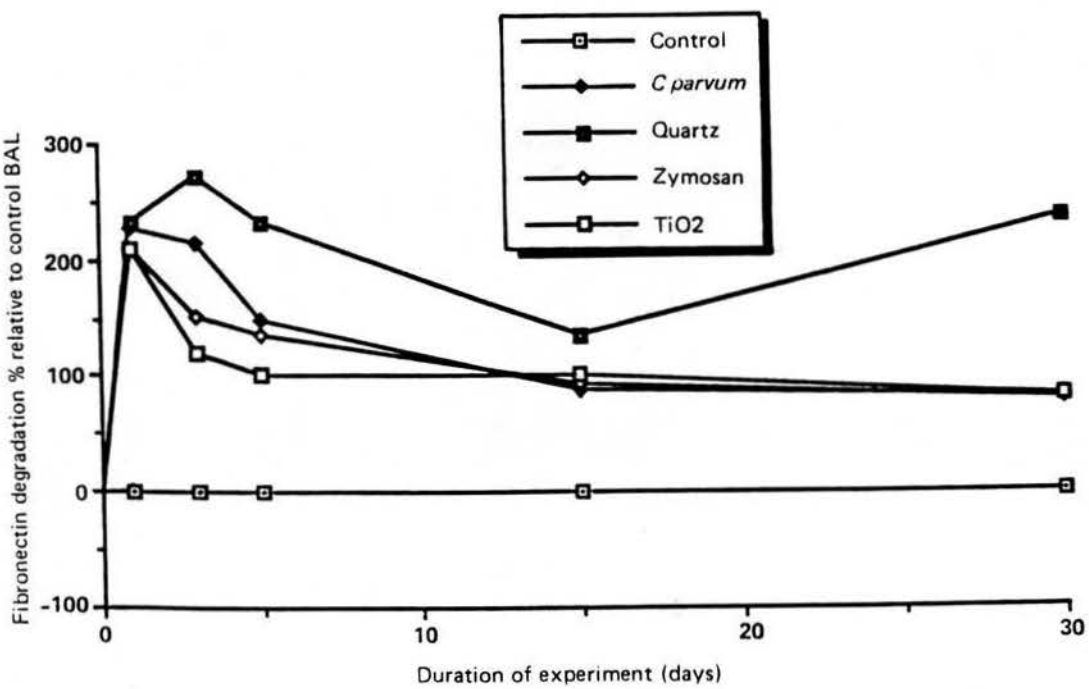


Figure 3 Proteolytic activity of inflammatory leucocytes with time after deposition of a single bolus of the inflammogen on day 0. Results are expressed as fibronectin proteolysis as a percentage of control cell activity and are the mean (SE) of three wells per sample and three animals per sample at each time point. BAL = bronchoalveolar lavage.

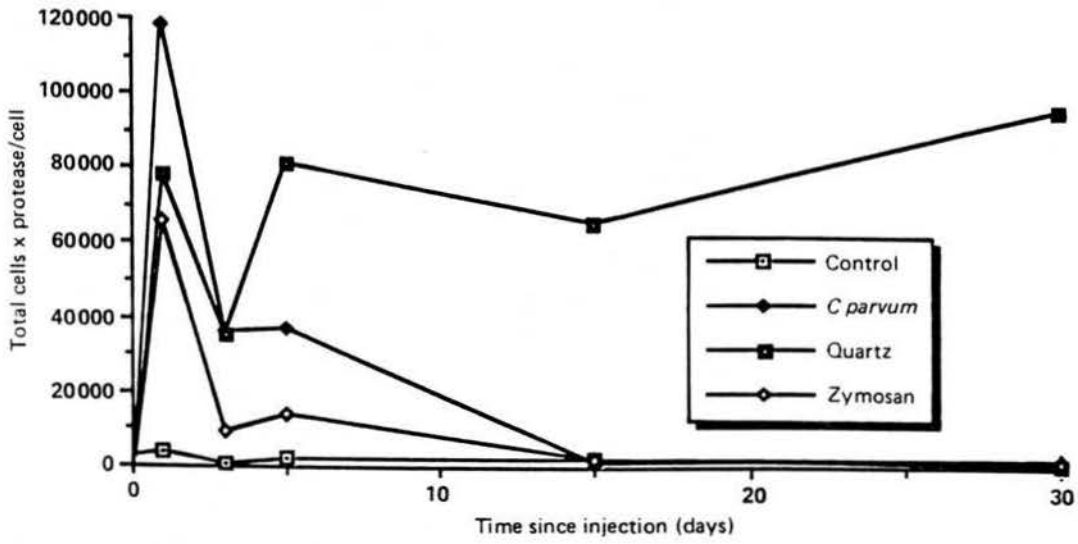


Figure 4 Bronchoalveolar leucocyte protease burden obtained by multiplying the total number of leucocytes in the lavage by the proteolytic activity per leucocyte. Results are expressed as total counts per minute of degraded fibronectin released.

potential protease burden in the alveolar region as there was substantial recruitment of inflammatory leucocytes. We therefore calculated the putative total protease burden of the lung by multiplying the proteolytic activity per leucocyte by the total number of leucocytes in the bronchoalveolar lavage (fig 4); this gave us a measure of the overall proteolytic potential of the lavage cells. The levels were greatest one day after injection, when the cellular influx was at its peak; *C parvum* had most activity at this time but quickly returned to much lower levels; only the quartz sample showed a prolonged increase which was 87-fold greater than all other samples 30 days after injection.

Exposing the control or inflammatory bronchoalveolar leucocytes to TiO_2, quartz, zymosan, and PMA in vitro had no effect on their proteolytic activity, irrespective of the preceding in vivo treatment (results not shown).

PLASMINOGEN ACTIVATOR ACTIVITY

Plasminogen activator activity was not increased by in vivo treatment with any of the particles, nor was there any increase following in vitro exposure of the bronchoalveolar leucocytes to TiO_2, quartz, zymosan, or PMA (results not shown).

ABILITY OF PARTICLES TO GENERATE CHEMOTAXINS IN SERUM

The two biological particulates, *C parvum* and zymosan, had the greatest effect in generating chemotaxins in normal rat serum (table). The sera treated with quartz and TiO_2 were considerably less active but still engendered three to four times more chemotaxis than untreated rat serum; the differences between the control and treated sera were significant at $p < 0{\cdot}001$.

TISSUE RESPONSE TO PARTICULATE EXPOSURE

At the 30 day time point no evidence of tissue damage was found in any of the treatment groups, except quartz. In the quartz dosed animals, however, there was considerable damage. Areas of alveolar lipoproteinosis were evident in conjunction with hyperplasia of the septal epithelium (fig 5).

Chemotaxis of control rat alveolar macrophages towards particle activated serum. Results are mean (SD) of migrated cells in 10 high power fields

	Particle treatment of serum				
	None	*C parvum*	*Zymosan*	*Quartz*	*TiO_2*
Cell number	3·7 (2·0)	59·4 (4·0)	73·2 (8·5)	16·3 (5·4)	12·0 (3·7)

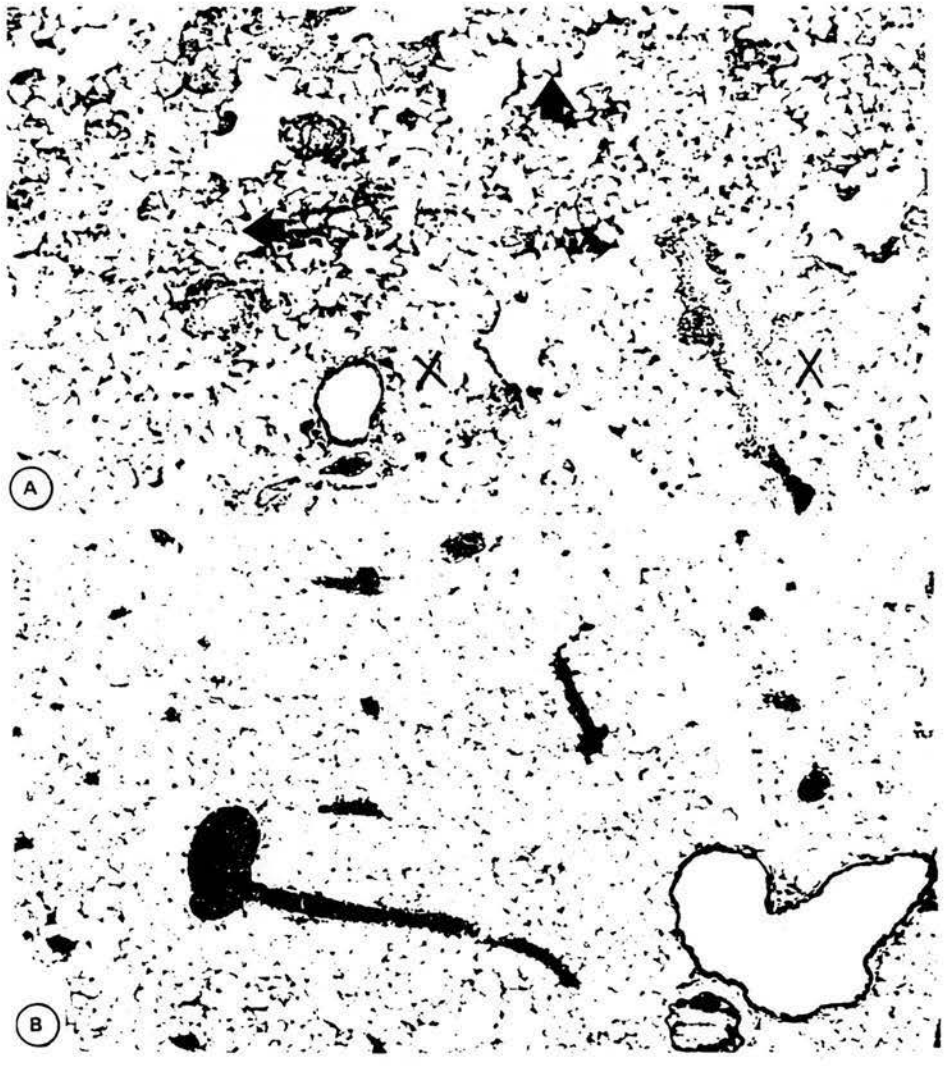


Figure 5 Tissue response 30 days after intratracheal instillation of 1 mg quartz (× 500 magnification). A, quartz dosed lung; B, control lung. Areas of alveolar lipoproteinosis (X) and type II cell hyperplasia (arrows) are extensive in (A).

Discussion

Three key factors could determine the persistence of lung inflammation in response to mineral dust: (1) intensity of inhalation exposure to particles (dose and duration of exposure), (2) deposition and retention of harmful particles within the alveolar region, and (3) persistence of the ability of particles retained in the lung to impart harm to the tissue. We have previously considered the effects of intensity of exposure to mineral dust in causing inflammation of the lung[21] and interstitial fibrosis[22] and have shown that both are time and dose related. We have also assessed the ability of particles to persist within lung tissue by measuring clearance rates following inhalation exposure and have shown that over the time scale we have used in the present study, little difference is evident in the clearance rates for harmful and non-harmful dusts.[23] Thus the major descriptor of the harmfulness of a dust may be the retention of its biological reactivity in the lung.

In this paper, we have considered the key parameters of persistence and reactivity of dust by assessing the magnitude and duration of the inflammatory response in rat lungs following a single intratracheal instillation of particles of different biological reactivity and durability. We have shown that deposition of particulate matter in the lung evokes an inflammatory response. This is true

whether the particulate is a typical biological inflammogen such as *C parvum* or zymosan (both of which are non-durable particles), or whether it is a harmful mineral dust, quartz, or the inert dust, titanium dioxide (both are non-degradable). Leukocyte chemotaxins are secreted by macrophages in response to a phagocytic stimulus[23 24] and this may account for the large influx of leucocytes one day after intratracheal injection. There may also be a direct role, however, for extracellular particles in influencing leucocyte recruitment as all the inflammogens could generate chemotaxins in serum. Our earlier work has shown that this chemotactic activity is generated by cleavage of the complement component, C3 and production of C5.[20] Because alveolar lining fluid contains C3,[25] particles may activate complement at the alveolar surface[7] and so contribute directly to recruitment of leucocytes. We have shown in the present study, however, that this mechanism is not the primary source of chemotaxins as the extent of complement activation by particles was not correlated with the magnitude of the response to those particles in vivo. Most particles depositing in the lung are cleared within one to two days by phagocytosis[26] or by extracellular transfer to lymph nodes.[27] The reduction in inflammation from one day onwards with three of the particulates thus supports the theory that phagocytosis (with attendant secretion of chemotaxins by the phagocytosing leucocytes) and complement activation by free particles may be important mechanisms in generating the initial acute inflammatory response. Inflammation in response to injection of quartz did not, however, subside after one day but increased up to 30 days after injection when there were 10-fold more leucocytes in the bronchoalveolar lavages of quartz exposed animals than in the controls or in the other exposed groups. We have recently produced evidence that the alveolitis persists up to 48 days after injection.[28] So, alternative explanations must be sought for the sustained inflammogenic properties of quartz.

A commonly invoked mechanism is that the pathogenic potential of a particle is related to its toxicity[29] but other mechanisms may also be implicated in dust induced inflammation in the lung. We have previously shown that leucocytes lavaged from rats exposed to quartz by inhalation were more than 99% viable[21] and remained so during 24 hours of culture in vitro. Similarly, in the present study, the lavaged leucocytes were also more than 99% viable for all particulates after intratracheal exposure (results not published). Studies exposing alveolar macrophages from control rats to low concentrations of quartz in vitro also showed that the cells remained viable, although increasing the concentration of quartz did lead to cell death.[16] We therefore propose a further mechanism for the persistence of quartz induced inflammation in the lung. The surface of quartz particles is highly reactive and interacts with cell membranes by acting as a hydrogen donor.[30] This may be the means whereby quartz kills cells at high concentrations, but at low concentrations, where the macrophages remain viable, interaction of intracellular quartz with cell components may lead to macrophage activation with its attendant consequences for enhancing the inflammatory response. We have shown previously that bronchoalveolar leucocytes from rats exposed to silica secrete increased amounts of interleukin-1.[31] Also, macrophages cultured in vitro in the presence of silica release interleukin-1,[31 32] leucocyte chemotaxins,[24] and fibroblast growth factor.[33]

Plasminogen activator is released by activated macrophages and is cited as being central to the inflammatory response.[5] In our rat model, there was no measurable change in production of plasminogen activator with any of the inflammogens at any time. This suggests that plasminogen activator plays a minor role in the inflammatory response to particulates in rat lung. As alveolitis and tissue damage in the quartz dosed rats were pronounced for up to 30 days after exposure, these results indicate that whereas plasminogen activator may play a part in the disease process in other models or in man, it need not be essential for tissue damage to occur. Other proteases released by inflammatory leucocytes may, however, play a part in the inflammatory response. These are the connective tissue proteases (elastase, collagenase, etc), which can act on connective tissue to generate fragments that are leucocyte chemotaxins.[34 35] Proteases may also enhance inflammation by activating components of complement[36] and in the long term, are thought to have a major part in the remodelling of connective tissue components that is associated with pathological change in the lungs.

In this study, we have shown that inflammatory bronchoalveolar leucocytes have enhanced ability to degrade the connective tissue component fibronectin compared with resident alveolar macrophages. We have also shown previously that other connective tissue molecules (collagen and laminin) can be similarly degraded by these cells.[12] The assay we used is representative of the type of interaction that might occur in the lung parenchyma where leucocytes come into close contact with the extracellular matrix. Increases in the observed proteolytic activity of the leucocytes tended to reflect the neutrophil content of the lavaged populations but there was residual increased proteolytic activity from 15 days onwards with all of the particles, when there were only neutrophils in the quartz elicited population. We have shown previously that inflammatory macrophages also exhibit enhanced proteolytic activity compared with resident alveolar macrophages.[12] Thus the persistently increased proteolytic activity from 15 days onwards is likely to be due to persis-

tence of activated macrophages. Our results are in agreement with previously published work where experimental exposure to particulates led to enhanced protease production by leucocytes in vitro both experimentally[37] and in bronchoalveolar lavage from occupationally exposed men.[38]

Our studies have shown a substantial increase in connective tissue protease per leucocyte during inflammation; also the number of leucocytes was dramatically increased. Taken together, these produce a potential leucocyte burden that is the product of the two variables. A calculation of the leucocyte protease burden (leucocyte protease burden = total cells × protease per cell) showed that 30 days after exposure to quartz there was an 87-fold increase in this parameter compared with controls. Whereas the leucocyte protease burden was increased during the acute inflammatory response to the biological particulates and TiO_2 as well as quartz, only quartz caused a sustained increase. The leucocyte protease burden of the four types of particle in this assay system are related in their pathological potential in man. Acute lung inflammation induced by bacterial infection is not usually associated with long term pathological change, even in pneumonia where there can be up to 70% neutrophils and substantial increases in total numbers of leucocytes in the lavage.[3] Similarly, TiO_2, despite widespread industrial use, is not associated with long term pathology in occupationally exposed workers,[39] nor did it evoke a persistent inflammatory response in our rat model. It was actually less inflammogenic than the two microbial particulates. The only material which elicited a persistent inflammatory response was quartz. Development of silicosis in exposed workers where there is a very low level of alveolar inflammation[3] is likely, therefore, to be related to the persistence of that alveolitis. Indeed, the progressive nature of the inflammatory response in our experiments following a single intratracheal injection of quartz may yield some information regarding the progressive nature of silicosis, even when exposure ceases.[1]

The proteolytic activity of the leucocyte populations in the present study was not enhanced by further exposure to particles for four hours in vitro. Thus, phagocytosis of dust is not the sole trigger of leucocyte proteolytic activity, but it may act in conjunction with other in vivo signals such as tumour necrosis factor (TNF) or interleukin-1 to activate the leucocytes; TNF has multiple activities in modulating leucocyte function and has been reported to be secreted in increased amounts by blood monocytes of miners with coalworkers' pneumoconiosis.[40]

In summary, there was an initial acute inflammatory response to all of the particulates deposited in the alveolar region of the lung. This rapidly subsided with degradable biological particulates. The durable, non-harmful particulate, TiO_2, also provoked only a transient inflammatory response but there was sustained inflammation in response to quartz. Thus durability of particles is not the major descriptor of their potential inflammogenicity. There is little evidence for differential clearance of TiO_2 and quartz in the lung[22] and so the difference in the inflammogenicity of TiO_2 and quartz particles may reflect, primarily, differences in their biological reactivity. This may be due to the toxicity of quartz, causing cell death and consequent leucocyte recruitment, at high concentrations. Alternatively, or perhaps also, as the particle:cell ratio alters with increasing leucocyte recruitment, the number of particles per leucocyte may reach such a low level that the cell is not killed; the quartz particles may then remain sequestered within macrophages, so activating them and thus prolonging the inflammatory response.

This study suggests that the difference between harmful and non-harmful particles in the lung is related to their ability to exert a continuing inflammatory stimulus on the lung tissue. The pathology associated with occupational exposure to quartz is likely to be related to the low level alveolitis seen in silicotic subjects, causing a persistent increase in the overall protease burden in the lungs. The progression of the quartz-induced alveolitis which we have described may have some bearing on the progressive nature of silicosis.

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Contrasting Bronchoalveolar Leukocyte Responses in Rats Inhaling Coal Mine Dust, Quartz, or Titanium Dioxide: Effects of Coal Rank, Airborne Mass Concentration, and Cessation of Exposure

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The aim of this study was to determine the bronchoalveolar leukocyte response to airborne coal mine dust; quartz and titanium dioxide were used as positive and negative controls, respectively. Groups of rats were exposed to airborne mass concentrations of 10 and 50 mg/m^3 of the dusts for 7 hr/day, 5 days/week and their bronchoalveolar space was lavaged at time points between 2 and 75 days of exposure, to assess the leukocyte response. This study revealed time-dependent and airborne mass concentration-dependent recruitment of neutrophils and macrophages into the bronchoalveolar region with coal mine dust inhalation but no real difference in the magnitude of the response between coal mine dusts from collieries mining coal of different rank and quartz content although the maximum quartz content in the dusts used was 6%. The inflammatory response was much less than that produced by quartz, at similar airborne mass concentrations, and more than that produced by titanium dioxide which was, in general, a poor inflammogen in the rat lung. Groups of rats were exposed to the airborne dusts for 32 or 75 days, then removed from the exposure chambers, and allowed to recover by breathing room air for a further 64 days. During this recovery period there was marked progression of the leukocyte response with quartz and persistence of the response with coal mine dust. Chronic recruitment of leukocytes to the lungs of individuals inhaling coal mine dust is likely to be an important factor in the development of coal workers' pneumoconiosis. © 1990 Academic Press, Inc.

INTRODUCTION

Occupational exposure to coal mine dust is associated with the respiratory disease coal workers' pneumoconiosis in which nodular fibrosis of the lung parenchyma is found (Morgan and Seaton, 1984); emphysema is also present in some coal workers (Ruckley *et al.*, 1984). Fibrous lesions are found in the lungs of persons occupationally exposed to pure quartz (silicosis) (Morgan and Seaton, 1984) and it has been suggested that the fibrogenicity of coal mine dust might be related to its quartz content since coal, while principally a carboniferous material, contains other minerals, including quartz, in different proportions. Coal is classified by the rank system, which is based mainly on carbon content but also takes into account other properties such as volatile matter content and caking quality. These differences in coal type arise from geological factors unique to any seam and so collieries mining different seams generate coal mine dust which is related to the rank of coal being mined. The prevalence of coal workers' pneumoconiosis

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varies and it has been suggested that rank of coal dust mined affects development of pneumoconiosis (Naeye *et al.*, 1971). There does not appear to be a simple relationship between the quartz content of the colliery dust and its toxicity; this is possibly due to the modulating influence of other minerals in coal mine dust (Le Bouffant *et al.*, 1977; Davis *et al.*, 1977; Walton *et al.*, 1977).

The response of the lung to the deposition of toxic mineral dusts includes accumulation of leukocytes in the bronchoalveolar space and these are important in bringing about subsequent pathological change (Gee and Walker Smith, 1984; Doll *et al.*, 1983). Little is known, however, about the effect of coal of different rank and quartz content on the early part of the pulmonary response since most experimental research concerning this aspect of the response to pneumoconiotic dusts has been confined to quartz and asbestos. The aim of the present study was therefore to examine, by bronchoalveolar lavage, the leukocyte response to coal mine dust inhalation in rats. For comparison, the toxic dust quartz was used as well as the nonpathogenic particulate titanium dioxide (TiO_2).

The specific objectives were (i) to assess the ability of airborne exposure to dust collected from the air of collieries to cause changes in the rat bronchoalveolar leukocyte population; (ii) to determine differences in activity between dusts collected from collieries mining coal of different rank; (iii) to examine the effect of increasing the airborne mass concentration; (iv) to determine the effect, on the leukocyte response, of ceasing exposure and allowing animals to breathe room air for a "recovery" period.

MATERIALS AND METHODS

Collection of Coal Mine Dusts

Three British collieries A, H, and L (Table 1) were selected to obtain airborne dust from seams of different coal rank and with different mineralogical contents. Coal mine dust was collected in the return roadway of a single face at each of the three collieries, into a Bondina sock mounted in a Wheatley cage with air drawn through at 1.5 m^3/s. The bulk dust was collected, stored under nitrogen, and irradiated before use to reduce the proliferation of contaminating microbes.

Analysis of Coal Mine Dust

The percentage ash content of the coal mine dust samples was obtained by ashing in a muffle furnace at 380°C for 3 hr. The ashed coal mine dusts were assayed for mineral content using the potassium bromide disk method of infrared analysis (Dodgson and Whittaker, 1973) which allows precise estimates of quartz, kaolinite, and mica.

Quartz and Titanium Dioxide Samples

A sample of pure quartz, Sikron F600 (A9950 Euro standard), was used as a positive control. Titanium dioxide (rutile; Tioxide Limited, Stockton on Tees) greater than 99% pure with no detectable quartz (R. Nolan, Mount Sinai, personal communication) was used as a control dust of low biological activity.

Rats

Syngeneic, PVG rats maintained under specific pathogen-free conditions at the Institute of Occupational Medicine's Animal Unit were used throughout. In the middle of the study the rats were microbiologically screened and no bacteriological or viral infection was detected. Male rats, 15 weeks of age at the commencement of dusting, were used throughout.

Exposure of Rats to Airborne Dust

Rats were exposed to airborne dust for 7 hr/day, 5 days/week in exposure chambers of the type described by Beckett (1975). The dusts were dispersed using either the Wright dust dispensers (Wright, 1950) or the Timbrell fibrous dust dispenser (Timbrell *et al.*, 1968) for coal mine dust containing relatively large particles among the fine dust. The target concentrations were 10 or 50 mg/m^3 of respirable dust (Orenstein, 1960) using the Casella MRE 113A dust sampler. The concentrations were measured daily and the day-to-day running mean concentration was kept on target by appropriate adjustment of the dust generation system and the chamber ventilation. As an aid to achieving the target concentration on each day, the rise in pressure across a filter sample was used as a semiquantitative guide to the amount of dust being sampled; a separate filter sample was taken daily specifically for this purpose. Although it was not measured in this study the similarity in size of the dusts (Table 1) means that similar masses of dust will have deposited in the lung for the three different coal mine dusts.

Bronchoalveolar Lavage

At 2, 4, 8, 16, 32, 52, and 75 days of exposure, rats were removed for bronchoalveolar lavage. In addition, groups of four rats exposed for 32 or 75 days were removed from exposure chambers and kept in cages in normal room air for 2 calendar months—these groups are hereafter referred to as "32 days plus recovery" and "75 days plus recovery." Rats were killed by injection with 2 ml Nembutal (Ceva, Ltd., Watford) and weighed and the lungs and trachea exposed by dissection. The lungs were exsanguinated using 30 ml of 0.85% NaCl at 37°C

TABLE 1
CHARACTERISTICS OF RESPIRABLE COAL MINE DUST USED IN THE STUDY

Coal mine dust	BCC coal rank code	Classification	% Ash	% In ash			Median aerodynamic diameter
				Kaolin	Mica	Quartz	
A	101	Anthracite	10.6[a]	0.8	4.3	1.4	4.6 (2.0)[b]
H	301	High rank coking coal	13.2	0.6	0.5	1.0	ND[c]
L	802	Low rank bituminous coal	53.2	18.1	0.0	6.7	5.0 (2.1)

[a] Mean of two separate analyses.
[b] Mean (SD) measured in a cascade impactor (see Materials and Methods).
[c] ND, not done.

and were then lavaged with 4 × 8-ml volumes of 0.85% NaCl at 37°C which was quickly withdrawn and decanted into ice-cold plastic tubes. The lungs were massaged by stroking outward toward the tip of each lobe on the second wash to increase the yield (Brain, 1971). The first 10 ml saline recovered was kept apart from the other washes to assess the levels of enzymes in the first wash, while the total cell yield from each rat was obtained by centrifuging, pooling, and counting. For each of the four dust-exposed rats and two control rats on any day, the total cell number was obtained using a Neubauer chamber and percentage viability assessed by trypan blue exclusion.

Quantitation of Leukocyte Type in the Bronchoalveolar Lavage

The proportion of different leukocyte types in 200 randomly selected cells was assessed using May–Grunewald-Giemsa-stained cytospin preparations.

Assay of Soluble Factors in the First 10 ml of Lavage Fluid

The following were assessed for their concentration in the first 10 ml of lavage fluid: lactate dehydrogenase (LDH) by the method of Wroblewski and Ladue (1955) and *N*-acetyl-β-D-glucosaminidase (NAG) by the method of Woollen *et al.* (1961).

Statistical Analysis

Results were obtained from four experimental and two control rats on each day. The data were transformed to the log scale before analysis. Initial assessment of the control data revealed the anticipated day-to-day variation, but no substantial differences between control rats and TiO_2-exposed rats.

Data from exposed rats were corrected by subtracting the control figures on that day to obtain a "standardized" figure for each rat in each assay. This figure was used in comparisons between each dust and TiO_2 using analysis of variance in the Genstat statistical computer package.

RESULTS

Analysis and Presentation of Results

Since preliminary analysis revealed no differences between control bronchoalveolar leukocytes and TiO_2-exposed leukocytes except at 75 days with 50 mg/m^3 when there was inflammation, the control data are omitted and all differences are related to TiO_2-exposed leukocytes.

The results from the analysis of variance showed that for each response variable there were significant differences between lengths of dusting time and between each dust and TiO_2. In addition, there was in each case a significant interaction between type of dust and length of dusting time, showing that the magnitude of the differences between dusts and TiO_2 varied over dusting times. The results below, therefore, focus on the differences between dusts and TiO_2 at specific dusting times, in order to highlight what times the response to the treatment dust was higher than the response to TiO_2. These differences were examined using Student's t test and values of $P < 0.01$ were taken to indicate a significant difference.

The standard error used in the comparisons was calculated using the residual mean square from the analysis of variance. In the figures, however, we felt it appropriate and necessary to show the "raw" data as they were obtained in the counts and assays. Thus the figures show untransformed data as means although any indicated statistically significant differences were obtained by analyzing data transformed and analyzed as described above.

Mineralogical Comparisons of the Coal Mine Dust Samples

The composition of the samples is given in Table 1.

Attained Airborne Mass Concentrations

The mean concentrations of dust in the air of the exposure chambers averaged over the 75-day exposure were all within 1 mg/m^3 of the target concentration with a standard error less than 3% of the mean.

Kinetics of the Bronchoalveolar Leukocyte Response

Control levels of the different parameters are not shown but there were no significant differences between TiO_2-exposed and control rats except at 75 days with 50 mg/m^3 TiO_2 and so all data are compared to those of the TiO_2-exposed controls. Figures 1 and 2 show the kinetics of the accumulation of macrophages and neutrophils (PMN), respectively, into the bronchoalveolar space of rats inhaling 10 or 50 mg/m^3 of coal mine dusts. Macrophage numbers in the coal mine dust-exposed rats were generally higher than in the TiO_2-exposed rats especially with increasing exposure. In terms of PMN recruitment there were substantial increases in numbers as from Day 52 at 10 mg/m^3 and from Day 32 at 50 mg/m^3, with all the coal mine dusts. Titanium dioxide inhalation had a negligible effect on leukocyte number except for a small increment in the number of neutrophils present at the longest time point with 50 mg/m^3. Quartz exposure produced a time-dependent increase in the number of both leukocyte types which was much greater than that produced by coal mine dust and much earlier in onset and greater in magnitude at 50 mg/m^3 than at 10 mg/m^3. The leukocytes found in the lavage came largely from the alveolar space as was clear in paraffin sections taken throughout the study, although there was also some bronchiolitis.

Kinetics of the Accumulation of Leukocyte Enzymes

Figures 3 and 4 show the kinetics of the accumulation of LDH and NAG in the bronchoalveolar lining fluid.

(*a*) *LDH*. There is clear evidence of a time-dependent increase in free lavage LDH in rats exposed to coal mine dusts H and A while dust L caused an early rise followed by a decline toward the end of exposure. At 50 mg/m^3 the LDH response was more variable but all of the coal mine dusts had caused a rise by 52 days although this had fallen again by Day 75. There were dramatic, significant increases in the levels of LDH on quartz exposure from 2 days onward at both 10 and 50 mg/m^3, and no effect on the levels of LDH was found in the TiO_2-exposed rats until the 75-day time point at 50 mg/m^3.

(*b*) *NAG*. The levels of the lysosomal enzyme NAG in the lavage fluid generally

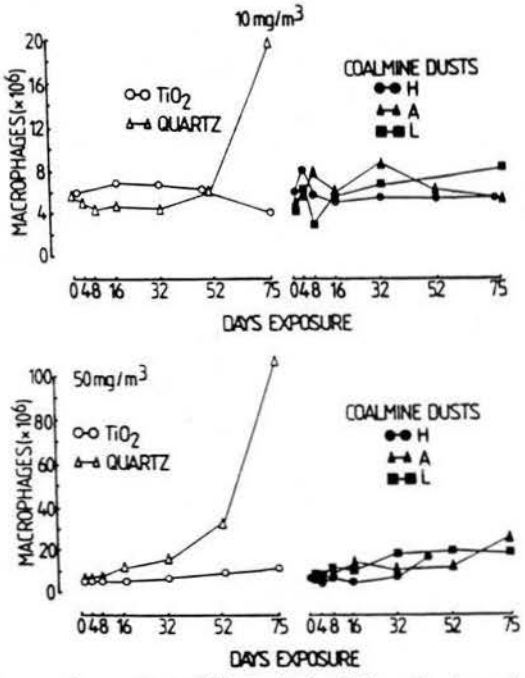


FIG. 1. Number of macrophages ($\bar{x} \times 10^6$/rat) obtained from the bronchoalveolar space of rats exposed to 10 mg/m^3 (upper panel) or 50 mg/m^3 (lower panel) of the indicated dusts up to 75 days of exposure. Data derived as the means of four rats at each time point. There were no significant differences between control and TiO_2-exposed rats except at 75 days with 50 mg/m^3 TiO_2. The error bars are omitted for the sake of clarity but these were less than 15% of the mean. All data are available in an IOM report by request.

followed the pattern described above for LDH with increased amounts accumulating as exposure to coal mine dusts progressed compared to TiO_2 at both 10 and 50 mg/m^3. Once again quartz exposure caused substantially more NAG to be present by 75 days with 10 mg/m^3 than was found with coal mine dust; the increase was earlier in onset but not substantially greater in magnitude at 50 mg/m^3.

The Effect of Coal Rank on Leukocyte Response

Reference to Figs. 1–4 shows that there were no consistent differences between the different coal mine dust samples in their ability to cause inflammation.

Effect of Increasing Airborne Mass Concentration

Table 2 shows data selected from Figs. 1–4 chosen to demonstrate the effect of increasing airborne mass concentration on three key parameters—macrophages, neutrophils, and LDH. Significant increases in these parameters were present withi increasing airborne mass concentration in almost every case except for TiO_2-exposed macrophages. The greatest increases were seen with quartz and the coal mine dusts, where increases ranged from approximately 5-fold to 10-fold in response to the 5-fold increase in the airborne mass concentration.

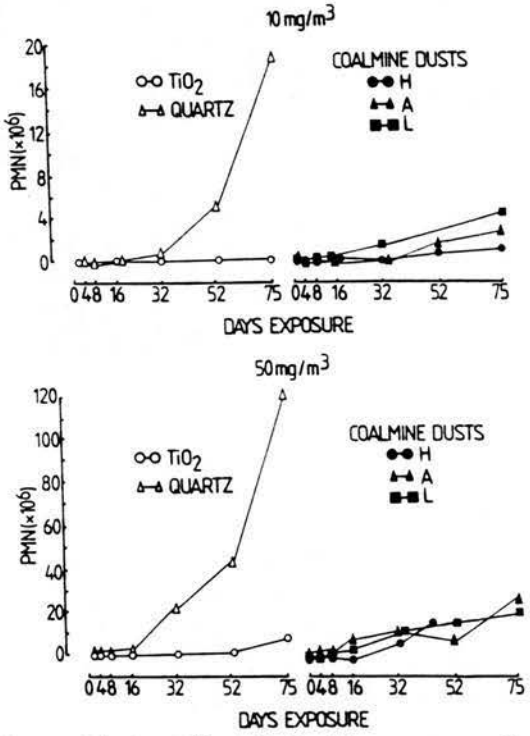


FIG. 2. Numbers of neutrophils ($\bar{x} \times 10^6$/rat) obtained from rats exposed to 10 mg/m^3 (upper panel) or 50 mg/m^3 (lower panel) of the indicated dusts. Data derived as described in legend to Fig. 1. There were no significant differences between TiO_2-exposed and control rats except at 75 days with 50 mg/m^3 TiO_2.

Effect of Cessation of Exposure on Leukocyte Response within the Lung

Table 3 shows the effect of cessation of exposure to airborne dust. In these experiments rats were maintained in room air for 64 days following inhalation exposure for 32 or 75 days. The single index chosen to represent the general response of the lung is neutrophil numbers. The data clearly show a dramatic significant increase in the extent of the inflammatory response continuing after cessation of exposure to quartz. For the coal mine dusts and TiO_2 at 50 mg/m^3/75 days there is no postexposure progression evident and, indeed, there is a suggestion of a general decrease in the extent of PMN recruitment. In general however even after 64 days breathing room air there was still inflammation present with all of the coal mine dusts and it was notable that the lowest level of inflammation in the recovery animals was found in the TiO_2-exposed group. A pattern similar to that found for the leukocyte response was found with other indices of bronchoalveolar leukocyte response such as NAG.

DISCUSSION

Many previous human and experimental studies on leukocyte responses in the lung parenchyma in disease have suggested that the quality and quantity of the

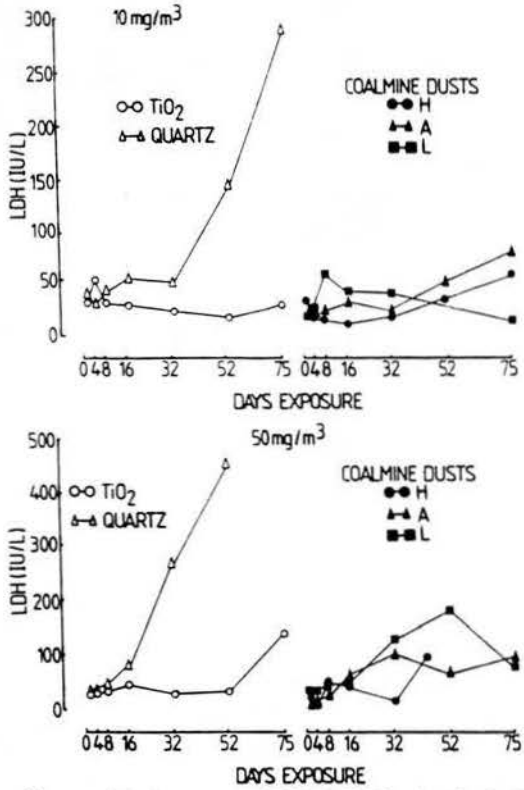


FIG. 3. Concentration of lactate dehydrogenase (LDH) ($\bar{x}$ IU/liter/rat) in the first 10 ml of lavage fluid obtained from rats exposed to 10 mg/m^3 (upper panel) or 50 mg/m^3 (lower panel) of the indicated dusts. Data derived as described in the legend to Fig. 1. There were no significant differences between TiO_2-exposed and control rats except at 50 mg/m^3 TiO_2 for 75 days.

leukocyte response are major factors in influencing disease type and progression. In the present study bronchoalveolar lavage was used to study the leukocyte response to inhaled coal mine dust in rats and to specifically address (a) whether dusts from the air of British collieries cause leukocyte responses detectable by bronchoalveolar lavage; (b) whether any differences could be detected in those responses in rats exposed to coal mine dust from different British collieries mining coal of different rank and mineralogical content; (c) the extent of the bronchoalveolar leukocyte response to inhaled coal mine dust compared to that elicited by the toxic dust quartz and the nontoxic dust titanium dioxide; (d) the effect of increasing the airborne mass concentration; (e) the effect of cessation of exposure on the leukocyte response.

The results revealed that inhalation exposure to coal mine dust collected from the air of British collieries elicited a time-dependent inflammatory response characterized by increased numbers of neutrophils and macrophages in the bronchoalveolar region and increased levels of free leukocyte enzymes in the lavage fluid.

There were no marked differences between the coal mine dusts of different rank

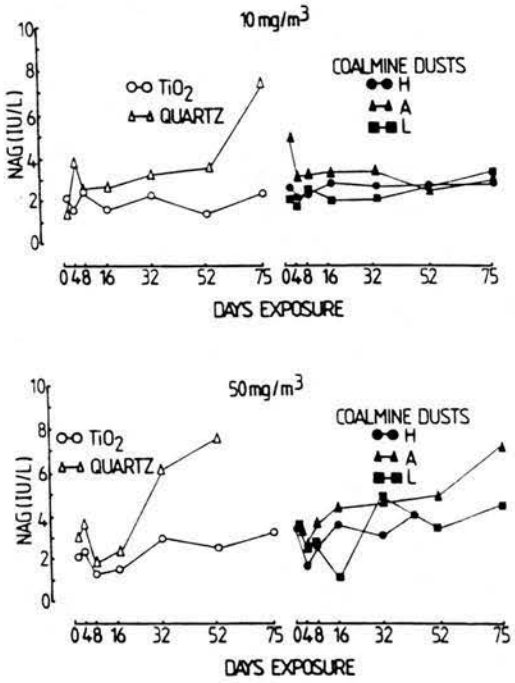


FIG. 4. Concentration of *n*-acetylglucosaminidase (NAG) ($\bar{x}$ IU/liter/rat) in the first 10 ml of lavage fluid obtained from rats exposed to 10 mg/m^3 (upper panel) or 50 mg/m^3 (lower panel) of the indicated dust. Data derived as described in the legend to Fig. 1. There were no significant differences between TiO_2-exposed and control rats except at 50 mg/m^3 TiO_2 for 75 days.

and the quartz content of the coal, in terms of ability to cause inflammation in the bronchoalveolar region following inhalation exposure in the present study. From this result it was evident that the percentage of quartz alone was not the main descriptor of the pathogenicity of the coal mine dust since anthracite had very low amounts of quartz (1.4% in dust) while the low rank dust had substantially more (6.7% in dust). The inflammogenicity of anthracite, with virtually no quartz, suggests that some factor present in coal mine dust, other than quartz, can imbue it with potential to cause inflammation. Additionally, the lack of difference between anthracite and low rank (containing about five times more quartz) could be due to the presence of other minerals which ameliorate the toxicity of quartz (Le Bouffant *et al.*, 1977; Davis *et al.*, 1977) in the high quartz dust. However, it is of note that the maximum level of quartz present within the coal mine dusts used in the present study was below 7.5%, a level reported in epidemiological studies at which quartz has no influence on the toxicity of coal mine dust (Walton *et al.*, 1977). This is backed up by animal studies where percentages of quartz below 10% had minimal pneumoconiotic potential but percentages of 10, 15, and 20% endowed dusts with clear pneumoconiotic potential (Ross *et al.*, 1962; Rotaru and Lazarescu, 1982). Additionally, an increase in quartz content of coal mine dust, from a British average of 5% to 8–13% in a Scottish colliery, was associated with marked progression of pneumoconiosis in the exposed coal miners (Seaton *et al.*,

TABLE 2

NUMBER OF MACROPHAGES, PMN, AND CONCENTRATION OF LDH, IN LAVAGE AT 32 AND 75 DAYS OF EXPOSURE TO THE INDICATED DUSTS AT 10 OR 50 mg/m³. THIS DEMONSTRATES THE AIRBORNE MASS CONCENTRATION DEPENDENCE OF THE RESPONSE EXCEPT WITH TiO_2 AT 32 DAYS WHEN THERE WAS NO SIGNIFICANT DIFFERENCE BETWEEN TiO_2-EXPOSED AND CONTROL RATS

	Macrophages[a]		PMN[a]		LDH[b]	
	10 mg/m³	50 mg/m³	10 mg/m³	50 mg/m³	10 mg/m³	50 mg/m³
			Response at 32 days			
TiO_2	6.67 (2.73)	6.31 (1.00)	0.00 (0.00)	0.14 (0.12)	24.50 (13.03)	29.00 (2.83)
Quartz	4.28 (1.42)	15.08 (3.83)	0.78 (0.57)	22.52 (4.18)	50.33 (9.50)	272.75 (41.33)
H	5.37 (0.88)	7.48 (2.16)	0.04 (0.03)	5.66 (2.43)	20.00 (10.42)	22.25 (7.37)
A	8.51 (1.44)	10.04 (5.09)	0.32 (0.53)	11.94 (3.49)	26.25 (8.02)	106.50 (34.14)
L	6.44 (3.18)	17.06 (4.44)	1.45 (0.60)	11.73 (4.78)	42.00 (22.06)	134.75 (17.35)
			Response at 75 days			
TiO_2	4.04 (1.17)	9.30 (3.39)	0.02 (0.02)	7.10 (3.29)	29.25 (12.84)	142.00 (30.43)
Quartz	19.79 (4.37)	105.43 (34.08)	18.78 (7.00)	119.18 (36.74)	285.50 (69.14)	ND
H	5.44 (1.07)	ND[c]	0.89 (0.43)	ND	57.25 (16.15)	ND
A	5.20 (1.75)	24.40 (5.88)	2.47 (0.34)	24.00 (14.72)	75.50 (21.36)	100.75 (12.12)
L	8.12 (1.31)	17.97 (4.28)	4.34 (0.28)	20.16 (4.38)	14.75 (18.03)	86.25 (4.65)

[a] Data given as mean (SD) × 10^6 cells/rat.
[b] Data given as mean (SD) International Units/liter.
[c] ND, not done.

1981). It would be of great interest therefore to compare a coal mine dust of high (>10%) and low (<10%) quartz content using the same assay systems as those described here. A more clear assessment of the role of quartz in coal workers' pneumoconiosis would then be obtained.

The fact that substantial inflammatory responses were caused by dust where the

TABLE 3

EFFECT OF CESSATION OF EXPOSURE (RECOVERY) ON THE NUMBER OF NEUTROPHILS OBTAINED BY BRONCHOALVEOLAR LAVAGE

Dust	32 Days	32 Days + recovery	75 Days	75 Days + recovery
		10 mg/m³		
TiO_2	0 (0)	0 (0)	0.02 (0.02)	0.03 (0.04)
Quartz	0.78 (0.57)	17.53 (0.90)	18.78 (7.00)	20.88 (11.45)
H	0.04 (0.03)	0.06 (0.06)	0.89 (0.43)	0.72 (0.67)
A	0.32 (0.53)	0.11 (0.08)	2.47 (0.34)	2.03 (0.34)
L	1.45 (0.60)	0.58 (0.24)	4.33 (0.28)	0.84 (0.12)
		50 mg/m³		
TiO_2	0.14 (0.12)	ND[a]	7.10 (3.29)	3.74 (2.13)
Quartz	22.52 (4.18)	71.38 (29.01)	119.18 (36.74)	213.66 (54.64)
H	5.66 (2.43)	3.24 (0.72)	ND	8.60 (0.60)
A	11.94 (3.49)	11.97 (1.69)	24.00 (14.72)	12.19 (5.28)
L	11.73 (4.78)	7.08 (1.54)	20.16 (4.38)	11.41 (4.10)

Note. Rats were exposed for 32 or 75 days to the indicated dusts at 10 or 50 mg/m³, then lavaged or allowed to recover by breathing room air for 2 months, and then lavaged. The mean (SD) number of neutrophils × 10^6/rat is shown.

[a] ND, not done.

quartz did not appear to contribute to the toxicity supports previous epidemiological studies showing pneumoconiosis in miners mining high rank coal (Bennett *et al.*, 1979; Naeye *et al.*, 1971). It also confirms that substances present in coal mine dust apart from quartz are capable of causing considerable inflammation even at 10 mg/m^3 airborne mass concentration.

The magnitude of the response to coal mine dust in the present study was markedly more than that produced by the nonpathogenic particulate, TiO_2, and less than that produced by quartz at the same airborne mass concentration. This no doubt reflects the ability of quartz to produce widespread severe pulmonary injury and fibrosis in exposed humans (Morgan and Seaton, 1984) and animals, and of TiO_2 to be largely nonpathogenic (Richards *et al.*, 1985) although at high lung dose TiO_2 has been reported to cause lung injury and pathological change (Lee *et al.*, 1985). Evidence that the 50 mg/m^3 regimen of TiO_2 could have led to pathological change in the long term was evident in the inflammatory response present in the lungs of rats inhaling 50 mg/m^3 TiO_2 for 75 days. Additionally the inflammation present in the lungs following this TiO_2 exposure was not resolved but was still present following recovery suggesting that there was ongoing lung injury during cessation of exposure.

Coal mine dust was substantially less toxic than quartz at the doses examined but the dose relatedness of the inflammation caused by coal mine dust suggests that biologically significant bronchoalveolar leukocyte recruitment and activation could occur during chronic exposure to lower levels of dust than 10 mg/m^3, at levels pertaining in coal mines.

In one previous study crushed coal was delivered to rats by inhalation at airborne mass concentrations similar to those used here and bronchoalveolar leukocyte responses assessed (Bingham *et al.*, 1977). In that study there was no marked recruitment of bronchoalveolar leukocytes to the lung in the long term. Several explanations can be put forward as to how this result was produced including the fact that airborne coal mine dust was not used and particle size and deposition characteristics of crushed coal are not necessarily relevant to coal mine dusts. In another study coal mine dust was instilled into rat lungs on four separate occasions over 2 weeks and there was increased recruitment of macrophages to the airspaces over the subsequent 6 months (Martin *et al.*, 1980). Coal mine dust instilled into the lungs of hamsters on repeated occasions also elicited a neutrophil/macrophage alveolitis (Ip *et al.*, 1986).

With quartz there was marked progression of the inflammatory response during the period when the rats were removed from exposure and breathed room air for 64 days. In the cases of the different coal mine dust samples there was no evidence of such a progression although the inflammation did persist and was not entirely resolved within the 64 days of follow-up. In rats inhaling 50 mg/m^3 of TiO_2 for 75 days inflammation waned during the period when the rats were not breathing dust but did not entirely resolve. By the end of the recovery period, at 50 mg/m^3, TiO_2-exposed rats did however have less severe alveolitis than either coal mine dust- or quartz-exposed rats. Both quartz and coal mine dust are capable of causing a progressive form of pneumoconiosis, silicosis and progressive massive fibrosis, respectively, and persistent alveolitis during a period when the animals

were not inhaling quartz and coal mine dust could be a factor in these progressive diseases. One previous study (Susskind *et al.*, 1988) describes persistently increased pulmonary permeability, measured as a more rapid clearance of Tc99m DTPA, in retired coal miners, suggesting that there is persistent pulmonary inflammation following cessation of exposure to coal mine dust in man.

Current views on the cellular events underlying development of pulmonary fibrosis and emphysema, diseases prevalent in coal workers, have arisen in large part from studies utilizing bronchoalveolar lavage in human and animal models (Hunninghake *et al.*, 1979; Crystal *et al.*, 1981; Janoff, 1985; Snider, 1986). The models which have evolved from these studies implicate the leukocytes—macrophages, PMN, and lymphocytes—as primary effector cells in tissue derangement in the alveolar region. Leukocytes recruited to the alveolar region under the influence of a range of stimuli, which are mineral dusts in the case of the pneumoconioses, along with the resident leukocytes of the alveolar space, are activated in the milieu of an inflammatory or immunologically stimulated lung. These activated leukocytes then mediate, to varying degrees, the contrasting processes of tissue injury and destruction, regeneration, and repair; the balance between these processes determines the course of the disease. A recent important study by Rom *et al.* (1987) described the bronchoalveolar lavage cell profile in coal workers' pneumoconiosis which showed an increase in total cell number and a modest increase in proportion of neutrophils. Rom and colleagues also demonstrated that the leukocytes from patients with coal workers' pneumoconiosis tended to produce increased amounts of oxidant and growth factor. The present study provides good evidence that the rat responds similarly to man, with sustained recruitment of inflammatory leukocytes to the alveolar region on exposure to airborne coal mine dust. The major difference between the rat and human response shown by Rom *et al.* (1987) is the high proportion of neutrophils found in the bronchoalveolar region of rats. This very likely reflects the relatively high levels of airborne dust used in the experimental situation.

The present study was limited in its assessment of the functional activity of the coal mine dust-elicited leukocytes. The only functional measure of leukocyte activity assayed in the present study was the presence of the lysosomal protease NAG in the lavage fluid and increased amounts were detected as inflammation developed in the rats inhaling coal mine dust. Both acid protease such as NAG and neutral proteases have been implicated in lung injury during persistent inflammation leading to fibrosis and emphysema (Fantone and Ward, 1984). In a companion study to the present one we have described the release of protease, with the ability to degrade the connective tissue component fibronectin, by bronchoalveolar leukocytes from rats inhaling coal mine dust (Brown and Donaldson, 1989). Similarly, increased levels of protease have been found in the bronchoalveolar lavage of coal workers (Sablonniere *et al.*, 1983). These leukocyte proteases could enhance inflammation by causing epithelial injury (Donaldson *et al.*, 1988) and attacking connective tissue components (Brown and Donaldson, 1987) so contributing to fibrosis and emphysema. However, as has been shown in other studies of mineral dust-induced inflammatory bronchoalveolar leukocytes, these

cells are also likely to be releasing, along with proteases, growth factors capable of enhancing mesenchymal cell activity (Sjostrand and Rylander, 1987) so leading to fibrosis.

In summary the present study has revealed airborne mass concentration and time-dependent accumulation of inflammatory leukocytes in the bronchoalveolar region of rats inhaling dust collected from the air of three British collieries mining coal of different rank. All of the different coal mine dusts caused pulmonary inflammation and there was no real difference between coal mine dust of different rank; the inflammation caused by coal mine dust persisted for 2 months after exposure had ceased. The low levels of quartz in the different coal mine dust samples emphasized that mineral components other than quartz are toxic to rat lung. The persistence of the lung injury after cessation of exposure has important implications for understanding disease progression. TiO_2 inhalation had little inflammatory effect except at 50 mg/m^3 for extended periods of time. Quartz caused substantially more inflammation than the coal mine dusts and this progressed dramatically during recovery when animals were inhaling clean room air; this may be related to the progression of silicosis found in some individuals.

ACKNOWLEDGMENTS

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IMPAIRED CHEMOTACTIC RESPONSES OF BRONCHOALVEOLAR LEUKOCYTES IN EXPERIMENTAL PNEUMOCONIOSIS

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SUMMARY

Rats were exposed to clouds of the following pneumoconiotic dusts: quartz, coal-mine dust, and chrysotile asbestos at 10 or 50 mg/m^3 for 8, 32, and 75 days; for comparison, rats were also exposed to the non-pathogenic dust titanium dioxide (TiO_2). The bronchoalveolar leukocytes (macrophages and neutrophils) from dust-exposed and control rats were obtained by lavage and tested for their ability to migrate toward zymosan-activated serum. Varying amounts of neutrophils were present depending on the ability of the dust to cause inflammation and the length of exposure. There was a marked loss of chemotactic ability in leukocytes from rats inhaling the pneumoconiotic dusts compared with controls; TiO_2-exposed leukocytes had some impairment of chemotaxis, but this was substantially less than that found with the pneumoconiotic dusts. The loss of chemotactic activity did not correlate with the percentage of neutrophils in the lavage cells except when there were very high levels of neutrophils, and there was substantial impairment of chemotaxis with negligible numbers of neutrophils, showing that macrophage chemotaxis was impaired. A phagocytic burden within the leucocytes was not sufficient alone to inhibit chemotaxis, nor was the loss of chemotactic activity due to occupied receptors, since incubation failed to restore chemotaxis. Loss to chemotactic activity by leukocytes from pneumoconiotic dust-exposed lung could be an important factor in the development of pneumoconiosis.

KEY WORDS—Pneumoconiosis, bronchoalveolar leukocytes, chemotaxis, phagocytosis, inflammation, quartz, coal-mine dust.

INTRODUCTION

Lavage of the bronchoalveolar space in humans and in laboratory animals has revealed that exposure to dusts commonly associated with pneumoconiosis (e.g., quartz and asbestos) results in recruitment of leukocytes to the lung parenchyma.[1-3] Once accumulated in the bronchoalveolar region, these leukocytes are considered to be important for the development of pathological change through altered functional activities and secretions. The definitive pathological change found in pneumoconiosis is fibrosis of the lung parenchyma, although emphysema has also been reported in the lungs of coal-miners[4] and in the lungs of rats exposed to quartz.[5]

Not all dusts depositing in the lung cause pneumoconiosis, and in experimental models, different dusts delivered at a similar airborne mass concentration are capable of causing different degrees of inflammatory cell recruitment into the lungs of experimental animals and this probably relates to their differential pathogenicity.[3] Factors other than those inducing increased numbers and altered secretory activity of leukocytes within the lung could operate in causing a dust to be pathogenic. One possibility is that, following exposure to pathogenic dusts, the bronchoalveolar leukocytes do not migrate as well as in normal lung or in lung exposed to non-pathogenic dust. If this were to be the case, then the pathogenic dusts might not be so well cleared from the lung and dust-activated leukocytes would be more likely to persist, releasing active substances contributing to pathological change. In addition, other aspects of host defence which rely on macrophage chemotaxis could be compromised.

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As part of a study into leukocyte recruitment into the lungs of rats exposed to pneumoconiosis-producing dusts, we examined the chemotactic activity of bronchoalveolar leukocytes lavaged from these animals.

METHOD

Rats

Syngeneic, PVG rats, SPF maintained and 15 weeks old at commencement of exposure, were used throughout.

Minerals

The dusts used in this study were (a) titanium dioxide (rutile form), obtained from Tioxide Ltd, Stockton on Tees; (b) the quartz standard DQ12; (c) coal-mine dust collected from the air of British collieries mining anthracite (A), high rank (coking) coal (H), and low rank (bituminous) coal (L). Airborne coal-mine dust samples were collected on dry Bondina socks mounted in the return airway of a single face at each of the three collieries; full details of the collection procedure are described elsewhere;[6] and (d) chrysotile asbestos (UICC standard sample 'A'). Details of the mineralogical and size characteristics of these dust samples are described elsewhere.[6,7]

Inhalation exposure

Groups of 48 rats were exposed to airborne dust for 5 days/week, 7 h/day in exposure chambers described by Beckett.[8] The dusts were dispensed using either Wright or Timbrell dust dispensers. The concentration of dust in the chamber was monitored as the mass concentration of respirable dust defined by the Johannesburg sampling criterion[9] using standard dust samplers. Full details of the exposure system are described elsewhere[6] and the target airborne mass concentrations of 10 or 50 mg/m^3 were attained.

Rats were exposed for 8, 32, or 75 days and then removed from the chamber; on each day, control rats of similar age were also used.

Intratracheal instillation

Rats under general anaesthesia had their trachea exposed by dissection and 0·5 ml of sterile saline containing 1 mg of quartz was introduced into the lung; the skin was closed with metal clips and the animals recovered within minutes.

Bronchoalveolar lavage

At 8, 32, and 75 days into inhalational exposure, groups of four experimental rats from each exposure group were removed from the chamber and subjected to bronchoalveolar lavage; two control rats maintained in room air were similarly treated. Lungs were exsanguinated and the bronchoalveolar space was lavaged with 4×8 ml volumes of saline at 37°C. Bronchoalveolar leukocytes were concentrated by centrifugation, and a differential leukocyte count was performed on May–Grunwald Giemsa-stained cytospin preparations. At each time-point, cells from four dust-exposed rats and two control rats were pooled for use in the chemotaxis assay.

Rats exposed to dust by intratracheal instillation, plus controls, were lavaged in the same manner.

Assay of bronchoalveolar leukocyte chemotaxis

Chemotaxis was assessed using Blindwell chambers as described elsewhere.[6] Briefly, 200 μl of 5 per cent zymosan-activated rat serum were placed in the lower compartment and a filter (Nucleopore, Pleasanton, CA) placed on top. The top compartment was screwed down and 6×10^5 bronchoalveolar leukocytes in 400 μl of RPMI medium (Gibco, Paisley), +1 per cent BSA (Sigma, Poole) were placed in the upper compartment. The filters used were 5 μm pore size and incubation was for 3·5 h at 37°C in 5 per cent CO_2 to allow migration of cells through the filter. At the end of the incubation period the filter was removed, washed, stained, and allowed to dry before being mounted on a slide in plastic mountant. Two chambers were set up for each condition and the number of cells that migrated to the underside of the filter in five high-power fields ($\times$ 1000) was assessed for each filter.

Statistical analysis of results

Data were analysed by analysis of variance and comparisons were made using a '*t*'-test.

RESULTS

Bronchoalveolar leukocyte response in dust-exposed rats

Table I shows the total numbers of leukocytes lavaged from the lungs of rats exposed to the dusts and the percentage of neutrophils (lymphocytes and

Table I—Percentage of neutrophils and total leukocytes in bronchoalveolar lavage of rats inhaling different dusts at the indicated airborne mass concentrations for the indicated number of days

Dust	Airborne mass concentration (mg/m^3)	Days' exposure					
		8		32		75	
		% PMN	Total	% PMN	Total	% PMN	Total
TiO_2	10	0 (0)	6·8 (0·7)	0 (0)	6·8 (2·8)	0·6 (0·5)	4·1 (1·1)
Quartz	10	1·0 (1·0)	4·3 (1·5)	16·0 (14·1)	5·3 (1·2)	44·7 (4·5)	33·5 (18·2)
Chrysotile	10	21·0 (3·4)	12·55 (4·34)	14·0 (1·2)	12·62 (7·83)	29·5 (6·7)	6·45 (1·51)
Coal-mine dust H	10	0·5 (0·4)	5·8 (1·2)	0·8 (0·5)	5·6 (0·8)	13·5 (6·4)	6·6 (1·1)
	50	5·6 (1·7)	8·8 (1·2)	40·2 (9·5)	40·9 (7·4)	ND	232·0 (68·7)
Coal-mine dust A	10	1·0 (0·7)	8·2 (0·8)	3·2 (5·2)	9·2 (1·6)	31·8 (9·0)	8·1 (1·4)
	50	23·5 (6·4)	11·4 (2·6)	52·3 (17·6)	21·0 (6·4)	45·5 (13·2)	50·0 (15·7)
Coal-mine dust L	10	2·8 (1·7)	3·0 (0·8)	18·0 (5·7)	8·4 (3·8)	34·5 (2·4)	12·6 (1·7)
	50	4·8 (1·3)	12·2 (4·6)	38·0 (11·5)	30·2 (6·8)	51·2 (4·1)	39·4 (7·7)
Control		0 (0)	6·3 (1·4)				

Data as mean, with standard deviation in parentheses, of the percentage of PMN or total cells × 10^6 for four rats. The cell type over and above the neutrophils was predominantly macrophages with less than a total of 5 per cent of lymphocytes, mast cells, basophils, and eosinophils in any population.

PMN = Neutrophil.

basophils comprised the remainder and were always less than 5 per cent). The data revealed that inhalation of TiO_2 had virtually no effect when given at 10 mg/m^3, but that at this airborne mass concentration the pneumoconiotic dusts also showed little effect initially, except for chrysotile which rapidly elicited an inflammatory response. Not until towards 32 and 75 days with quartz and coal-mine dust was there increased recruitment and increased proportions of neutrophils, both changes indicative of inflammation. At 50 mg/m^3 with the coal-mine dusts, these inflammatory effects were earlier in onset and greater in magnitude.

Chemotaxis versus chemokinesis in leukocyte migration

To ensure that chemotaxis was the dominant activity being measured in each sample, and not chemokinesis, we used a modified 'checker board' method. Typical results were as follows (all subsequent data given as mean ± SD migration cells/high power field): spontaneous migration, 0·0 ± 0·0; chemokinesis (measured as migration with 5 per cent zymosan-activated serum in both the upper and the lower compartments) 14·8 ± 6·6; chemotaxis (measured with zymosan-activated serum in the lower compartment), 46·4 ± 4·8. These results

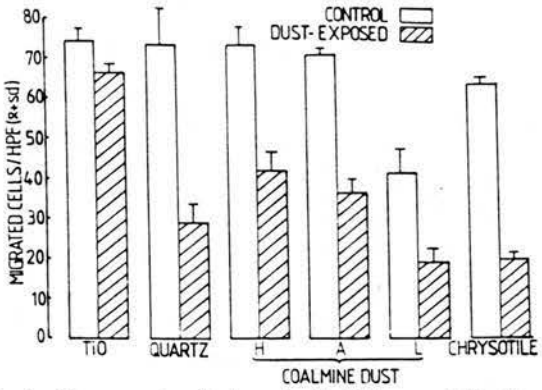


Fig. 1—Mean number of migrated cells/high power field of bronchoalveolar leukocytes from control rats and rats inhaling the indicated dusts at 10 mg/m³. Open bars show controls and hatched bars show dust-exposed. Error bars represent 1 SD. Data obtained by pooling all data from 8, 32, and 75 days. Data derived from two chambers, five high power fields/chamber at each time-point for each dust. Significant reductions in dust-exposed ($P < 0.05$) with all dusts except coal-mine dust L and TiO_2.

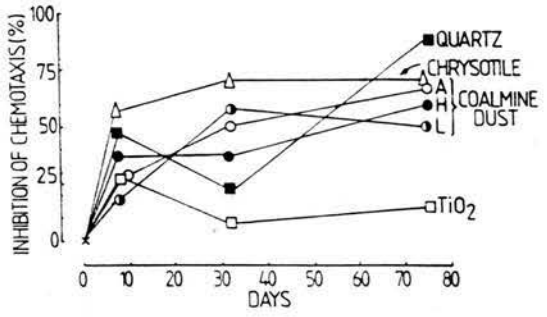


Fig. 2—Inhibition of chemotaxis caused by inhalation exposure to the indicated dusts at 10 mg/m³ with time of exposure. Data calculated as

$$\frac{\text{mean chemotaxis in dust-exposed}}{\text{mean chemotaxis in control}} \times \frac{100}{1}$$

obtained from two chemotaxis chambers, five high power fields/time-point for each dust. Significantly more inhibition, compared to TiO_2, with quartz and chrysotile at 8 days and with all pneumoconiotic dusts at 32 days and 75 days ($P < 0.05$)

confirm that, whilst there was some chemokinetic activity, the majority of the migration was in fact chemotaxis; migration in the Blindwell chambers will henceforth be referred to as chemotaxis.

Effect of dust exposure on chemotaxis of bronchoalveolar leukocytes

Figure 1 shows the data obtained for all chemotaxis experiments with leukocytes from rats exposed, by inhalation, to 10 mg/m³ of the five dusts. These data clearly show that inhalation exposure to TiO_2 had very little effect of the ability of cells to chemotact, whereas exposure to quartz, coal-mine dusts, and chrysotile asbestos was associated with a marked reduction in the ability of the bronchoalveolar leukocytes to chemotact (see legend to Fig. 1 for statistical significance). The data shown in Fig. 1 are the mean and standard deviation of the pooled results obtained on days 8, 32, and 75.

In Fig. 2, and in the subsequent figures, the data are expressed as per cent inhibition of chemotaxis compared with controls on the day, to highlight more clearly the effects of dust exposure on chemotaxis at 10 mg/m³. Figure 2 shows the time dependence of the inhibition of chemotaxis. Although inhibition of 25 per cent was present with TiO_2 at 8 days, the inhibitory effect did not exceed 17 per cent thereafter and was substantially less than with all the other dusts at subsequent time-points. In the case of quartz, the coal-mine dusts, and chrysotile asbestos, there was a greater loss of chemotactic activity with time. At 8 days, the increase was

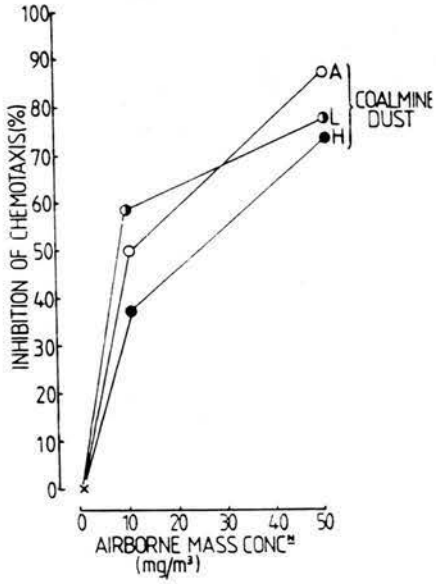


Fig. 3—Inhibition of chemotaxis caused by inhalation of coal-mine dusts at 10 and 50 mg/m³ airborne mass concentration. Data calculated as given in the legend to Fig. 2. Significantly more inhibition caused at 50 compared with 10 mg/m³ with all three coal-mine dusts

already substantial; thereafter, the increases were not as dramatic but with all the pneumoconiotic dusts there was 50–80 per cent inhibition of chemotaxis by day 75.

Figure 3 shows the effect of increasing the airborne mass concentration of coal-mine dusts. The

increase from 10 to 50 mg/m^3 airborne mass concentration was associated with a dramatic increase in the impairment of chemotaxis observable in the coal-mine dust-exposed bronchoalveolar leukocytes, reaching 70–90 per cent at 50 mg/m^3 (the data shown are for day 75).

Effect of intratracheal instillation of pneumoconiotic dust on chemotaxis

Intratracheal instillation of quartz also produced a significant reduction in the chemotactic activity of the bronchoalveolar leukocytes (all data given as $\bar{x}$ (SD) of migrated cells/high power field): control bronchoalveolar leukocytes (99 per cent macrophages, 1 per cent lymphocytes), 36·3 (10·0); quartz-exposed bronchoalveolar leukocytes, (61 per cent macrophages, 36 per cent neutrophils, 3 per cent lymphocytes), 6·8 (2·5).

Attempts to elucidate the mechanism of dust-related impairment of leukocyte chemotaxis

Limited experiments were carried out to try to elucidate the mechanism whereby dust deposition in the lungs of rats, as described above, caused loss of ability to chemotact.

(a) Role of neutrophils in loss of chemotaxis As shown in Table I, many of the leukocyte populations obtained from the bronchoalveolar space of rats inhaling dust were inflammatory and were characterized by the presence of variable numbers of neutrophils. It seemed possible, therefore, that neutrophils migrate less than alveolar macrophages in the system we use and so we examined the role of neutrophils in the loss of chemotaxis. When acutely exudated neutrophils (83 per cent pure and obtained from the lungs of rats exposed to 1·4 mg of a heat-killed preparation of *Corynebacterium parvum*) were tested for ability to migrate in an identical system, the following result was obtained ($\bar{x}$ (SD) of ten high-power fields): control macrophages, 36·3 (10·0); neutrophil-enriched population 17·8 (2·6). Thus, at high proportions of neutrophils up to 50 per cent of the loss of chemotaxis could be attributable to the fact that these cells did not chemotact so well as macrophages. Figure 4, however, shows the percentage of neutrophils plotted against the percentage of inhibition for data obtained throughout the entire study for all the dusts. It clearly shows that there is no relationship between the percentage of neutrophils present and the loss of chemotaxis

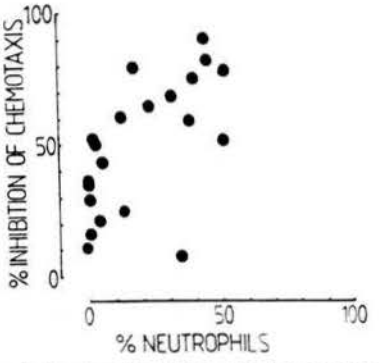


Fig. 4—Graph showing the relationship between the percentage of neutrophils in the bronchoalveolar leukocytes and inhibition of chemotaxis in that population. Inhibition data derived as in the legend to Fig. 2

at the lower proportions of neutrophils. Thus, with less than 10 per cent neutrophils in some samples, there was up to 60 per cent inhibition, whereas increasing the percentage of neutrophils to between 10 and 50 per cent only caused a maximum further 20 per cent inhibition. Thus, when there were large numbers of neutrophils present, up to half of the observed impairment of chemotaxis could be attributed to the contaminating neutrophils; however, the impaired chemotaxis is clearly present in macrophages, as shown by the substantial loss of chemotaxis present at negligible levels of neutrophils.

(b) Effect of ingested dusts on macrophage chemotaxis Control rat alveolar macrophages were allowed to adhere to filters and then incubated with quartz, TiO_2, or chrysotile asbestos for 1 h to allow phagocytosis; by microscopic examination, particles could be seen within the macrophages. A chemotaxis gradient was then set up by placing the filters in a chamber with zymosan-activated serum in the bottom compartment. Chemotaxis was then allowed to proceed and the results were as follows: untreated macrophages, with no phagocytic burden, 54·4 ± 11·3; TiO_2-exposed, 51·8 ± 6·2; quartz-exposed, 59·8 ± 6·0; chrysotile-exposed, 61·4 ± 7·5. Clearly, the mere presence of a phagocytic burden inside the macrophages was not sufficiently detrimental to the cell to cause impairment of chemotaxis.

(c) Effect of incubation for 4 h on chemotaxis Allowing dust-exposed macrophages, with impaired chemotaxis (obtained after 75 days of exposure to coal-mine dust), to incubate for 4 h in

medium to allow recycling of chemotaxis receptors had no effect on the impaired ability of the cells to chemotact: control alveolar macrophages, freshly derived, 55·0 ± 7·0; incubated for 4 h, 48·2 ± 11·0; dust-exposed bronchoalveolar leukocytes, freshly derived, 12·6 ± 3·6; incubated for 4 h, 9·1 ± 2·4.

DISCUSSION

This and many previous studies confirm that there is inflammatory leukocyte recruitment to the lungs of humans[1,2,10] and experimental animals[3] exposed to pneumoconiotic dusts. The present study on the chemotactic activity of bronchoalveolar leukocytes from rats exposed to different dust types by inhalation demonstrates an impairment of chemotaxis which is consistent with the ability of dust to cause pneumoconiosis. Thus, titanium dioxide caused minimal inflammation, does not cause pneumoconiosis[11] and caused negligible impairment of leukocyte chemotaxis. Quartz, coal-mine dust, and chrysotile asbestos, on the other hand, all cause alveolitis to varying degrees, are well known to cause pneumoconiosis and caused impaired chemotactic activity. There were no well-defined differences between the three coal-mine dusts, having different mineralogical compositions, in terms of their ability to impair chemotaxis. The effect was present when dust was delivered to rat lungs by inhalation or intratracheal instillation, an important finding in view of the widespread use of instillation as a cheaper, more convenient alternative to inhalation as a means of experimental exposure to pathogenic dusts.

One obvious candidate for explaining this loss of chemotactic activity is the occupation of the chemotaxin receptors by chemotaxin in the process of being recruited to the dust-exposed lung; this could then render the cell temporarily refractory to further chemotaxin exposure, a phenomenon frequently described and named chemotactic deactivation.[12,13] Allowing leukocytes from coal-mine dust-exposed lung to incubate for 4 h so that these receptors could be recycled[12] had no effect in restoring chemotaxis. Further evidence discounting chemotactic deactivation is found in the figures of chemotaxis inhibition at 8 days with 10 mg/m^3 compared with the numbers of leukocytes present at these time-points. Out of the three coal dust-exposed groups, two were below the control in total number and the third (coal-mine dust A) was only slightly above the control level. There was thus no marked recruitment to these lungs and so presumably no high levels of chemotaxin to cause chemotactic deactivation, yet there was impairment of chemotaxis of between 28·4 and 51·1 per cent.

Neither was the loss of chemotaxis due to the dust burden physically preventing passage of the phagocytes through the pores of the filter, since macrophages with a phagocytic burden taken up *in vitro* were able to chemotact to the same extent as normal phagocytes. It appears, therefore, that the milieu of a dust-inflamed lung is able to induce loss of chemotaxis in the local phagocytes. It can be presumed that this loss of chemotaxis is due to an effect on the locomotory system of the cell, although there could be a loss of receptors, and this will form the subject of future study.

In the present study, chemotaxis was measured in the entire bronchoalveolar leukocyte population from the dust-exposed rats. Some populations contained very few neutrophils and there was still substantial impairment of chemotaxis. Up to 50 per cent inhibition could be seen with less than 10 per cent neutrophils present, confirming that alveolar macrophage chemotactic activity must be impaired. At high proportions of neutrophils, however, the decreased ability of neutrophils to migrate in the chemotaxis assay may influence the outcome of the assay.

Our own studies have provided evidence that neutrophils release substances capable of causing some inhibition of the chemotactic activity of macrophages.[14] These effects were, however, modest and our finding that neutrophil number did not correlate with loss of chemotactic activity until the proportion of neutrophils was more than 50 per cent supports the notion that the loss of chemotaxis is not simply a result of the presence of neutrophils acting on the macrophages.

Inhibition of chemotaxis of bronchoalveolar leukocytes, similar to that described here, has been described previously with quartz[15,16] and chrysotile asbestos[17] exposure. The present report is the first, to our knowledge, demonstrating loss of chemotaxis in leukocytes from coal-mine dust-exposed animals and the first showing this effect with three different pneumoconiotic dusts in the same system. Myrvik *et al.*[18] have reported inhibition of the migration of rabbit alveolar macrophages which had phagocytosed asbestos *in vitro*: the impairment of migration could not be attributed to toxicity, but inert dusts were not included as controls. No experiments into the basis of this loss of chemotactic activity *in vitro* were carried out in the study.

The ability of alveolar macrophages to carry out their normal host defence functions is reliant on their ability to respond to chemotactic gradients which effect movement of the leukocytes around the lung parenchyma. Thus, chemotactic gradients are likely to be involved in the location of particles for phagocytosis and movement of the dust-laden leukocytes to the mucociliary escalator for clearance, or to the regional lymph nodes. Several factors, in dust-inflamed lung, could therefore lead to loss of overall efficiency of dust clearance. Alveolar macrophages from pneumoconiotic dust-exposed lung may have decreased ability to find particles and phagocytose them; they may then have impaired ability to carry this dust to the mucociliary escalator for clearance. In addition, ability to respond to the gradients which attract them to the lymph nodes for concomitant immune responses, which may be necessary in dust-exposed lung as in normal lung, could be substantially impaired. Dust-exposed individuals could therefore be more susceptible to infection, which could influence the pneumoconiotic response to dust.[19] This could, of course, be offset to some extent by the increase in leukocyte numbers evident in inflammed alveoli which would enhance the general host defence system. At the single cell level, however, cells activated by ingestion of pathogenic dust may remain for a longer period in the alveolar region, if chemotaxis is impaired, and will be available to release factors such as interleukin-1[20] and fibroblast growth factors[21] which could affect the lung tissue leading to fibrosis. These 'slowed down' leukocytes may also release proteases[22] which could contribute to the emphysema which is found in coal-miners[4] if the overall protease: anti-protease balance is tipped in favour of proteolytic destruction of connective tissue components. The finding of impaired chemotaxis in alveolar macrophages from lungs of rats exposed to three different pneumoconiotic dusts, but not in rats exposed to the largely innocuous dust TiO_2, suggests this to be a phenomenon of relevance to the development of pneumoconiosis.

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Inflammation in the lungs of rats after deposition of dust collected from the air of wool mills: the role of epithelial injury and complement activation

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Abstract

In a previous study assessing respiratory symptoms in individuals employed in wool textile mills in the north of England relations between symptoms of chronic bronchitis, breathlessness and wheeze, and rhinitis and current exposure to airborne mass concentration of dust were shown. As preliminary steps in defining the potential hazard associated with dust from the air of wool mills the ability of inspirable dust, collected from the air of wool textile mills, to cause inflammation when injected into the lungs of rats was determined. Dusts were collected from the beginning of wool processing (opening) in one factory and from the middle (combing) and late (backwinding) stages of the process in two other factories. Ability of the dusts to cause inflammation was assessed by instillation into the lungs of rats followed by bronchoalveolar lavage. All the dusts caused some inflammation which peaked on day 1 and did not persist beyond one week. A distinctive aggregation response of mononuclear cells in the lavage, however, had a different time course, peaking at day 7. An attempt was made to determine how the wool mill dusts caused inflammation and experiments showed that the dusts themselves had no inherent chemotactic activity but that they did have a pronounced ability to generate chemotaxins in serum and so could activate complement in lung fluid. In addition, dust collected from ledges in the mills had the ability to injure epithelial cells in vitro which could also contribute to inflammation. A role for endotoxin in the inflammatory activity of the dusts was not discounted and a leachate of the dust had the ability to cause inflammation when injected into the lungs of rats. Wool mill dust is likely to be a complex mixture of materials and these experiments represent a preliminary approach to understanding the biological activity of the whole unfractionated dust and further studies are in progress to define more accurately the toxic material(s) in the dust.

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The Institute of Occupational Medicine studied the prevalence of respiratory symptoms in individuals employed in wool textile mills in the north of England.[1–3] Clear relations have been shown between current exposures to airborne mass concentrations of inspirable dust in these mills and the prevalence of symptoms, such as chronic bronchitis, breathlessness and wheeze, rhinitis, and conjunctivitis, suggesting that inhalation of the dusts causes inflammation in the upper and lower respiratory tract.[1] Complementary studies of the lung functional and radiographic effects of occupational exposure to wool mill dusts have also been carried out.[3] As an adjunct to the epidemiological studies on symptoms, respirable dust was collected from the air of selected wool mills with the objective of testing the dusts in rat lung to measure their ability to cause inflammation. Similar in vitro studies have been carried out on other organic dusts associated with bronchitic symptoms. These have shown evidence of chemotactic activity, complement activating activity, and epithelial injuring activity in cotton dust components[4,5] and chemotactic activity, complement activating activity, and ability to induce neutrophil chemotaxin release from macrophages in grain dust.[6]

The present paper reports preliminary work aimed at defining the biological activity of unfractionated respirable wool mill dust and describes the leukocyte responses, assessed by bronchoalveolar lavage, to the deposition of the dust or soluble factors thereof in the lungs of laboratory rats. The ability of the wool mill dust to activate complement and cause epithelial injury has also been shown and this may explain, in part at least, how wool mill dust engenders inflammation in the lung experimentally and causes inflammation of the respiratory tract in exposed workers.

Materials and methods

RATS

Syngeneic, PVG rats, SPF maintained, were obtained from the Institute of Occupational Medicine breeding unit.

DUSTS

Dust samples were collected using IOM static inspirable dust samplers from the air of three mills chosen to represent the start (S), middle (M), and end (E) of the industrial processing of wool in the vicinity of opening (carpet yarn), combing (worsted process), and backwinding (carpet yarn) processes respectively from three mills in west Yorkshire. Each process was sampled in a different mill (in other IOM reports these are referred to as mills 15, 4, and 11 respectively). These samples, on filters, were sent by post to the Institute of Occupational Medicine where they were stored at −20°C for up to six months before use. Samples from each mill were removed from the filter with a brush, pooled, and coarse material was removed by hand. The remaining pooled samples of fine dust were mixed overnight on a rotating mixer to ensure homogeneity and stored at −20°C. Samples of the three wool dusts were then irradiated at 150 KRad at the National Engineering Laboratory, East Kilbride, to reduce the number of live contaminating fungal spores and bacteria. Non-irradiated samples of each dust were retained as a reference. Titanium dioxide (TiO_2; rutile form; Tioxide Limited, Stockton-on-Tees) was used as an inert control dust.

INTRATRACHEAL INJECTION

Wool dusts and TiO_2 were prepared at 5 mg/ml in sterile phosphate buffered saline (Gibco, Paisley) and 0·5 ml (2·5 mg) aliquots were introduced into the lungs of groups of three rats. Instillation was carried out by surgical exposure of the trachea in anaesthetised rats followed by direct injection of dust suspension into the lung through a small incision in the trachea; the overlying skin was then closed with metal clips. Animals were conscious within two minutes and completely recovered within hours; no animal died after injection of the dusts. Rats were kept for one, three, seven, or 14 days at which point the experimental group was killed and the bronchoalveolar space lavaged.

BRONCHOALVEOLAR LAVAGE

To assess inflammation, three rats were killed by Nembutal (Ceva, Watford) overdose and the lungs were lavaged with 4 × 8 ml volumes of saline at 37°C. The cells were concentrated by centrifugation, counted, and the differential count carried out on a May-Grunwald Giemsa-stained cytospin (Shandon Runcorn) preparation. From these, the totals of each cell type were calculated. Data is presented as mean and standard error of macrophages, neutrophils, and lymphocytes.

SOLUBLE PRODUCTS (LEACHATE) FROM WOOL MILL DUST

A leachate was prepared, using sterile pyrogen free saline, from dust M (5 mg/ml) by mixing overnight in a rotating mixer; the particulate fraction was centrifuged out to yield the soluble components, or leachate, of the dust. This was injected as 0·5 ml (equivalent to 2·5 mg of dust) as described above.

ENDOTOXIN

Endotoxin (Gibco) was injected intratracheally into the lungs of a group of three rats as 2·5 μg in 0·5 ml of saline and the lungs lavaged three days later.

ACTIVATION OF CHEMOTAXINS IN SERUM BY TREATMENT WITH WOOL DUST

Rat serum (10%) was treated with wool dust M (1 mg/ml), mixed in a water bath at 37°C for 30 minutes, and then heat inactivated (56°C for 30 minutes). Previous studies have shown that treatment of serum with particulate in this manner causes activation of complement to yield the chemotactic anaphylatoxin C5a.[7]

CHEMOTAXIS

Chemotaxis was carried out using conventional Blindwell chambers. Zymosan activated serum (200 μl) or serum treated with wool dust, at 5%, or leachate of wool mill dust was placed in the lower compartment of a Blindwell chamber and a 5 μm pore filter (Nuclepore, Pleasanton, California) placed on top. In the upper compartment were placed 400 μl (6×10^5 cells) of control bronchoalveolar lavage cells (>95% macrophages). Blindwell chambers were then incubated at 37°C in 5% CO_2 for 3·5 hours. Filters were removed and stained with Diff-Quik and the number of migrated cells assessed by high power light microscopy. Results are expressed as migrated cells/high power field (mean of five fields on two filters—that is, n = 10). To test for chemokinesis a modified "checkerboard" analysis was performed with 5% zymosan activated serum in both the upper and lower compartments.

ASSAY OF INJURY TO EPITHELIAL CELLS IN VITRO

The assay is that described previously.[8] Briefly, cells of the alveolar epithelial cell line A549 were prelabelled with ^{51}Cr (Amersham International, Amersham) by incubating overnight in microtitre plate wells at 5×10^4 in 100 μl of MEM + 10% fetal calf serum (Gibco, Paisley). To these plates were added varying dilutions of a leachate prepared, as described above, from fine ledge dust obtained from wool mills M and S, to a total volume of 200 μl. Ledge dust was used because all the respirable

material was used for the above experiments and ledge dust was therefore the best alternative to airborne dust; it was stored as for the airborne dust. In some experiments dust was leached into lung lavage fluid as well as medium to determine whether leaching was likely to occur in the milieu of the lung. After four hours of incubation at 37°C in 5% CO_2 the wells were sampled to obtain the counts released from lysed (dead) cells and counts within cells which had detached from the substratum.

STATISTICAL ANALYSIS

Results were analysed by analysis of variance and differences between treatment groups tested for statistical significance using a Student's *t* test.

Results

BRONCHOALVEOLAR LEUKOCYTE RESPONSE TO DEPOSITION OF WOOL DUST

Figure 1 shows the kinetics of the bronchoalveolar leukocyte response to the injection of 2·5 mg of dust from three different wool mills and TiO_2. With two of the dusts the macrophage counts did not increase substantially, although with dust S the numbers were significantly higher than the TiO_2 response. In the case of dust M, however, there was pronounced recruitment on days 1 and 3. Neutrophil response shows clearly that all four dusts caused some degree of inflammation on day 1 with the following order of activity: M > S > E > TiO_2. By day 3 all dusts were producing low levels of neutrophil influx and by days 7 and 14 levels approached 0 the norm for control rats.

The lymphocyte reponse was in keeping with the general pattern of the leukocyte response but it was noticeable that by day 14 the lymphocytes present in wool exposed lung exceeded those present in TiO_2 exposed lung.

The total number of cells present in the lavage is not completely reflected in the free cells described above in view of the fact that a small proportion of

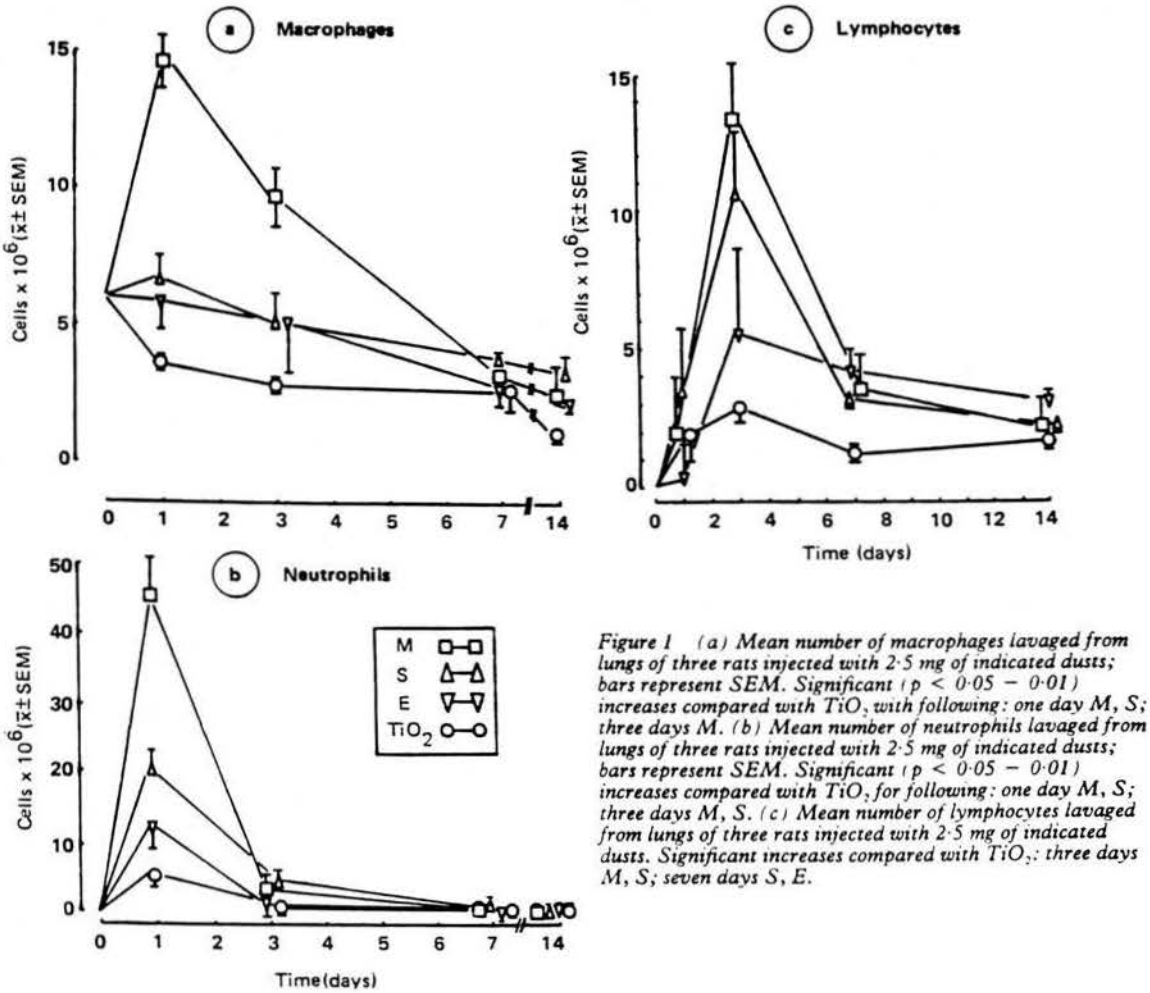


Figure 1 (a) Mean number of macrophages lavaged from lungs of three rats injected with 2·5 mg of indicated dusts; bars represent SEM. Significant ($p < 0·05 - 0·01$) increases compared with TiO_2 with following: one day M, S; three days M. (b) Mean number of neutrophils lavaged from lungs of three rats injected with 2·5 mg of indicated dusts; bars represent SEM. Significant ($p < 0·05 - 0·01$) increases compared with TiO_2 for following: one day M, S; three days M, S. (c) Mean number of lymphocytes lavaged from lungs of three rats injected with 2·5 mg of indicated dusts. Significant increases compared with TiO_2: three days M, S; seven days S, E.

cells were bound up in cell clumps (see below).

CELL CLUMPS

Populations of cells obtained from the bronchoalveolar space of rats treated with wool dust contained unusual aggregates (or clumps) of cells. These clumps often seemed to be gathered around particles of wool mill dust visible by light microscopy but sometimes there was no particle clearly visible at the centre of the aggregate. These cells were predominantly mononuclear and often comprised layers of cells, some making contact with the particle but some making contact with other cells only. The appearance of these clumps did not coincide with the peak of inflammation (one day) but peaked between days 3 and 14 (seven days in our time course) (fig 2).

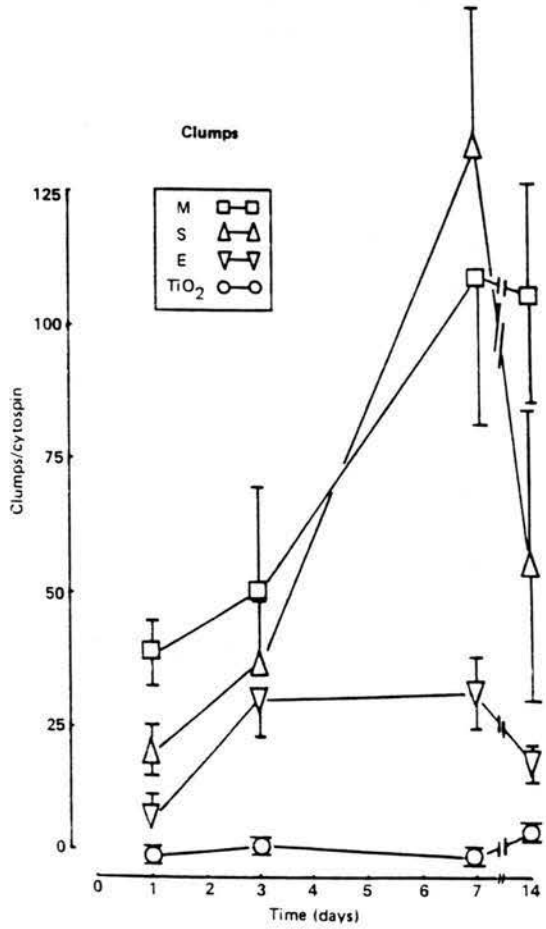


Figure 2 Mean number of clumps per cytospin preparation of bronchoalveolar cells collected from three rats injected with 2·5 mg of indicated dusts. Significantly ($p < 0·05 - 0·001$) increased numbers of clumps compared with TiO_2 at following: one day M, S; three days M; seven days M, S, E; and 14 days M, S, E.

In all cases the number of aggregates had fallen substantially by day 14 except in the case of dust M where the number remained high.

ROLE OF DIFFUSIBLE COMPONENTS IN THE WOOL DUST

To test whether soluble components of wool dust were diffusing from the particles and causing inflammation, a particulate free leachate of dust M (2·5 mg of dust equivalent) was injected intratracheally into the lungs of rats and the rats lavaged three days later. As shown in table 1 some, but not all, of the inflammatory potential of whole wool dust could be reproduced with a particle free extract, strongly suggesting that soluble factors do have a role in causing inflammation. It was noticeable, however, that these diffusible components did not have the ability to produce clumps.

Endotoxin (2·5 μg) caused only moderate neutrophil recruitment and had little effect on macrophage recruitment (table 1).

EFFECTS OF IRRADIATION ON THE ABILITY OF WOOL DUST TO CAUSE INFLAMMATION

Irradiation of wool mill dusts did not substantially affect their ability to generate inflammation in rat lungs. The irradiated dusts did, however, cause slightly more macrophage and neutrophil recruitment than the non-irradiated: results given are $\bar{x}$ (SD) of cells $\times 10^6$ for groups of three rats, three days after instillation—non-irradiated macrophages 4·6 (0·5), neutrophils 3·7 (0·7); irradiated macrophages 7·8 (1·1), neutrophils 5·1 (0·9).

GENERATION OF CHEMOTAXINS BY WOOL DUST

As shown in table 2 the leachate of pooled dust M had no chemotactic activity so precluding the possibility that products released by the wool dust itself were directly attractive to leukocytes and could contribute to the recruitment of inflammatory cells. As shown in table 3, however, wool dust was highly active in generating chemotaxins in rat serum and, by analogy in alveolar fluid. The modified "checkerboard" analysis (table 3) showed that although some

Table 1 Inflammation assessed by brochoalveolar lavage three days after intratracheal instillation of soluble components derived from wool dust M and by endotoxin. Significantly ($p < 0·001$) increased numbers of both macrophages and neutrophils with all treatments compared with saline

	Cells ($\times 10^6$)	
	Macrophages	*Neutrophils*
Saline	2·6 (0·4)*	0·1 (0·0)
Leachate (equiv to 2·5 mg wool dust M)	7·2 (0·7)	1·3 (0·4)
Wool dust M (2·5 mg)	10·3 (1·2)	3·0 (0·4)
Endotoxin (2·5 μg)	5·5 (0·5)	0·6 (0·1)

*$\bar{x}$ ± SEM; n = three rats.

Table 2 Chemotactic activity in wool dust M leachate. No significant increase in chemotaxis with leachate compared with saline alone

	Chemotaxis (Migrated cells/high power field)		
	Saline	*Wool dust leachate*	*ZAS* (Pos control)*
Experiment 1	0·4 (0·7)†	0 (0)	86·3 (11·8)
Experiment 2	1·2 (1·2)	0 (0)	

*Zymosan activated serum.
†(SEM) of 10 high power fields on two filters.

chemokinetic activity was present in the activated serum most of the migration was true chemotaxis.

EPITHELIAL INJURY CAUSED BY DUST FROM WOOL MILLS

Figure 3 shows that the leachate from wool mill ledge dust was capable of causing direct epithelial injury of both the lytic and detachment type. Lysis was present to the greatest extent in neat and 1/10 dilutions whereas detachment was present at all concentrations even down to 1/100, with some samples of dust E showing pronounced activity at this dilution. Detachment and lysis were also caused, whether the dust was leached into fluid lavaged from rat lung or into medium. On the whole, dust M caused more measurable detachment to epithelial cells than dust E with an average of 5·1-fold more detachment with undiluted leachate compared with control ($p < 0·05$); dust E by comparison showed an average of 2·1-fold more detachment than the medium control. Detachment is underestimated in cases where there are high concentrations of lysis as with dust E, since lysed cells are not available for detachment. Dust E was most toxic causing more lysis, 2·9-fold more than control with the undiluted leachate ($p < 0·05$) whereas this figure was 1·9 for dust M. Significant increases in detachment were also caused by dust E at 1/10 and 1/100 dilution ($p < 0·05$).

Discussion

The most obvious response in rat lungs to instilled wool mill dust was acute inflammation. The inflammation was short lived, since, despite the fact that a considerable insult of 2·5 mg of dust was used, the bronchoalveolar lavage cellular profile had returned to near normal by day 7. Presumably daily inhalation exposure to wool dust would result in chronic inflammation, though we have not investigated this. In addition to inflammation an immune response was suggested by the presence of aggregates of leukocytes, peaking at seven days after exposure. We have not observed this response with any of the mineral dusts we have tested previously using a similar assay system. A continuing immune response in the alveolar region could contribute to chronic inflammation through immune complex mediated injury in the case of humoral immunity or, in the case of a cell mediated immune response, lymphokine mediated inflammation. The presence of persistently, although modestly, increased free lymphocytes in the bronchoalveolar lavage tends to support the idea that the immune system is affected. The type(s) of cells comprising the clumps was not ascertained but is to be the object of further study. A parallel study of the effects of wool dust on the immune system will be reported separately.

The inflammation caused by the wool dusts was not due simply to a phagocytic burden since TiO_2 was substantially less active than wool mill dust. We investigated two possible mechanisms whereby wool mill dusts could have generated inflammation; (*a*) endotoxin, or some other component within the dust, could possess chemotactic activity or directly activate the complement cascade to generate chemotaxins; (*b*)

Table 3 Generation of chemotactic activity by the action of wool dust on rat serum. Significant ($p < 0·01$) increase in chemotaxis with wool treated serum compared with control serum

	Chemotaxis (Migrated cells/high power field)			
	Control serum	*Wool treated serum*	*ZAS* (Pos control)*	*Chemokinesis*
Experiment 1	23·9 (3·2)†	61·4 (8·0)	86·3 (11·8)	16·9 (6·5)
Experiment 2	31·9 (5·7)	62·3 (9·4)		12·3 (3·2)

*Zymosan activated serum.
†$\bar{x}$ (SEM) of 10 high power fields on two filters.

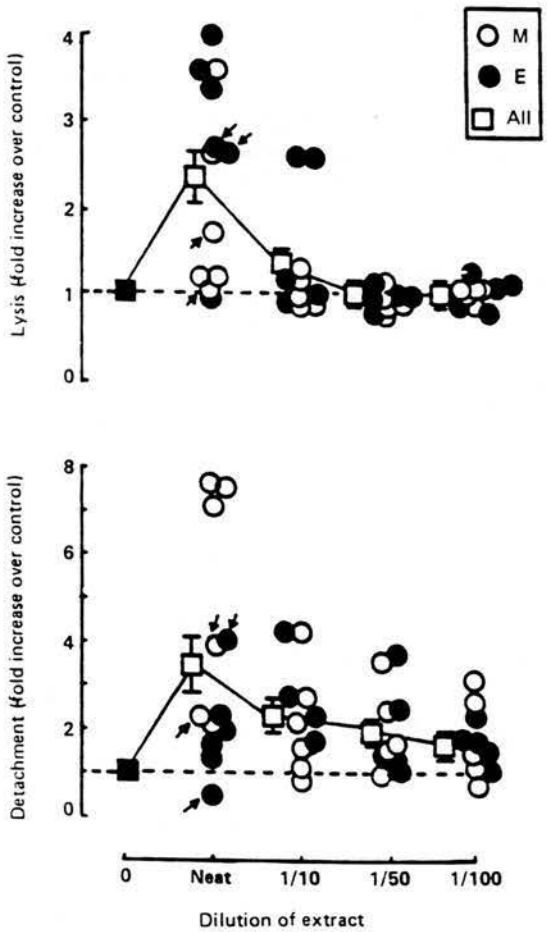


Figure 3 Alveolar epithelial cell-lysis (upper panel) and detachment (lower panel) shown by extracts of dust from mill M (open circles) and mill E (closed circles). Results from six (undiluted) or five (other dilutions) separate experiments. Arrows denote experiments where diluted lung lavage fluid was used for extraction. Squares show mean ± SEM from all data at that dilution. Black squares and dotted line represent unity—that is, spontaneous detachment or lysis—all data obtained as

$$\frac{\text{cpm in presence of wool extract}}{\text{cpm spontaneous.}}$$

See results section for statistical analysis

component(s) of the wool mill dust could injure epithelial cells. Wool mill dust samples possessed no intrinsic chemotactic activity but did activate complement and injure epithelial cells in vitro. The potential role of endotoxin in causing these inflammatory/immune effects in the lungs of workers and rats exposed to wool dust deserves attention because of its inflammatory activity[9 10] and because endotoxin was present in other samples of wool mill dust collected from the air of the mills studied here.[1] Endotoxin may contribute to the respiratory symptoms observed in workers from a variety of industries exposed to airborne dusts[11] and has been implicated in symptoms such as those of byssinosis in workers in a wool carpet factory in Turkey.[12]

Some, but not all, of the inflammatory activity of the wool dust could be accounted for by soluble components that could be leached into saline. Some of this leached material could have been endotoxin but a relatively large dose of endotoxin (2·5 μg) was not very active in causing inflammation in the rat lung. This was a commercially obtained sample of endotoxin, however, and may not have been representative of the endotoxin in the samples of the wool mill dust. Endotoxin may also have been involved in the generation of chemotaxins in serum. Since alveolar fluid is, in part, a transudate of plasma and contains complement,[13] then wool dust depositing in the lung could activate complement leading to inflammation.

Our finding that a soluble product from wool mill ledge dust caused both lysis and detachment of epithelial cells indicates another possible mechanism by which wool dust could contribute to inflammation. Epithelial injury is a powerful stimulus to inflammation, since dead cells are chemotactic and exposed basement membrane activates complement.[14]

The dust collected from the carding room (M) was the most active of the dusts in causing inflammation; the backwinding dust the least. Since the three dusts were collected from different factories we cannot be sure that these differences are related to the stages of the manufacturing process rather than the differences between the factories. Size distribution could be important in determining what mass of dust reaches the alveoli and is phagocytosed. In this respect the ranking of activity exactly follows the ranking of the dusts with regard to particle size. The proportions by mass of fine (respirable) dust in each sample were M–21·8%, S–16·6%, and E–10·4%.[2] Since the dusts in this study were instilled, however, aerodynamic behaviour is unlikely to influence deposition of the dusts and increased particle number per unit mass in the finer dusts is a more likely explanation for the differences in inflammatory response.

This predominantly inflammatory response to wool mill dust is broadly consistent with what is known of dust related illness in wool textile workers. The symptoms we found to be associated with exposure to dust in workers in the industry included conjunctivitis, rhinitis, chronic bronchitis, and wheeze. There was little evidence that wool dust caused asthma, the most dramatic allergic reaction in the lung, and atopic individuals were not more susceptible to symptoms related to wool dust than other subjects.[3] There was no evidence on the chest radiographs of lung fibrosis or alveolitis. Lung functional deficits have not so far been convincingly shown to be related to exposure to wool dust, though

we do not consider this possibility to have been excluded.[3]

Thus the symptoms conform to what is called "non-specific" lung disease, analogous to the chronic bronchitis associated with smoking, air pollution,[15] and exposure to coalmine dust[16] and some other industrial dusts, including organic dusts such as grain dust.[17] The mechanisms of this non-specific response are not well understood, but inflammatory cell recruitment has been reported in the lung lavage of subjects with chronic bronchitis[18] and in smokers[19] and rats exposed experimentally to coalmine dust.[20] Experimental studies similar to those described in the present paper have been carried out with grain dust in view of its ability to cause bronchitis.[6] This study showed that the grain dust had strong inherent chemotactic activity for neutrophils which we did not find in the wool dust sample we tested; however, like wool dust, grain dust had powerful ability to activate complement and also caused alveolar macrophages to release neutrophil chemotaxins. Grain dust was not tested for ability to injure epithelial cells but cotton bract extract has been found to directly injure epithelial cells[4] and to activate complement.[5] Thus wool dust may be seen as generally fitting into the pattern of activity shown by other organic dusts with multiple non-specific effects on the pulmonary epithelium and leukocyte populations that result in inflammation in the airways.

Exposure to inorganic industrial dusts has been reported to cause recruitment of macrophages and neutrophils to the lungs of workers and experimentally exposed rats.[21-23] Exposure to these dusts is associated in the long term with structural changes in the lung parenchyma and airways that include fibrosis, small airways disease (in response to asbestos), and possibly emphysema in response to coal dust. The non-specific effects of these dusts have received attention and they have been found to activate complement[7] and injure macrophages.[24] The ability of inorganic mineral dusts to cause pronounced parenchymal lung disease (fibrosis and emphysema) while wool mill dust injury appears to be confined to the airways could be related to (*a*) the size of the dust, with more small dust particles depositing in the alveoli in the case of inorganic dusts or (*b*) the non-biodegradability of natural mineral dusts causing increased leukocyte activation in the alveolar region.

The study was conceived as a preliminary approach to understanding the biological potential of whole unfractionated respirable dust collected from the air of wool mills. The study showed that this dust has the ability to cause inflammation in the rat lung in excess of that caused by a phagocytic stimulus alone (TiO_2). In relation to pulmonary disease in wool workers, we would suggest that if airborne wool mill dust is inhaled regularly in sufficient quantities it could cause epithelial injury in the airways or respiratory zone so contributing to inflammation. In addition complement could be activated by the dust, causing some recruitment via C5a and also coating the wool dust particles with C3b. Macrophages may thereby be stimulated through their complement receptors to release chemotaxins and other mediators leading to further leukocyte recruitment. A combination of epithelial injury generated inflammation and leukocyte recruitment via complement activation could lead to further epithelial injury due to the injurious effects of the leukocytes themselves.[8] The involvement of the specific immune response could also be a factor in enhancing inflammation.

No attempt has been made to characterise this complex mixture of materials likely to be present in the dust but further work is in progress.

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1 International Steering Committee of Medical Editors. Uniform requirements for manuscripts submitted to biomedical journals. *Br Med J* 1979;**1**:532-5.
2 Soter NA, Wasserman SI, Austen KF. Cold urticaria: release into the circulation of histamine and eosino-phil chemotactic factor of anaphylaxis during cold challenge. *N Engl J Med* 1976;**294**:687-90.
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INFLAMMATORY RESPONSE TO PARTICLES IN THE RAT LUNG: SECRETION OF ACID AND NEUTRAL PROTEINASES BY BRONCHOALVEOLAR LEUCOCYTES

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Abstract—Leucocyte proteinases are considered to be central to the tissue damage that is associated with chronic inflammatory lung disease. Both acid and neutral proteinases have the ability to degrade connective tissue molecules and both may therefore play a part in tissue proteolysis in inflamed lungs. In this study we have used a rat model of lung inflammation to investigate levels of acid and neutral proteinase activity in the bronchoalveolar region of control and inflamed lungs. We assessed the ability of proteinases, secreted by resident and inflammatory bronchoalveolar leucocytes, to damage the connective tissue molecule fibronectin. Inflammatory bronchoalveolar leucocytes had greater ability to mediate connective tissue damage than had resident alveolar macrophages and the tissue proteolysis was mediated, in the main, by proteinases active at neutral rather than at acid pH. The increased secretion of proteinase by inflammatory leucocytes was dependent on *in vivo* stimulation; exposing resident or inflammatory bronchoalveolar leucocytes *in vitro* to particulate or soluble stimuli had no effect in increasing their ability to degrade fibronectin or secrete the inflammogenic proteinase, plasminogen activator. Thus, neutrophils in the bronchoalveolar region of the rat lung have proteolytic activity which is mediated largely by neutral proteinases.

INTRODUCTION

PROTEINASES released by inflammatory leucocytes are considered to be of major importance in the tissue damage that is associated with chronic inflammatory lung disease (HUNNINGHAKE *et al.*, 1984). Activated leucocytes secrete proteolytic enzymes with activity at acid and neutral pH (FANTONE and WARD, 1984) and both types of proteinase have been implicated in causing tissue damage (VAES, 1985; PADILLA *et al.*, 1988). Neutral proteinases damage connective tissue components *in vivo* (JANOFF, 1985) and *in vitro* (BROWN and DONALDSON, 1988) and acid proteinases can also damage connective tissue molecules *in vitro* (VAES, 1985). Bronchoalveloar lavage studies of patients with chronic inflammatory lung disease have demonstrated increased amounts of both acid (SHIOTA, 1987; PADILLA *et al.*, 1988) and neutral proteinase activity in the lavage fluid (CHRISTNER *et al.* 1985; O'CONNOR *et al.*, 1987) and have thus reinforced the hypothesis that these proteinases contribute to the pathogenesis of the disease. Plasminogen activator is a neutral proteinase that has been implicated indirectly in tissue injury in inflamed lung through its action in converting plaminogen to plasmin (HAMILTON, 1980). Plasmin can act to enhance inflammation through its action in the clotting, complement and fibrinolysis pathways. The relative importance of these acid and neutral proteinases in diseases arising from pulmonary inflammation is not yet defined.

Previous work has demonstrated the proteolytic activity of intact bronchoalveolar leucocytes (CAMPBELL *et al.*, 1982; BROWN *et al.*, 1989) and has implicated both acid (CHAPMAN and STONE, 1984) and neutral proteinases (BROWN and DONALDSON, 1988)

in connective tissue proteolysis. The normal pH of lung fluid is neutral and therefore might facilitate neutral proteinase activity. However, secretion of lysosomal contents may acidify the site of close contact between cells and matrix and so permit proteolysis of connective tissue by acid proteinases. In the present study, we addressed the relative contribution of acid and neutral proteinases to fibronectin degradation by measuring the proteolytic activity of supernatants produced by bronchoalveolar leucocytes from control or inflamed rat lungs. The ability of the supernatants to degrade a fibronectin matrix was tested at neutral and acid pH. Secretion of the lysosomal acid proteinase, β-glucosaminidase was also assessed.

Leucocyte activation is characterized by increased secretion of various enzymes and proteins and, in particular, proteolytic enzymes (BAGGIOLINI and DEWALD, 1988). In inflamed lung such activation may be mediated by phagocytosis of inhaled particles (SCHNYDER and BAGGIOLINI, 1978) or by soluble mediators present in the inflammatory milieu, such as interleukin-1 or tumour necrosis factor (BILLINGHAM, 1987). In this study, we addressed this aspect of leucocyte activation by exposing control and inflammatory bronchoalveolar leucocytes, *in vitro*, to soluble or particulate stimuli and then measuring their ability to degrade fibronectin and to secrete plasminogen activator.

MATERIALS AND METHODS

Animals

Specific pathogen-free male rats of the PVG strain, obtained from the breeding unit of the Institute of Occupational Medicine, were used throughout the study.

Intratracheal dosing

The particulates used in this study were zymosan (a preparation of yeast cell walls—Sigma Ltd, Poole, Dorset, U.K.), titanium dioxide (TiO_2—Tioxide Ltd, Stockton-on-Tees, U.K.) or DQ12 standard quartz. Particles were administered intratracheally as follows: rats of 300 g weight were anaesthetized with 0.3 ml Diazepam (10 mg ml^{-1}, Lagap Pharmaceutical, Bordon, Hampshire, U.K.) intraperitoneally and 0.1 ml Hypnorm (Janssen Pharmaceuticals, Grove, Oxford, U.K.) intramuscularly. The trachea was exposed by dissection and a small incision made; 1 mg of TiO_2, quartz or zymosan in 0.5 ml sterile saline was then injected as a single bolus and the skin was then closed with metal clips. Nine rats were injected in each treatment group and three rats per treatment were killed at each time point. Recovery of the animals was initiated by intramuscular injection of 0.2 ml naloxone hydrochloride (Sigma Ltd, Poole, Dorset, U.K., 0.3 mg ml^{-1} in PBS); the animals were conscious within 1 h of treatment. Previous work from this laboratory has demonstrated that intratracheal injection of saline alone has no effect on bronchoalveolar lavage leucocytes at the time points used in the present study (unpublished results) and so the age-matched control rats were untreated.

Bronchoalveolar leucocytes

Leucocytes were obtained by bronchoalveolar lavage 1, 5 and 15 days after intratracheal treatment; each experiment was carried out in duplicate giving $N = 6$ per

test at each time point. At each time point three animals in each treatment group were killed by intraperitoneal overdose with phenobarbitone, the chest cavity was opened, and the trachea cannulated with a blunt 16 g (1.5 mm diameter) needle. The lungs were dissected free of the chest cavity and lavaged with four 6 ml aliquots of sterile saline, warmed to 37°C, being massaged gently during each lavage to increase the cell yield; the pooled cells were placed immediately on ice. They were then washed with phosphate-buffered saline, counted and finally centrifuged and resuspended in Ham's F10 medium (Gibco, Paisley, U.K.) containing 2% bovine serum albumin (BSA—Sigma, Poole, Dorset, U.K.). Cytospin preparations were stained with May–Grunwald Giemsa for the differential cell count.

Preparation of leucocyte supernatants

Bronchoalveolar leucocytes from untreated and from 1- and 15-day quartz- and TiO_2-treated rats were set up in 24 well plates (Sterilin, Fletham, Middlesex, U.K.) at 1×10^6 cells ml^{-1}, 1 ml $well^{-1}$, in F10/2% BSA and were incubated at 37°C for 24 h. The supernatants were then harvested and spun at 2500 rev min^{-1} in an MSE super minor centrifuge (Fisons, Crawley, U.K.) to remove particulate material. Neutral proteinase activity in the supernatants was measured by assessing their ability to degrade fibronectin at the pH at which they were harvested (around pH 8.0). To measure acid proteinase activity, the supernatants were acidified with 0.1 M HCl to a pH of 3.8–4.0 and then tested for ability to degrade fibronectin.

In vitro *activation of leucocytes*

To assess the effect of *in vitro* triggering in eliciting leucocyte activation, leucocytes, obtained from untreated rats or from rats exposed to TiO_2, quartz or zymosan for 5 days, were cultured for 3 days *in vitro* in presence or absence of exogenous triggers. The triggers used were quartz, TiO_2, zymosan and the soluble trigger of leucocyte activation, phorbol myristate acetate (PMA); (zymosan and PMA were obtained from Sigma Ltd, Poole, Dorset, U.K.). These were added to give final concentrations of 1.0 and 10 $\mu g\ ml^{-1}$ for quartz and TiO_2, 0.1 and 1.0 mg ml^{-1} for zymosan and 1 $\mu g\ ml^{-1}$ for PMA. All supernatants were then harvested and centrifuged as above and the content of plasminogen activator and fibronectin proteolytic activity in each supernatant was assessed.

Proteinase assays

(1) *Plasminogen activator assay.* The plasminogen activator activity of the bronchoalveolar leucocyte supernatants was assayed as previously described (DONALDSON *et al.*, 1987) by measuring plasminogen-dependent breakdown of a ^{125}I fibrin matrix adhered in microtitre plates. The supernatants were incubated on the fibrin matrices for 24 h at 37°C. Fibrinolysis was measured as counts min^{-1} of degraded ^{125}I fibrin in three wells per sample, released into the supernatant medium.

(2) *Fibronectin proteolysis.* ^{125}I-labelled fibronectin matrices in microtitre plates were set up as previously described (BROWN and DONALDSON, 1988). Two-hundred microlitres of leucocyte supernatant were then added to triplicate wells for each test sample and the plates incubated for 4 h at 37°C.

(3) *Glucosaminidase assay.* *N*-Acetyl glucosaminidase activity in the leucocyte supernatants was measured spectrophotometrically as breakdown of the phenylated glucosaminide substrate according to the method of WOOLLEN *et al.* (1961).

Statistical analysis

Analysis of variance was carried out on the results using the Minitab Statistical Package.

Differences between means were tested using Student's *t*-test.

RESULTS

(1) *Acid and neutral proteinase in bronchoalveolar lavage leucocyte supernatants*

Supernatants of bronchoalveolar leucocytes retrieved from rats either 1 or 15 days after *in vivo* treatment with quartz and TiO_2, and then cultured for 1 day *in vitro*, were tested for proteolytic activity at acid or neutral pH. At neutral pH, both TiO_2 and quartz-elicited cell supernatants from the 1-day-treated animals had increased ability to degrade fibronectin compared with controls; this was significant only with the quartz supernatants ($P < 0.005$) (Fig. 1). By 15 days after treatment, the activity of the TiO_2 supernatants had declined to control levels. The activity of the quartz supernatants remained greater than the controls at 15 days but the difference was not statistically significant.

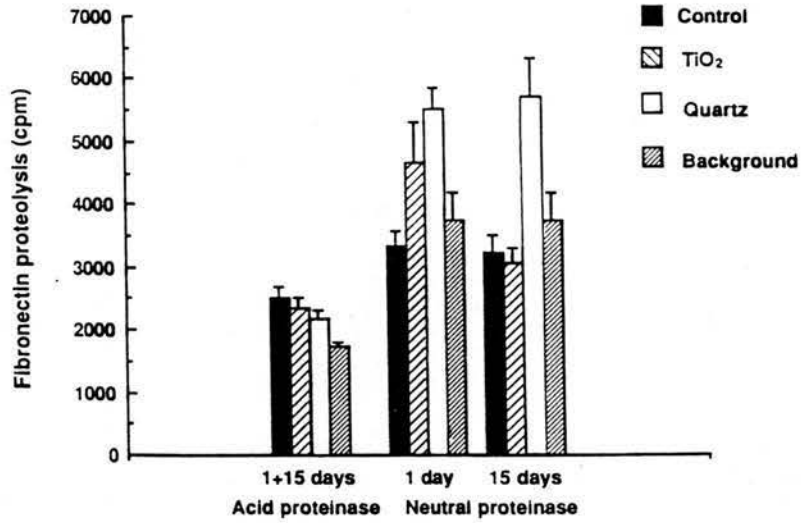


FIG. 1. The proteolytic activity of bronchoalveolar leucocyte supernatants at acid and neutral pH expressed as mean (SEM) degraded [^{125}I] fibronectin released in triplicate wells per sample, with three rats per group and two separate experiments at each time point. Medium control represents background counts due to medium alone.

There was no increase in fibronectin proteolysis at acid pH with either TiO_2 or quartz supernatants at 1 or 15 days and so the 1- and 15-day acid proteinase data are shown pooled in Fig. 1. Although all of the supernatants showed some ability to degrade fibronectin at acid pH, the two inflammatory cell supernatants were less active

than the controls; the difference was, however, not statistically significant. There was no increase in the level of *N*-acetyl glucosaminidase in the 1- or 15-day TiO_2 or quartz supernatants compared with controls (Fig. 2). In the quartz supernatants, there was a decrease in glucosaminidase at both time points but this was not statistically significant.

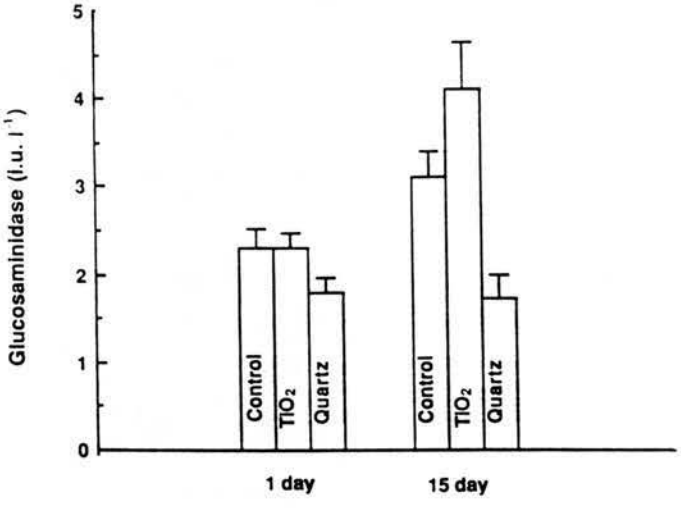


FIG. 2. Secretion of glucosaminidase *in vitro* by bronchoalveolar leucocytes. Results are expressed as mean (SEM) i.u. $l.^{-1}$ of glucosaminidase into the supernatant medium for triplicate samples in two separate experiments.

(2) In vitro *triggering of leucocyte proteinase activity*

Culturing control or inflammatory bronchoalveolar leucocytes (obtained 5 days after intratracheal treatment with quartz, TiO_2 or zymosan) *in vitro*, in the presence of TiO_2, quartz or zymosan particles or PMA had no effect in increasing the secretion of leucocyte proteinase into the supernatant medium. Supernatants of bronchoalveolar leucocytes treated *in vitro* with soluble or particulate triggers showed no difference in their ability to degrade fibronectin (Table 1). Supernatants produced in the same series of experiments were also tested for plasminogen activator activity and again there was no difference between untreated leucocytes and those triggered *in vitro* (data not shown) for any of the *in vivo* treatment groups. These experiments were carried out over a number of weeks and so half-life effects of the ^{125}Iodine have influenced the results. Direct comparisons between the *in vivo* treatment groups are therefore invalid.

DISCUSSION

Numerous studies have documented secretion of acid and neutral proteinases (e.g. FANTONE and WARD, 1984) by leucocytes but little is known about the relative contribution of each to epithelial and connective tissue damage in the lung. In the present study, we have used three models of inflammation in rat lungs, one of which (quartz injection) leads in the long term to pathological change (BROWN *et al.*, 1989)

TABLE 1. EFFECT OF *in vitro* TRIGGERS ON FIBRONECTIN PROTEOLYSIS BY BRONCHOALVEOLAR LEUCOCYTES ELICITED BY THE INDICATED *in vivo* TREATMENTS. RESULTS (MEAN [SEM] counts min^{-1} RELEASED) ARE DERIVED FROM TRIPLICATE WELLS PER SAMPLE IN THREE EXPERIMENTS PER TREATMENT FOR CONTROL LEUCOCYTES AND TWO EXPERIMENTS PER TREATMENT FOR TiO_2-, QUARTZ- AND ZYMOSAN-ELICITED CELLS

In vitro trigger	Control $N=3$* Mean	SD	Quartz $N=2$ Mean	SD	TiO_2 $N=2$ Mean	SD	Zymosan $N=2$ Mean	SD
0	1303	(102)	1114	(94)	666	(268)	1275	(176)
PMA 1.0 $\mu g\ ml^{-1}$	1434	(269)	1152	(94)	637	(248)	1157	(107)
Zymosan 1 $\mu g\ ml^{-1}$	1298	(79)	1373	(481)	574	(282)	1191	(80)
Zymosan 10 $\mu g\ ml^{-1}$	1287	(89)	1308	(421)		—	1175	(154)
TiO_2 1 $\mu g\ ml^{-1}$	1279	(191)	1365	(379)	574	(572)	1231	(102)
TiO_2 10 $\mu g\ ml^{-1}$	1306	(141)	1784	(917)		—	1227	(52)
Quartz 1 $\mu g\ ml^{-1}$	1388	(116)	2036	(1039)	622	(260)	1420	(392)
Quartz 10 $\mu g\ ml^{-1}$	1311	(93)	1586	(698)		—	1206	(126)

*N = No. of experiments.

and the others (TiO_2 and zymosan injection) which have no long-term pathological consequences. We have shown that, over a period of up to 15 days exposure, leucocytes from the bronchoalveolar region of rats exposed to particles *in vivo*, secreted increased amounts of proteolytic enzymes, compared with resident bronchoalveolar leucocytes. The proteolytic activity of the inflammatory leucocyte supernatants was manifest at neutral and not acid pH. This suggests that neutral proteinases may be a major source of proteolytic lung injury in our rat model and is in agreement with the current view that neutral proteinase activity is central to the development of both emphysematous (SNIDER *et al.*, 1986) and fibrotic (FANTONE and WARD, 1984) lesions. These results also lend support to our previous findings that fibronectin proteolysis (BROWN and DONALDSON, 1988) and epithelial injury (DONALDSON *et al.*, 1987), caused by inflammatory bronchoalveolar leucocytes, was likely to be mediated by neutral proteinase activity.

The relative importance of neutral and acid proteinases to the disease process remains equivocal, however. Secretion of acid proteinase *in vitro* was reduced in the inflammatory leucocyte populations in this and in one other study (FOGELMARK *et al.*, 1983) but we (DONALDSON *et al.*, 1988a) and others (SJÖSTRAND and RYLANDER, 1984) have previously shown that there is increased acid proteinase activity in the bronchoalveolar lavage fluid of rats exposed to pneumoconiotic dusts. The decreased *N*-acetyl glucosaminidase secretion by inflammatory bronchoalveolar leucocytes *in vitro* may therefore be a result of prior discharge of the enzyme within the lung. However, the proportion of the neutrophils in the inflammatory population could also be important in the reduced acid proteinase activity per cell *in vitro*, since neutrophils have been shown to secrete significantly less acid proteinase than inflammatory macrophages (PADILLA *et al.*, 1988). In inflamed lung, the proportion of macrophages is frequently decreased relative to neutrophils but the absolute number of macrophages is often markedly increased. Therefore, the increased numbers of macrophages in inflamed lung may be sufficient to account for the elevated levels of acid proteinase in lavage. Although the pH of the lung extracellular matrix is around neutral, local accumulation of secreted lysosomal contents may acidify the surrounding tissue and so allow connective tissue proteolysis by acid proteinases in inflamed lung.

The increased neutral proteinase activity released by the inflammatory broncho-

alveolar leucocytes *in vitro* indicated that these cells were activated. We investigated the mechanisms whereby such activation might be mediated by culturing bronchoalveolar leucocytes from control or treated rat lungs, *in vitro*, with the same particles that had caused activation *in vivo* and with the soluble trigger, PMA. We then compared the fibronectin proteolytic and plasminogen activator activities of supernatants from treated and untreated cells. None of the leucocyte supernatants showed increased proteolytic activity following *in vitro* triggering for 3 days. We have shown previously that bronchoalveolar leucocytes do not respond to short-term (4 h) *in vitro* triggering with particles by increased fibronectin proteolysis, even when the particles are present during the course of the proteolysis assay (BROWN *et al.*, 1991) nor are they activated by cigarette smoke *in vitro* (BROWN *et al.*, 1991). Therefore, activation of proteinase secretion by bronchoalveolar leucocytes in this rat model is not due simply to phagocytosis of particles but is likely to be mediated by the multiplicity of stimulatory factors such as cytokines, which occur at inflammatory sites (BILLINGHAM, 1987) and which are likely to be present in the milieu of inflamed lungs.

In the lungs of individuals and laboratory animals exposed to toxic dust there is inflammatory cell recruitment (BEGIN *et al.*, 1986; DONALDSON *et al.*, 1988b), both macrophages and neutrophils being present in increased numbers. This study, and our previous work (DONALDSON *et al.*, 1988a; BROWN and DONALDSON, 1988), suggests that acid proteinases are released into the bronchoalveolar region during inflammation but that the ability to secrete neutral proteinase is a more sustained property of the inflammatory cells. Both acid and neutral proteinases are capable of causing extracellular matrix injury and our results indicate that both may have the potential to cause pathological change in inflamed lungs.

The particles themselves are incapable of enhancing control bronchoalveolar leucocytes to the increased proteolytic state found in inflamed lungs: therefore the role of co-factors such as cytokines and chemotactic factors in priming neutrophils and macrophages for enhanced secretion are emphasized. Studies on the role of mediators such as interferon, TNF and interleukin-1 in matrix degradation are therefore warranted.

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Epithelial and Extracellular Matrix Injury in Quartz-Inflamed Lung: Role of the Alveolar Macrophage

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The bronchoalveolar leukocytes from quartz-inflamed lung were separated into macrophage-enriched and neutrophil-enriched populations on density gradients. Neutrophil-enriched populations showed the greatest activity in causing injury to epithelial cells and fibronectin *in vitro*. Inflammatory macrophage-enriched populations from quartz-exposed lung had the ability to cause fibronectin degradation but could not cause detachment injury to epithelial cells over and above that caused by control alveolar macrophages. Fibronectin damage *in vivo* could be an important factor in disordering the connective tissue scaffold of the lung, thereby favoring fibrosis. *In vitro* quartz stimulated more production of cytokines by alveolar macrophages than the inert particulate titanium dioxide. Cytokines could be important in upregulating adhesion molecules in the membranes of lung cells *in vivo*; this process could aid leukocyte/lung cell contact, allowing epithelial injury to be expressed, and could also be a factor leading to pathological change.

Introduction

Leukocytes perform an important defensive role in the lung, where they act to keep the alveolar epithelial surface free from particles. There is, however, evidence to suggest that leukocytes can also be harmful to the lung if they are recruited there in large numbers and become activated, as is found in workers in industries where the dust causes pneumoconiosis (*1*). Experimental inhalation of pneumoconiotic dusts such as quartz, asbestos, and coal mine dust causes macrophage and neutrophil recruitment (*2,3*). These leukocytes then have the potential to release a range of toxic products, including proteases, which we have demonstrated are able to injure epithelial cells (*4*) and extracellular matrix components (*5*) *in vitro*. In addition, leukocytes may also release fibroblast growth factors such as interleukin-1 (IL-1), which could contribute to fibrosis. In the presence of such growth factors, proteolytic injury to the connective tissue matrix of the lung after quartz exposure may lead to abnormal repair and fibrosis if the connective tissue scaffold is disordered. Epithelial injury can lead to disruption of the normal balance between the interstitium and the alveolar space. In addition, by stimulating proliferation of type II epithelial cells, quartz exposure can lead to the pathological lining of the alveoli with cells inappropriate to the gas transfer function of the alveoli and also accumulation of type II cell product.

In our own rat model of silicosis, both fibrosis and type II cell proliferation are evident (*6*) and, as described above, leukocytic protease has the potential to play a major role. It is clear from our studies that neutrophils are a major source of the connective tissue protease (*5*), but alveolar macrophages also provide a potential source of harmful protease and are present generally in greater numbers than neutrophils. We therefore set out to determine whether macrophages from quartz-inflamed lung were able to injure epithelial cells and break down fibronectin *in vitro*. We also assessed the effect of different dusts on the ability of macrophages to release tumor necrosis factor (TNF) and interleukins 1 and 2 (IL-1 and IL-2).

Materials and Methods

Leukocyte Populations

Leukocyte populations were obtained from control PVG rats (<98% macrophages) or rats exposed to 1 mg of quartz by instillation 5 days previously (50% macrophages and 50% polymorphonuclear neutrophils [PMN]). Cells were collected by bronchoalveolar lavage as previously described (*2*). Cell populations were either kept whole or separated into macrophage and neutrophil-rich fractions by density gradient centrifugation through Sepracell medium according to the manufacturer's instructions (Sepra-tech Corporation, Oklahoma City, OK).

Epithelial Injury

Epithelial injury was assessed by radiolabeling cells of the alveolar epithelial cell line A549 with ^{51}Cr and incubating them with effector leukocytes for 6 hr at an effector: target ratio of 5:1. At the end of this time, two types of injury were assessed: lytic injury and detachment injury (*4*).

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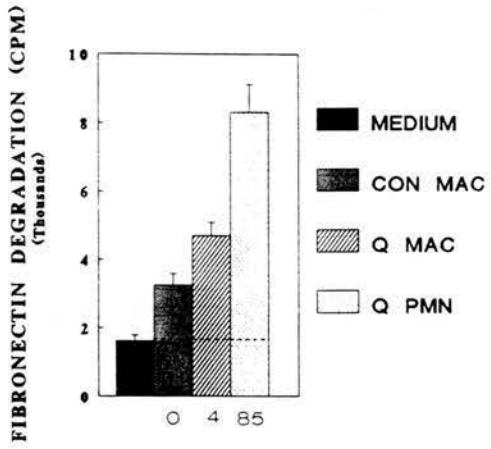


FIGURE 3. Fibronectin degradation caused by control alveolar macrophages (CON), macrophage-enriched (Q MAC), or PMN-enriched (Q PMN) populations from quartz-inflamed lung. Spontaneous release of ^{123}I-fibronectin counts are shown as the bar marked "medium." Numbers under the bars indicate the mean percentages of PMN. Results are the means +SDs of five separate experiments, wtih two to three rats used in each experiment. Significantly more fibronectin degradation with all populations compared to medium alone ($p < 0.01$) and with Q PMN compared to the other conditions.

here that the two functions are not necessarily associated and that macrophages have the ability to break down fibronectin in the absence of the potential to cause detachment injury to epithelial cells *in vitro*.

It seems likely that close-range interaction, possibly comprising membrane–membrane contact with target cells or adherence to extracellular matrix components, is a necessary prerequisite for inflammatory leukocytes to cause injury. Cell-to-cell attachment involves the integrin class of molecules and associated adhesion molecules in the cell membrane (*7*). Clearly, both macrophages and neutrophils possess the receptors that allow close interaction between these cells and fibronectin. However, only neutrophils may possess the receptors that allow close interaction with epithelial cells *in vitro*.

We have found that the inflammatory dust quartz, which was used as a model injuring agent in the present study, is able to directly stimulate the release of the cytokines IL-1 and TNF from alveolar macrophages in excess of that produced when the macrophages are treated with the relatively harmless dust titanium dioxide (*2*). Cell adhesion molecules can be upregulated by exposure to cytokines (*8*). Thus, in the lung itself, when the epithelial cells are exposed to the cytokine for longer than the 5 hr used in the assay here, this time may allow upregulation of adhesion molecules on the epithelial cells, allowing macrophage-mediated short-range epithelial injury to occur *in vivo*.

We intend to expose epithelial cells to cytokine *in vitro* and then assess the ability of the macrophages to cause injury. This may allow the epithelial cells to upregulate their adhesion molecules, which could promote close contact between the effectors and the targets, possibly allowing injury to be expressed. The fact that PMN can cause the injury may be related to expression of the appropriate adhesion molecules or their much increased production of protease, which may be sufficient to cause injury without the necessity for direct contact. We have, however, demonstrated

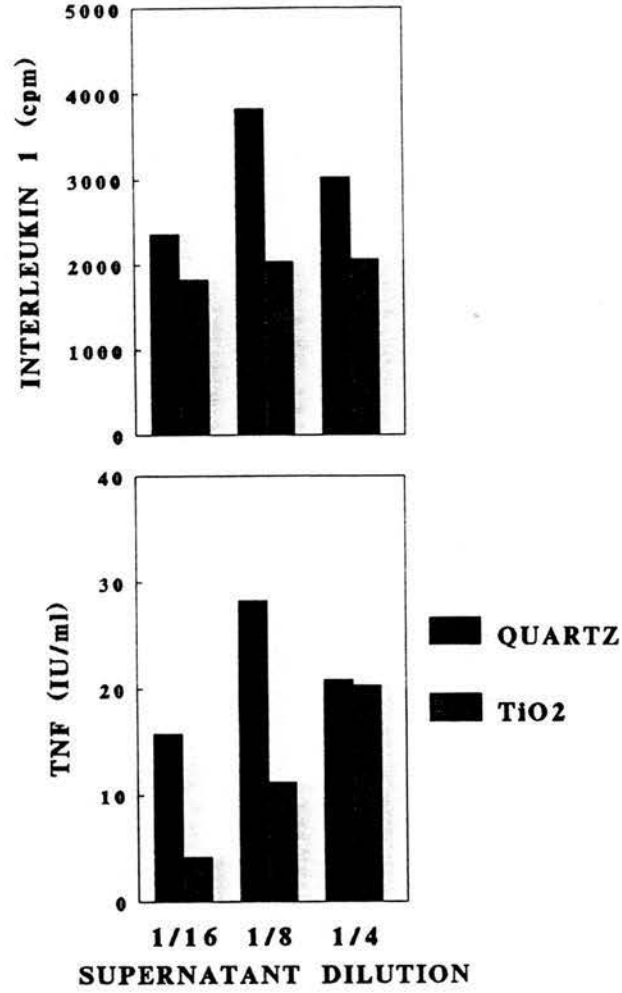


FIGURE 4. Production of interleukin-1 (IL-1) and tumor necrosis factor (TNF) by alveolar macrophages exposed to quartz and titanium dioxide (TiO_2) *in vitro*. Results are expressed as thymocyte proliferation in counts per minute (IL-1) or international units per milliliter (TNF). Results are shown as the means of three to five experiments with significant differences between TiO_2 and quartz at 1/8 and 1/16 dilutions ($p < 0.05$). Pooled cells from one to three rats were used in each experiment.

the production of high levels of IL 1 by neutrophils from inflamed lung (*9*), and this may also play a role.

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Fibronectin Degradation

The ability of leukocytes to break down the extacellular matrix component fibronectin was assessed using fibronectin labeled with ^{125}I. ^{125}I-fibronectin was dried on to the base of microtiter plate wells and leukocytes were incubated on this matrix for 4 hr, and the release of ^{125}I-fibronectin breakdown products into the supernatant was measured (*5*).

Cytokine Production

Alveolar macrophages were exposed *in vitro* to various types of dust for 24 hr at 25 μg/mL and the supernatants were collected. IL-1 in supernatants was measured as enhanced stimulation of suboptimal lectin-treated mouse thymocytes. TNF was measured using the L929 cell line.

Statistical Analysis

Data from repeat experiments were analyzed by analysis of variance to determine whether there were any treatment effects and to obtain a measure of estimated standard error. Differences between treatments were tested for statistical significance using this estimated error in a *t*-test.

Results

Epithelial Injury

As shown in Figure 1, the separated, macrophage-enriched populations from quartz-inflamed lung were able to cause detachment of epithelial cells. However, when individual experiments were assessed and detachment was related to the proportion of contaminating neutrophils (Fig. 2), it was clear that the detachment was attributable to the neutrophils. Results were obtained from four separate experiments using pooled cells from one to three rats in each experiment.

Fibronectin Degradation

As shown in Figure 3, the inflammatory macrophage-enriched population was capable of degrading large quantities of the extracellular matrix component fibronectin compared to control macrophages. However, in this case the increased proteolytic injury could not be explained by the 4% of contaminating PMN. This level of PMN would be anticipated to increase the proteolytic activity of the macrophage population by 12%, whereas the actual increase over the control macrophages was 45%. Results represent means and standard deviations from five separate experiments, with cells from one rat used in each experiment.

Cytokine Production

We have demonstrated, as shown in Figure 4, that quartz caused more release of cytokine from alveolar macrophages *in vitro* than titanium dioxide. On dilution, the supernatants showed a sigmoid curve of activity, as is normally seen with crude supernatant in a bioassay of this sort. It is presumed that the differential effect of dilution on the response is due to conflicting activities present in the supernatant, which dilute out at different rates to the TNF activity.

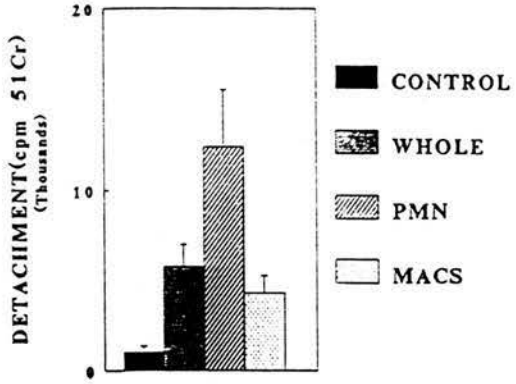


FIGURE 1. Detachment injury caused to epithelial cells by control bronchoalveolar leukocytes and cells from quartz-exposed lung. Whole, unseparated; PMN, PMN-enriched population; MACS, macrophage-enriched population. Significantly more detachment caused by all quartz populations compared to control macrophages ($p < 0.01$). Significantly more detachment caused by PMN than MACS or whole ($p < 0.05$).

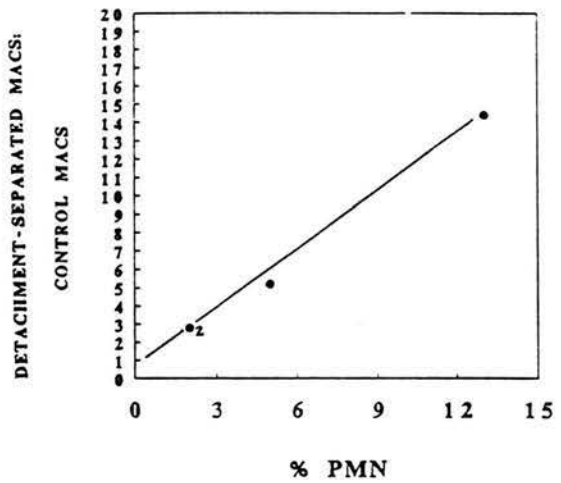


FIGURE 2. Detachment injury, expressed as detachment caused by macrophage-enriched populations divided by detachment caused by control macrophage populations, versus percentage of contaminating PMN. The line, fitted by eye, passes through 1, suggesting that inflammatory macrophages cause as much detachment as control macrophages when no PMN are present. Data from four separate experiments. The "2" represents two data points that were identical.

Discussion

We and others have demonstrated the ability of PMN to cause injury to epithelial cells and extracellular matrix components (*1*). Macrophages are present in normal lung but are present in increased numbers and in an activated state in dust inflamed lung (*2*) and so could also contribute to injury. We have shown here that macrophages from inflamed lung are unlikely to be able to cause epithelial damage, at least in the rat model of silicosis that we used here. In contrast, the inflammatory macrophages have the ability to break down fibronectin in increased amounts compared to control macrophages.

In the case of PMN, we have previously associated the ability to break down fibronectin with the ability to cause epithelial injury, both of which are protease-mediated events (*4*). We report

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Persistent Inflammation and Impaired Chemotaxis of Alveolar Macrophages on Cessation of Dust Exposure

by Geraldine M. Brown,[1] David M. Brown,[1] and Kenneth Donaldson[1]

Rats were exposed by inhalation to coal mine dust, titanium dioxide, or quartz. The magnitude of the consequent inflammatory response was assessed by counting numbers and types of leukocytes in the bronchoalveolar lavage fluid. The magnitude of the inflammatory response reflected the toxicity of the dusts, with quartz eliciting the greatest recruitment of inflammatory leukocytes, coal mine dust less than quartz, and titanium dioxide eliciting no inflammation. To assess the persistence of the inflammation, groups of rats were maintained in room air for 30 or 60 days after cessation of dust exposure and then numbers of leukocytes were assessed. Bronchoalveolar leukocytes in rats exposed to coal mine dust were reduced after exposure, but in the quartz-exposed rats the numbers increased with time after exposure. The chemotactic responses of bronchoalveolar leukocytes from rats inhaling coal mine dust and quartz were reduced and remained so after a 30-day recovery period. Their reduced ability to chemotact did not fully prevent macrophages from leaving the bronchoalveolar region of dust-exposed rats. However, it is likely that the delayed removal of inflammatory leukocytes with the potential to injure the lung tissue may contribute to septal damage and so contribute to the pathogenesis of pneumoconiosis.

Introduction

In recent years there has been considerable interest in the immunological/inflammatory role of leukocytes after dust deposition. Of particular importance is the function of the alveolar macrophage in clearing the lung of inhaled particles. An area where both particle clearance and the immunoinflammatory roles of bronchoalveolar leukocytes overlap is chemotaxis. Particles depositing in the alveolar region are phagocytized by alveolar macrophages and then transported intracellularly to the mucocilliary escalator (*1*). There is also firm evidence that particles are cleared from the alveolar space by transport to the lung lymph nodes (*2*). It is in such a situation that interactions between dust-laden macrophages and lymphocytes are likely to occur. Such interactions could have important consequences for the disease process, for example, through the generation of cytokines and growth factors.

A key feature of pneumoconioses is the presence of inflammatory leukocytes in the bronchoalveolar region (*3*). We have previously shown that inflammatory leukocytes have the potential to injure cells (*4*) and connective tissue molecules (*5*) of the alveolar septum. The chemotactic activity of macrophages is thus likely to be of key importance to lung defense because it may influence their ability to move out of the alveolar region and thus be of importance in limiting the extent of leukocyte-mediated tissue damage after dust exposure. We therefore assessed the persistence of the inflammatory response and the chemotactic activity of bronchoalveolar leukocytes from lungs of rats exposed to dust by inhalation.

Materials and Methods

Male SPF rats of the HAN strain were exposed to airborne mineral dusts for 8 hr/day, 5 days/week in 1-m^3 inhalation chambers as previously described (*6*).

Dusts. Coal mine dusts were sampled from the air of British Collieries using dry fabric filters and then generated as a cloud using a Timbrell dust generator. Details of coal mine dust mineralogy have been published elsewhere (*6*). The dust cloud was passed through a cyclone to produce a respirable fraction, which was then dispersed into the chamber at an airborne mass concentration of 50 mg/m^3 in experiment 1 and 10 mg/m^3 in experiment 2, as previously described (*7*). Titanium dioxide (TiO_2; rutile) was obtained from Tioxide Ltd. (Stockton on Tees, England). Quartz was the DQ12 standard preparation.

Cell Preparation. Rats were removed from the chambers at various time points after the start of dust exposure. "Recovery" animals were also removed from the chambers at selected time points and were then maintained in room air for a further 60 days (experiment 1) or 30 days (experiment 2). The animals were killed, and bronchoalveolar leukocytes (BAL) were obtained by

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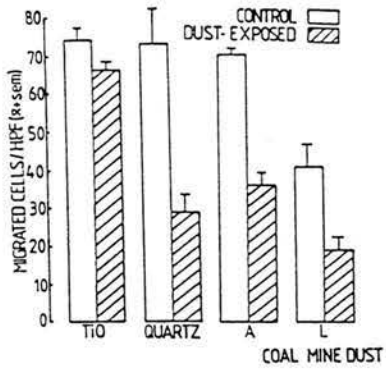


FIGURE 3. Chemotaxis of bronchoalveolar leukocytes from control rats and rats inhaling the indicated dusts at 10 mg/m^3. Results are the means (SD) of pooled data for 8, 32 and 75 days of exposure. Data were obtained from two filters, with five high-power fields per filter at each time point for each dust. Quartz and both coal mine dusts (A,L) show a significant reduction in chemotaxis ($p < 0.05$).

Table 1. Total cells and differential count in the bronchoalveolar lavage fluid of untreated rats, immediately on cessation of exposure to 10 mg/m^3 coal mine dust (dust), and following a further 3 days breathing room air (recovery rats).

	Total BAL leukocytes, $\times 10^6$ [a]		%PMN in BAL[a]	
	Control	Dust	Control	Dust
3 days	4.8 (0.5)	4.5 (1.0)	0 (0)	0 (0)
3 days + recovery	7.8 (0.1)	7.3 (0.7)	1.3 (0.9)	0.5 (0.4)
7 days	5.4 (0.7)	7.8 (1.2)	0 (0)	13.7 (5.9)
7 days + recovery	6.9 (0.2)	7.9 (0.6)	0 (0)	1.3 (0.7)
15 days	4.3 (0.4)	12.2 (1.0)	0 (0)	29.0 (5.0)
15 days + recovery	6.9 (1.6)	14.9 (2.1)	0 (0)	17.3 (1.7)
30 days	5.3 (0.1)	29.5 (4.5)	0.7 (0.6)	41 (5.8)
30 days + recovery	4.6 (0.8)	14.4 (1.7)	1.3 (2.7)	27.3 (2.7)

Abbreviations: BAL, bronchoalveolar lavage; PMN, polymorphonuclear neutrophils.
[a]Values are means; SEM in parentheses.

Table 2. Chemotaxis of bronchoalveolar leukocytes from rats inhaling coal mine dust and then breathing room air for an additional 30 days. [a]

	Control		Coal mine dust	
Time	Mean	SEM	Mean	SEM
3 days + recovery	37.0	3.3	43.2	4.7
7 days + recovery	66.9	7.8	40.6	6.3
15 days + recovery	21.7	3.2	5.4	1.7
30 days + recovery	26.5	3.9	3.7	0.9

[a]Results are the counts of five fields per filter and two filters for each rat, with three rats per group at each time point.

Discussion

In this study, we have confirmed previous work demonstrating the recruitment of inflammatory leukocytes to the bronchoalveolar region in response to the inhalation of mineral dust in man (*5*) and in experimental animals (*9*). The magnitude of the inflammatory response was related to the pathogenic potential of the dusts, with quartz proving to cause more inflammation than coal mine dust. The failure of titanium dioxide to elicit an inflammatory response in the present study reflects the innocuous effects of this mineral, which is widely used in industrial processes but is not associated with pathology in man (*10*). We have also demonstrated reduced chemotactic responses of bronchoalveolar leukocytes obtained from rats inhaling pneumoconiotic dusts but not TiO_2. These results are consistent with previous reports of impaired chemotaxis after exposure to quartz (*11*) and chrysotile asbestos (*12*).

The mechanisms governing the reduction in chemotaxis are as yet unclear, but we have previously reported that the chemotaxis deficit was largely due to impaired alveolar macrophage function and was not due to the presence of PMN in the BAL (*8*). Further evidence that PMN do not contribute substantially to the impaired chemotaxis of inflammatory bronchoalveolar leukocytes was obtained in the present study where reduced chemotaxis was observed in macrophages from rats allowed to recover in room air for 30 days after dust exposure, by which time there were no PMN remaining in the BAL. Interestingly, there was also decreased chemotaxis of alveolar macrophages from rats exposed to coal mine dust for 7 days and then allowed to recover for a further 30 days. In these animals there was no evidence of PMN or macrophage recruitment to the bronchoalveolar region at any time. This suggests that the changes that occur to alter macrophage chemotactic responses after mineral dust exposure are subtle and can occur in the absence of overt inflammation. The failure of TiO_2 to elicit any such changes in macrophage chemotaxis suggests that the effect may be due to a direct interaction between toxic dust particles and the macrophages. However, we have shown that *in vitro* exposure to dust does not alter chemotaxis (*8*), and so a direct effect of the dust *in vivo* is unlikely.

One of the interesting findings of the present study was that, although quartz caused more inflammation than coal mine dust, there was no significant difference in the extent of the chemotaxis deficit between the quartz and coal mine dust-elicited leukocytes. Taken together with the reduced chemotaxis of leukocytes before the onset of bronchoalveolar inflammation in rats exposed to coal mine dust, this suggests that a factor similar to migration inhibition factor may be released as part of the early response to any toxic mineral dust.

In this study, bronchoalveolar macrophage numbers decreased on cessation of exposure to coal mine dust and by 60 days had returned to normal control levels. Impaired chemotaxis was therefore not sufficient to fully abrogate macrophage clearance. However, the delay in clearance may be of importance in the development of pneumoconiosis. Activated alveolar macrophages release proteases that can damage the alveolar septum (*5*). In addition, we have demonstrated recently that inflammatory macrophages from dust-exposed rats secrete increased amounts of interleukin 1 (*13*) and tumor necrosis factor (Brown et al., manuscript in preparation). These cytokines can generate chemotaxins and cause increased recruitment of inflammatory leukocytes to the alveolar region. They can also stimulate proliferative responses in mesenchymal cells. The delayed removal of inflammatory macrophages may thus contribute to the persistence of inflammation in the bronchoalveolar region of the lung and in the long term may play a role in the pathogenesis of pneumoconiosis.

The authors are grateful to A. Jones, H. Cowie, J. M. G. Davis, R. E. Bolton, J. Slight, and D. Brown for their involvement in various aspects of this study.

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lavage as previously described (*5*). Total cells and differential counts were performed on the BAL before use in the chemotaxis assay.

Chemotaxis Measurement. Chemotaxis was assessed by measuring the directed migration of BAL through micropore filters in Boyden chambers as previously described (*8*). Zymosan-activated serum was used as the chemoattractant, and the modified checkerboard technique was used to check that the migration was true chemotaxis and not chemokinesis. The chemotactic activity of the bronchoalveolar leukocytes was measured at days 8, 15, and 32 in experiment 1 and after 30 days of recovery in rats exposed for 3, 7, 15, and 30 days in experiment 2. As a measure of chemotaxis, we counted the number of migrated cells per high power field in five fields per filter and two filters per condition.

Statistical Analysis. Differences between means were tested using Student's *t*-test.

Results

Experiment 1

The pneumoconiotic dusts (quartz and coal mine dust) elicited an inflammatory response in rat lungs after inhalation exposure at 50 mg/m³. Quartz produced the most marked response, whereas TiO_2, which is not associated with pathology in man, failed to induce inflammation except in the recruitment of polymorphonuclear neutrophils (PMN) at the latest time point. The total number of macrophages in the BAL was significantly greater ($p < 0.01$) than that in the TiO_2-exposed rats by 8 days of quartz exposure and by 16 days with the coal mine dusts (Fig. 1). On cessation of exposure to coal mine dust, macrophage numbers returned to normal, but in those animals exposed to quartz, the inflammation not only persisted but progressed markedly. The PMN response in the quartz-exposed rats reflected the macrophage response in that there was time-dependent recruitment of PMN and continuing increases in PMN numbers on cessation of dust exposure (Fig. 2). In rats exposed to coal mine dust, PMN numbers decreased during the recovery period but did not return to control levels, thus indicating persistence of the inflammatory response in these animals. Chemotaxis was assessed immediately on cessation of dusting at 8, 32, and 75 days after the start of dust exposure; at each time point there was a significant reduction in the chemotactic response of the quartz and coal mine dust-exposed leukocytes compared with controls ($p < 0.05$), but TiO_2 had no effect (Fig. 3).

Experiment 2

Having demonstrated impaired chemotactic responses of alveolar macrophages during dust exposure in experiment 1, we then went on to assess the persistence of the reduced chemotaxis. In experiment 2, inhalation of coal mine dust at 10 mg/m³ produced an inflammatory response by 15 days of exposure (Table 1). Although the increase in numbers of macrophages in the BAL was reduced when the animals were allowed to breathe room air for a further 30 days, numbers of macrophages in the BAL did not reach control levels, thus there was evidence of persistent inflammation. Chemotactic responses, measured in the recovery animals (Table 2), were impaired after 15 and 30 days of dust

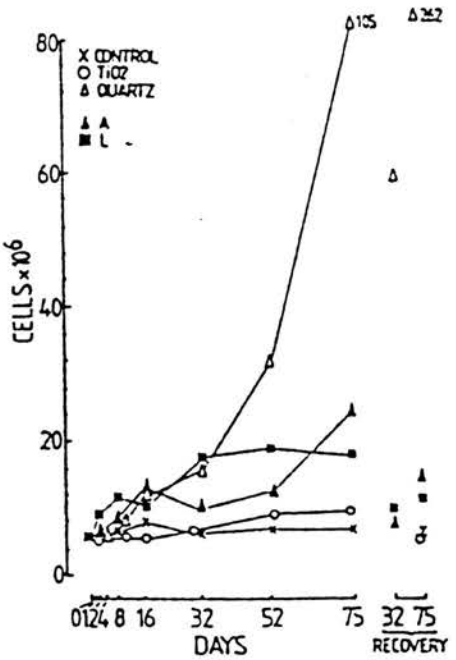


FIGURE 1. Total number of macrophages in the bronchoalveolar lavage of rats inhaling TiO_2, quartz, or coal mine dust (A,L). The last two time points represent, respectively, 32 days plus 64 days of recovery and 75 days plus 64 days of recovery. Results are the means of four rats at each time point for the dust-exposed animals and two for the controls.

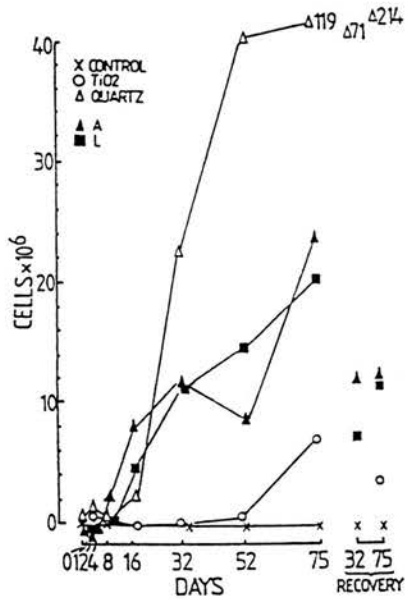


FIGURE 2. Percentage of neutrophils in the bronchoalveolar lavage with time of exposure to 50 mg/m³ of TiO_2, quartz, or coal mine dust (A,L). The last two time points are, respectively, 32 days of exposure plus 64 days of recovery and 75 days of exposure plus 64 days of recovery. Results are the means of four rats at each time point for the dust exposed animals and two for the controls.

exposure. This result was consistent with the presence of an inflammatory response in the bronchoalveolar region. However, there was also impaired chemotaxis of BAL leukocytes after only 7 days of dust exposure, where there had been no evidence of an inflammatory response.

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SHORT-TERM BRONCHOALVEOLAR LEUKOCYTE RESPONSES IN RATS INHALING GLASS MICRO-FIBRE AND AMOSITE ASBESTOS AT EQUAL AIRBORNE FIBRE NUMBER AS MONITORED BY PHASE CONTRAST OPTICAL MICROSCOPY

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ABSTRACT

he present study compared the short-term inflammatory effects in rats, of inhaling 000 fibres/ml (monitored by phase contrast optical microscopy) of a special purpose ass microfibre preparation - Johns Manville Code 100/475, and a control long fibre nosite asbestos preparation. Rats inhaled the fibres for 7 hours/day for 5 ays/week, for up to 14 days and inflammation was assessed by bronchoalveolar lavage. ccasional monitoring of the clouds by Scanning Electron Microscopy revealed that ere were many more respirable fibres in the case of the glass microfibre; however, flammation was greatest in response to inhalation of the amosite asbestos. This was pparent as increased recruitment of neutrophils and high free lactate dehydrogenase in e lavage fluid from 1 day of exposure onwards. The glass microfibre did not cause significant increase in recruitment of neutrophils to the alveolar space compared to ontrol rats, but did result in elevated lactate dehydrogenase. Although elevated, ctate dydrogenase was significantly lower in concentration in the lavage fluid from ass microfibre-exposed rats than in asbestos-exposed rats. Since there were more espirable fibres in the cloud of glass microfibre than in the amosite asbestos cloud, nd the fibre length distributions were similar, these experiments suggest that the rface of glass microfibre is less active in causing inflammation than the surface of e amosite asbestos sample.

INTRODUCTION

xposure to asbestos fibres is associated with the development of interstitial fibrosis and ancer of the lung. Man-made fibres having similar uses to asbestos have been less ell studied but there is evidence that these can cause similar lesions in experimental nimals (eg Davis *et al* 1984). Production of inflammation in the alveolar region of e lung is a property of inhaled fibres that is very likely to be an important factor in e the long term pathology described above.

n previous studies at the Institute of Occupational Medicine we have utilised ronchoalveolar lavage as a method of detecting the early, inflammatory effects of sbestos fibre inhalation (Donaldson *et al* 1989), but there is little information on such esponses in rats exposed to man-made fibres. In the present study we have used ronchoalveolar lavage to assess the early lung response in rats inhaling a special urpose glass microfibre sample - Johns Manville Code 100/475; comparison was made ith a long fibre amosite asbestos sample as a positive control. Clouds of both bres were adjusted to a target of 1000 fibres/ml airborne fibre number concentration, s assessed by Phase Contrast Optical Microscopy. Both fibre samples had a similar ngth distribution. Rats exposed to these clouds were examined for inflammation in e broncholaveolar region using lavage (BAL) at 1,3,7 and 14 days.

MATERIALS AND METHODS

Rats. Wistar-derived rats of the HAN strain 12 weeks of age at the start of exposure, were used throughout.

Inhalation exposure: Rats were placed in $1m^3$ whole-body inhalation chambers and exposed to fibres produced using Timbrell dust generators as described extensively previously (Davis *et al* 1982). Rats had access to food and water ad libitum and were exposed for 7 hours/day, 5 days/week. The fibres used were a long fibre amosite asbestos sample (long amosite), which we have reported on extensively (Davis *et al* 1986; Donaldson *et al* 1989) and a special purpose glass microfibre sample - Johns Manville glass microfibre Code 100/475 (Code 100/475). The ratio of the fibre number concentration to fibre-mass concentration was estimated at the start of the exposures by taking 8 short period samples for fibre counting during a day (7 hours) of exposure. The mean fibre number concentration was estimated from these short period samples, and the number to mass ratio calculated as the ratio of these mean concentrations. The consistency of the result was checked by repeating the exercise on at least two further days. This number to mass ratio was used to estimate the mass concentration which would correspond to the target number concentration of 1000 fibres/ml as measured by the standard phase contrast optical microscope method. The concentration in each chamber was monitored daily for mass concentration using the Casella MRE 113A respirable dust sampler, and the flow rates though the chamber adjusted to achieve the target mass concentration.

Bronchoalveolar Lavage Analysis: Following the requisite number of days exposure, rats were removed from the chamber and BAL was carried out as previously described (eg Donaldson *et al* 1988) to sample the cells of the broncholaveolar region. The cells were counted in a Neubauer chamber using standard methods and cytocentrifuge preparations were made which were stained with May Grunwald Giemsa, followed by differential counting. The lavage fluid itself was assayed for the cytoplasmic enzyme lactate dehrogenase (LDH)(Wroblewski and Ladue 1953).

Statistical Analysis: All experiments were repeated 3 times with 2 rats in each treatment group. All data were analysed by 3 Factor Analysis of Variance (General Linear Model) using the Minitab Statistical Package. The response variables were total cells, total macrophages, total PMN and LDH. The classifying variables were treatment (control, long amosite, Code 100/475), days exposed (1, 3, 8 and 14 days) and experiments (Numbers 1-3). Where a significant ($P<0.05$) F test was present for any of the classifying variables, t tests were carried out to determine which individual treatments differed significantly from controls.

RESULTS

Exposure conditions: Table 1 shows the average airborne mass concentrations and the conversion factors for fibres/ml, attained during the exposures used in the experiments described here. Figure 1 shows the PCOM size distributions of the fibres present in the chamber clouds.

BLE 1 Airborne mass concentrations (mg/m^3) of respirable fibre attained in the rt-term exposures used in the present study.

	DAYS EXPOSED	LONG AMOSITE	GLASS MICRO-FIBRE
	1	6.1 *	4.9**
	3	4.7	4.4
	8	4.7	4.8
	14	4.9	4.6
	mean mg/m3	5.1	4.7
iversion factor	mg/m3 to f/ml	178	194
	mean fibres/ml	908	912

al cells: In terms of the total number of lavageable cells, there was no difference ween the untreated group and the rats exposed to long amosite or Code 100/475 0.18:P>0.25). The mean numbers of total cells ranged between 7.1 and 10.3 x in the controls and 5.2 and 11.4 x 106 in the fibre-exposed groups.

crophages: Analysis of variance revealed no difference between any of the ıtments in the total number of lavageable macrophages (F=0.70: P>0.25: Figure 2).

utrophils: There was a significant treatment effect in the total number of ageable neutrophils (F=22.41: P<0.01: Figure 3). There was no significant effect days exposure (P> 0.25), nor any significant interaction between treatment and days osed (P>0.25). When the source of the treatment difference was examined using ests, it was found that the amosite treatment had produced a statistically significant rease over the controls (P<0.01)): Code 100/475 was not statistically different from control.

nphocytes: Total lymphocyte number obtained by lavage is shown in Figure 4. spite the appearance of a substantial increase on day 1, there was no significant ect of treatment (F=1.34;P>0.05), nor a significant interaction between days exposed l treatment (F=1.0;P>0.25).

H: There was a significant effect of treatment on the concentration of the oplasmic enzyme LDH present in the BAL fluid (F=13.236: P<0.05: Figure 5), as l as a significant effect of days exposed (F=6.87:P<0.05) although the effect did increase beyond 8 day; t tests revealed a significantly greater level of LDH in lavage fluid of both the long amosite-treated and Code 100/475-treated rats than the control rats (P<0.001). There was also significantly more LDH in the age fluid of the long amosite animals than in the Code 100/475 animals (P<0.001).

DISCUSSION

e rats in the present study were exposed to a target airborne fibre concentration of 0 fibres/ml, attained by daily gravimetric monitoring backed up by regular PCOM re number counts, to ensure an accurate conversion of the daily gravimetric results airborne fibre number. Using this standard method, the actual mean fibre number ıcentration, across all of the short-term exposures in the present study was slightly ve 900 for both fibres. Side-by-side samples for PCOM and Scanning Electron croscopy counting were taken on a single day for both fibre clouds. These ealed that, for fibres longer than 5 m, the PCOM counts closely reflected the

ual number of fibres for long amosite but under-estimated the Code 100/475 by out 60%. For all fibres longer than 0.4 m the SEM counts on that day showed mean figure of 1748 for long amosite and 3396 for Code 100/475. The increased lmonary cell response seen with long amosite inhalation was therefore produced in e face of a higher airborne fibre number of the Code 100/475; both clouds were 30% respirable.

e increase in total number of neutrophils with long amosite exposure was found at 1 y and continued throughout the 14 days of exposure. The effects of dissolution e unlikely to come into play in such a short time and so it must be concluded that, r fibre, the Code 100/475 is less inflammogenic in the rat lung than long amosite. e PCOM size distributions, for fibres longer than 5 m, revealed a striking similarity tween the two fibre samples so precluding a fibre-length-related difference. SEM ing of the fibres will be more informative on the actual fibre exposure and will be ported in due course. Although there was no evidence of significant effect of time e decrease in the total neutrophils could be a result of the pattern of dusting which eant that a weekend (2 days of non-dusting) intervened between day 3 and day 8. is may have allowed some recovery and a similar disruption to the exposure, curring between day 10 and day 14 was also present.

creased neutrophil recruitment was found even after a single day of exposure to long nosite. This suggests that the deposition of even a relatively small number of these res is capable of setting up a chemotactic gradient sufficient to cause the tra-vasation of neutrophils and their accumulation in the alveolar space. The igin of the chemotactic gradient, whether cell or complement-derived was not vestigated. The neutrophil recruitment was, however quite specific and no increase macrophage recruitment could be detected. We have no explanation for the peak lymphocyte recruitment seen only in the rats exposed for 1 day to long amosite. e have identified inceased lymphocytes previously in chrysotile asbestos-exposed rats t a transient response, of the type seen here has not been seen.

ssection of terminal airways and alveolar ducts and visualisation by SEM showed that e fibres of long amosite and Code 100/475 both deposited preferentially at these sites d were phagocytosed by the local alveolar macrophages (data not shown). In a mpanion paper in this volume we describe rapid and greatly increased release of the tokine Tumour Necrosis Factor (TNF) in vitro from alveolar macrophages agocytosing long amosite fibres compared to Code 100/475 (Brown *et al* ibid). The plication of Tumour Necrosis Factor in the inflammation and pathogenicity caused by number of mineral dusts (Piguet *et al* 1990; Driscoll *et al* 1991), combined with r own data on the relative potency of long amosite compared to Code 100/475 in mulating TNF release from rat alveolar macrophages, suggests that this cytokine may important in the long amosite-induced inflammation described here.

e main conclusion from this study is that, since both long amosite and Code 0/475 are both highly respirable, are of similar length distribution and deposit in the veolar region (and that more fibres of Code 100/475 were in effect present in the st cloud), the surface of the Code 100/475 must be considered to be less active than at of the long amosite. Code 100/475 may not be without activity and may have used an apparent increase in the total number of neutrophils at all of the points yond 1 day, although this did not represent a statistically significant effect. In ldition Code 100/475 caused a significant increase in LDH, indicating that there was deed some toxicity towards lung cells, either macrophages or epithelial cells. The ng-term pathology associated with inhalation of these two fibre types will be certained in due course as part of the Colt Fibre Research Programme, and the lationship to the inflammogenic activity will be apparent. However from the

present results and from previous experimentation with Code 100 fibres (McConnell *et al* 1984) and long amosite (Davis *et al* 1986) we anticipate tumours and fibrosis with the amosite and little pathology with Code 100/475.

Acknowledgement

This research was undertaken with funding from the Colt Fibre Research Programme.

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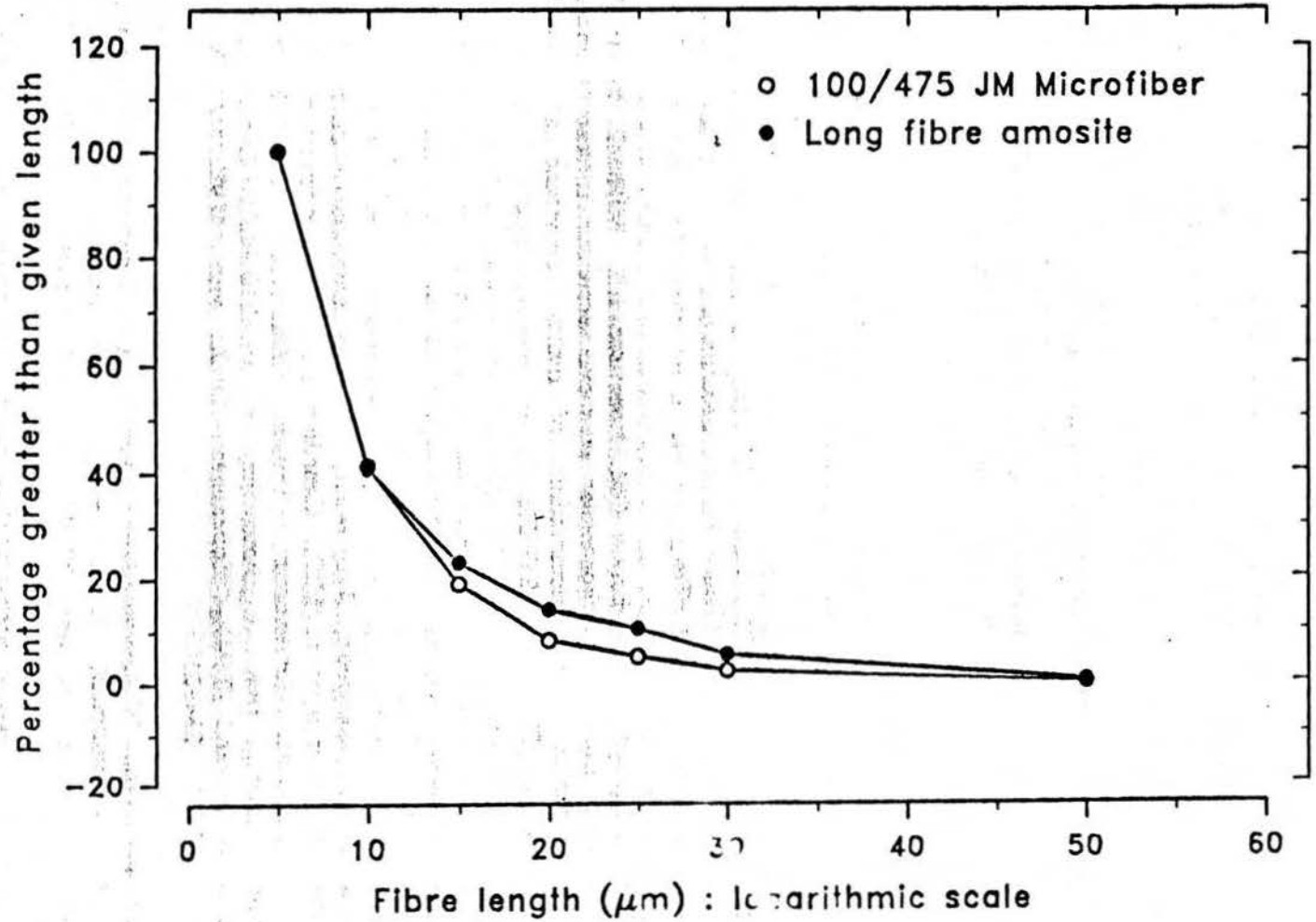


Figure 1. Length distribution of fibres present the inhalation chambers. PCOM sizing according to WHO guidelines (see Materials nd Methods).

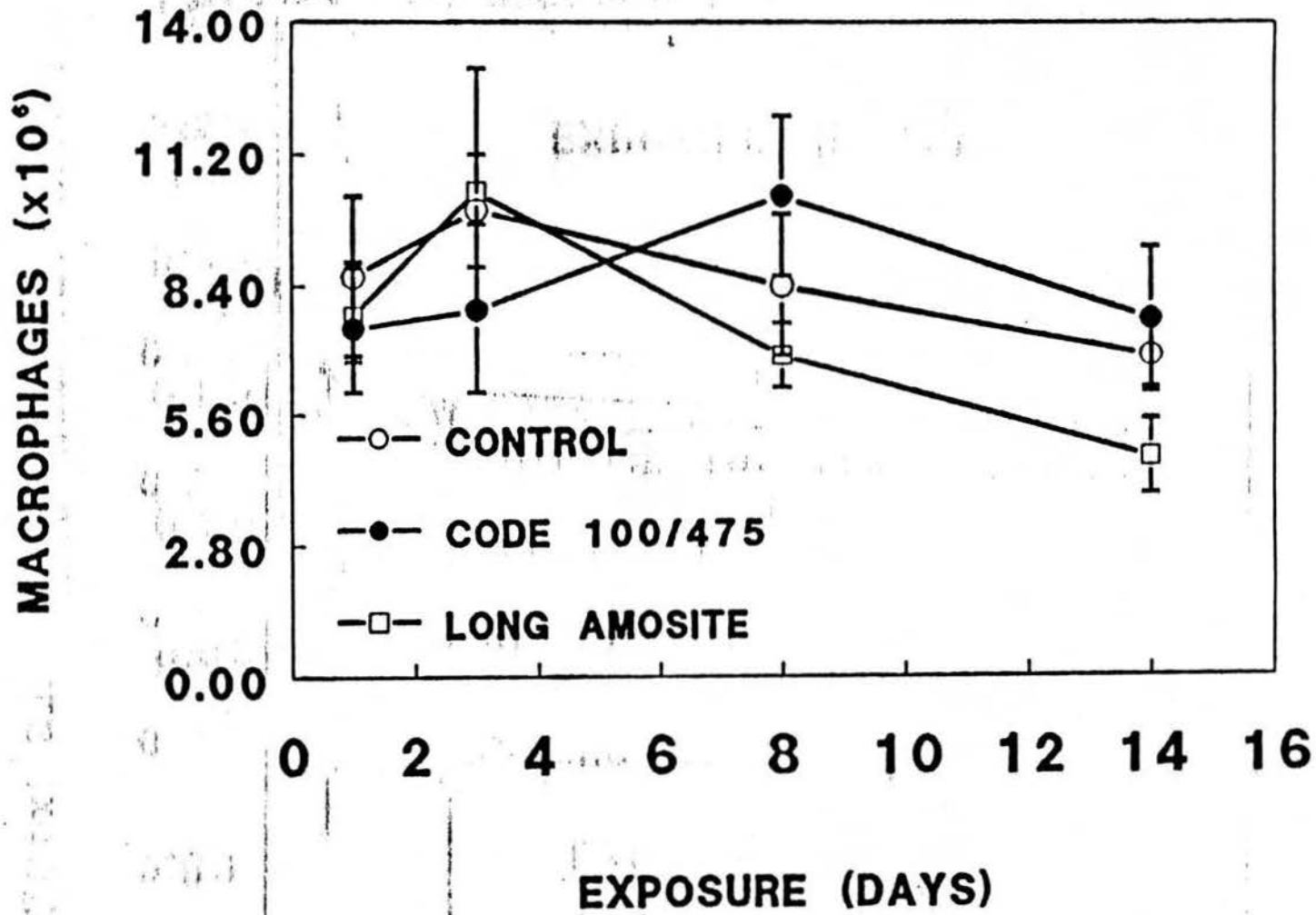


Figure 2. Total numbers of macrophages lavaged from the lungs of rats inhaling long amosite or Code 100/475 for the indicated number of days. Data is derived as the mean and SEM of 6 rats in 3 separate experiments.

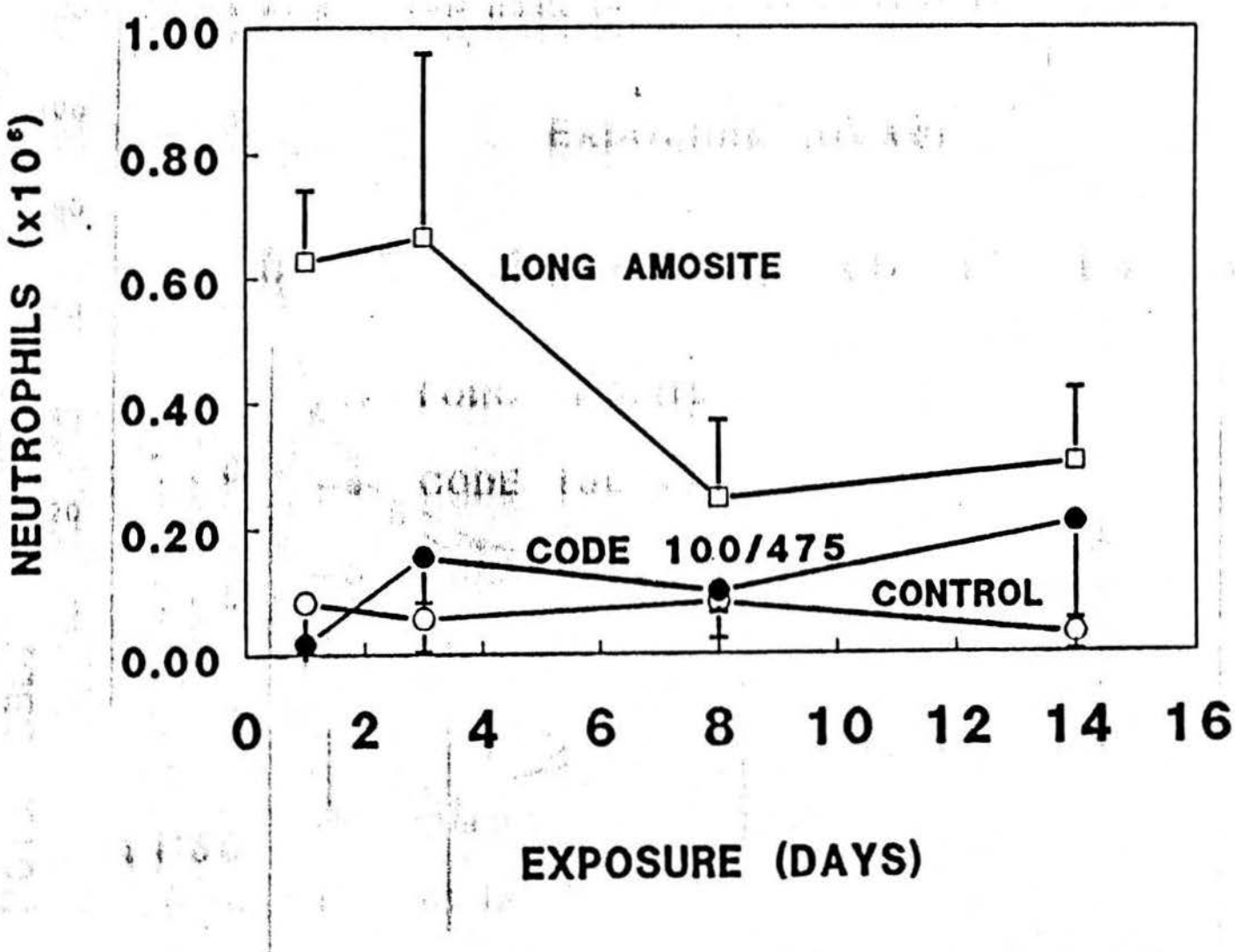


Figure 3. Total numbers of neutrophils lavaged from the lungs of rats inhaling long amosite or Code 100/475 for the indicated number of days. Data is derived as the mean and SEM of 6 rats in 3 separate experiments.

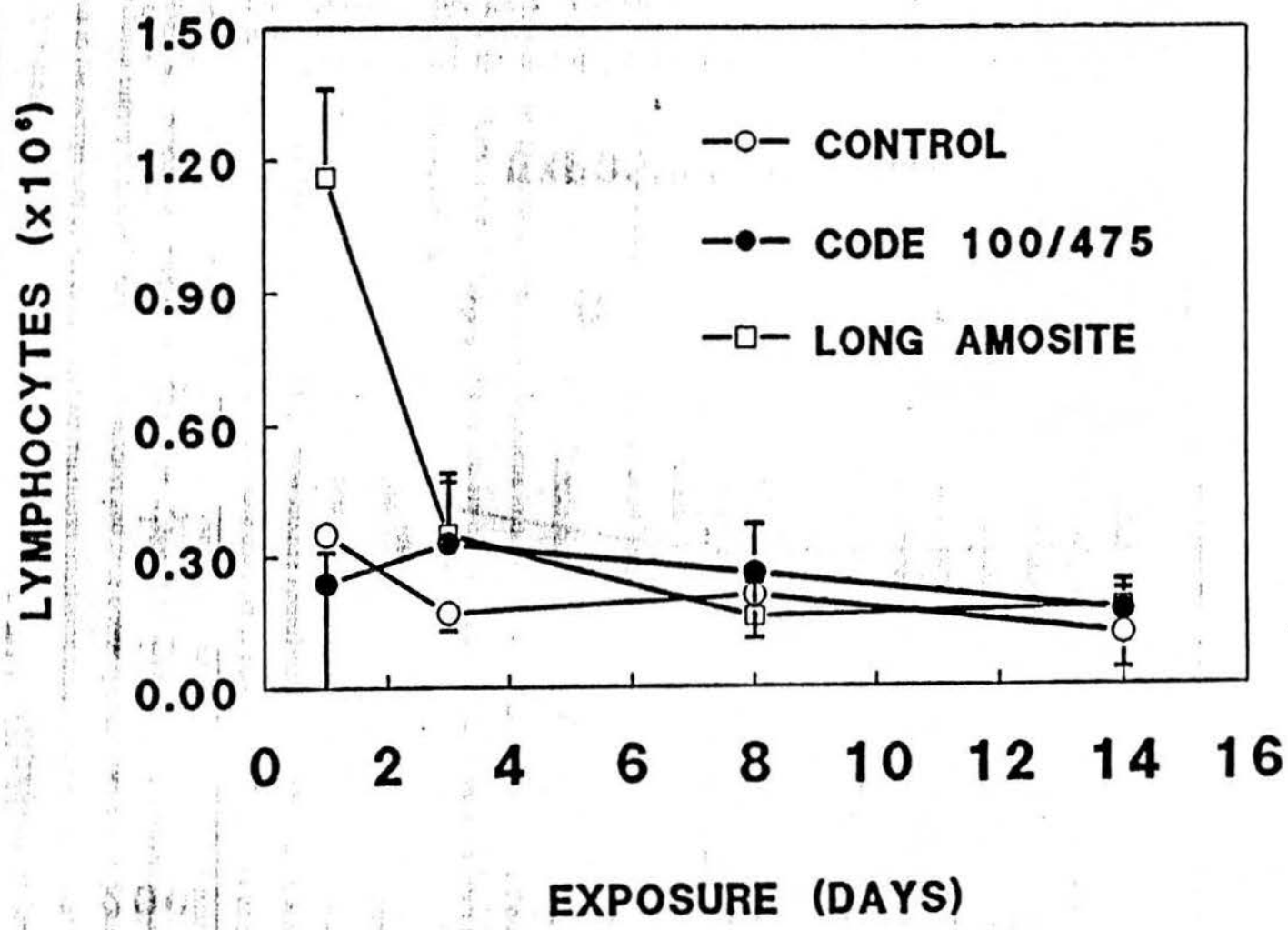


Figure 4. Total numbers of lymphocytes lavaged from the lungs of rats inhaling long amosite or Code 100/475 for the indicated number of days. Data is derived as the mean and SEM of 6 rats in 3 separate experiments.

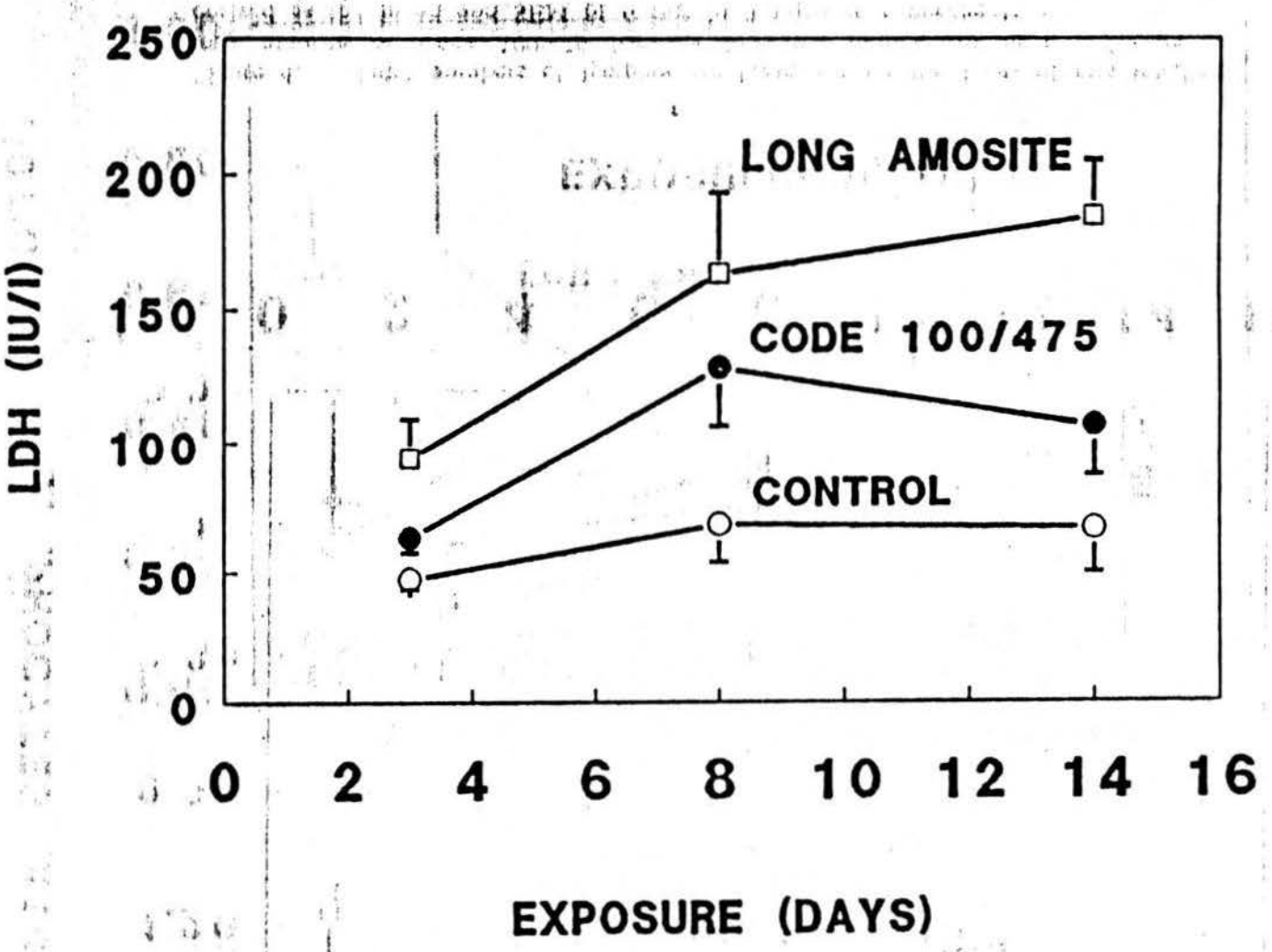


Figure 5. Levels of lactate dehydrogenase (LDH) in the lavage fluid from rats inhaling long amosite or Code 100/475 for the indicated number of days. Data is derived as the mean and SEM of 6 rats in 3 separate experiments.

INFLAMMATION IN THE MOUSE PERITONEAL CAVITY IN THE INVESTIGATION OF FACTORS DETERMINING THE BIOLOGICAL ACTIVITY OF RESPIRABLE INDUSTRIAL FIBRES.

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ABSTRACT

hirteen different respirable man-made fibres were treated for their ability to cause nflammation in the mouse peritoneal cavity. Vitreous, refractory ceramic and silicon arbide fibres, and asbestos fibres as controls, all caused inflammation and there was a narked similarity in the magnitude of the response. Exceptions to this were a rocidolite sample, which contained many fine fibres and produced a greater than verage response, and one of the refractory ceramic fibres which was shorter than the thers and produced less of a response than the fibres as a whole. Treatment of a mple of vitreous fibre with oxalic acid caused a reduction in its ability to induce eritoneal inflammation. Inflammation in the peritoneal cavity caused by fibres is ery likely to be a result of a toxic effect on the peritoneal mesothelial cells and thus iffers, we believe, from the mechanism of inflammation in the alveolar space, which elies on stimulation of alveolar macrophage secretion. Inflammation in the peritoneal avity may provide an assay for the ability of fibres to damage the mesothelium and hus, combined with a method of pre-treating the fibres to mimic dissolution rcocesses in the lung, may provide useful data on the potential of fibres to cause nesothelial pathology.

INTRODUCTION

Exposure to asbestos fibres has been shown to be associated with fibrosis, cancer and oth pleural and peritoneal mesothelioma. Using the rat peritoneal cavity we have emonstrated that a range of asbestos fibres cause mesothelioma in a dose responsive nanner and that there are differences between samples (Bolton *et al* 1982; Davis *et al* 991). However for amosite asbestos samples which were in relatively well-defined hort and long categories, there was a marked reduction in ability to cause nesotheliomas in the short compared to the long sample (Davis *et al* 1986).

We have also utilised the mouse peritoneal cavity to assess the short-term nflammatory response to fibres. As for mesothelioma in the rat peritoneal cavity, a elationship was found between presence of long fibres and ability to cause nflammation in the mouse peritoneal cavity (Donaldson *et al* 1989).

n the present paper we describe the activity of a large range of different respirable ibre types including vitreous, refractory ceramic and silicon carbide fibres, of pproximately similar length, to cause inflammation in the peritoneal cavity.

MATERIALS AND METHODS

Mice. Male mice of the C57Bl6 strain were used in all experiments.

Fibres. The fibre samples utilised in the study are shown in Table 1. All of the samples were largely respirable as judged by fibre diameter. All of the samples had many fibres longer than 10μm but there were some differences in the proportion of longer fibres. For instance, for fibres longer than 15μm, Code 100/475 and RCF 4 at 33.3% and 37.2% respectively, had only approximately half of the fibres that MMVF 11 had in this category (74%). As a group, with the exception of RCF 4, the RCFs were longer than the asbestos group. With the exception of MMVF 22, the MMVFs were longer than the RCFs and the SiCs were both relatively short. Samples from the TIMA repository were a gift from the Thermal Insulation Manufacturers of America who have made these samples available for research.

Assessment of fibre number. Fibre number/unit mass was determined by Phase Contrast Optical Microscopy according to WHO rules for regulated fibres (all fibres >5μm long, <3μm in diameter and with an aspect ratio of >3:1).

Intra-peritoneal instillation. The main study was a comparison of the inflammation produced in the peritoneal cavity by all of the different fibre types when instilled at similar fibre number. The fibre samples were adjusted to 8.2×10^7 phase contrast optical microscopy (PCOM) fibres (the number of fibres in 0.5 mg of long amosite) in 0.5 ml of saline and this was instilled into groups of 3 or 4 mice. Four days later the peritoneal cavity was lavaged with 4 x 2 ml volumes of saline and the cells enumerated differentially. Results are expressed as the number of macrophages and the number of granulocytes (neutrophils + eosinophils + basophils); lymphocytes comprised less than 5%.

Peritoneal inflammation by Code 100/475 microfibre treated with oxalic acid. To treat fibres in a manner that would mimic residence in lung tissue, Code 100/475 fibres were treated *in vitro* with oxalic acid pH 1.4 for 14 days. Assuming that the original concentration of fibres was present, a 0.5 ml volume was injected containing 8.2×10^7 fibres. As a control, fibres were treated identically with distilled water.

RESULTS

Inflammation in the peritoneal cavity. Given the wide range of fibre masses that were instilled, there was an impressive similarity in the extent of the inflammatory response to a wide range of fibre types. There were, however, differences within certain categories. The two SiC types were similar in their activity and the same was generally true for MMVFs, although MMVF21 (a rockwool type of fibre) tended to show a higher granulocyte response; there was only one fibre type in the microfibre category and so there were no internal comparisons. For the asbestos and the RCFs, however, there were substantial differences between fibre types within category. For the asbestos group this took the form of very large response to TIMA crocidolite, and for the RCFs, RCF 4 and RCF 3 produced relatively small responses.

Response of the peritoneal cavity to the mass of fibre instilled. Because of the different nature of the fibre samples used, different masses were needed to attain the target fibre number of 8.2×10^7 as shown in Table 1. Since there was wide variation in the mass of fibres instilled into the peritoneal cavity, we addressed the question of whether there was a mass-related recruitment of cells into the peritoneal cavity. When the data for all 13 fibre types on the recruitment of granulocytes, as

a general measure of inflammation, plotted against the mass instilled. There was no systematic relationship between mass of fibre instilled into the peritoneal cavity and the extent of recruitment of inflammation.

Effect of dissolution treatment on the activity of fibres. As shown in Figure 3, treatment of Code 100/475 vitreous fibres in an oxalic acid milieu for 14 days resulted in a pronounced decrease in biological activity as measured by both macrophage and neutrophil recruitmen

DISCUSSION

In these experiments we have utilised inflammation in the mouse peritoneal cavity as a measure of the short-term toxicity of a range of mineral fibres. The dose of each fibre type was adjusted to 8.2×10^7 fibres ($<3\mu m$ in diameter, $>5\mu m$ in length and with an aspect ratio of >3:1, as judged by phase contrast optical microscopy). Because of the fineness of the long amosite, virtually all of the other fibres were injected at a mass that exceeded 0.5mg. Some explanations can be offered for the fibres that showed the greatest deviation from what was a relatively consistent response. TIMA crocidolite, which caused a much greater than average response, was a finer sample than the long amosite and was probably composed of many fibres that were not detected by PCOM; subsequently many more than 8.2×10^7 long TIMA crocidolite fibres were instilled. For other fibres, in general, the number of PCOM fibres is probably quite similar to the number $>5\mu m$ that would have been visible by SEM; SEM counts of the same materials are planned and this data should be available soon. The low response to RCF4 was possibly due to its relative shortness and its tendency to form clumps so diminishing the effective dose. No real explanation is apparent fo the low response to RCF 3, which was similar in length to RCF 1 and RCF 2.

It was clear that inflammation in the mouse peritoneal cavity at 4 days was not a simple function of the mass of instilled fibres, since there was no correlation between mass instilled and either of the response variables. We have already demonstrated that compact pathogenic dusts, such as quartz and coalmine dust, are substantially less active than fibres (Donaldson *et al* 1988). Furthermore, we have demonstrated that the assay is responsive to long fibres with decreasing sensitivity to shorter fibres (Donaldson *et al* 1989).

The basis of the sensitivity of the assay to fibres is likely to be a direct toxic effect of the long fibres to mesothelial cells which are 10-fold and 200-fold more sensitive to the toxic effects of fibres than bronchial epithelial cells and fibroblasts respectively (Lechner *et al* 1985); the inflammation is thus very likely to be a direct result of this toxicity (Goodlick and Kane 1990).

The present study argues strongly for a unified mechanism of fibre toxicity in the mouse peritoneal cavity, since the toxicity was very similar across a wide range of fibre types regardless of composition. However, in another paper in this volume we used long amosite asbestos and Code 100/475 microfiber and demonstrated that the short-term inhalation of similar airborne fibre concentrations of these two fibres leads to rapid onset of inflammation in the case of long amosite but little response with the vitreous fibre (Donaldson *et al* ibid). The fact that the peritoneal assay does not discriminate between these two fibres confirms the likely difference in target cell; the alveolar macrophage in the case of inhaled fibres and the mesothelial cell in the case of intra-peritoneal fibres. Using alveolar macrophages we demonstrate, in a another paper in this volume that alveolar macrophage production of the pro-inflammatory cytokine tumour necrosis factor (TNF), is stimulated by long amosite but not by the

Code 100/475 vitreous fibres (Brown *et al* ibid). This may be an explanation of the inflammogenicity of the long amosite by inhalation. Thus the factors determining fibre-induced inflammation in the peritoneal cavity and in the alveolar space, appear to be quite different.

Identifying the factors that lead to mesothelioma is difficult but the data from our assay suggest that any fibre that is sufficiently durable and of the right size to be transported to the mesothelial surface, is likely to have the ability to cause injury to the mesothelial cells. The peritoneal inflammation assay described here may yield information on the ability of fibres to cause mesothelial injury, provided it is modified to include a step, prior to instillation, involving the treatment of test fibres in conditions mimicking residence in the lung that could cause dissolution. As demonstrated here, treatment of fibre with oxalic acid caused a reduction in its ability to cause inflammation in the peritoneal cavity and this was accompanied by a reduction in fibre number, as assessed by PCOM count, and a loss of Si into solution (data not shown).

We believe that the results of this study are illuminating for several reasons:-

1) across a wide range of fibre types there is similar degree of toxicity to mesothelial cells as measured by short term peritoneal inflammation; where there are exceptions to the general rule (TIMA crocidolite and RCF 4) can be explained although there is no explanation for the low activity of RCF 3;

2) taken together with our experience from inhalation and macrophage cytokine release studies with two of the fibres (Code 100/475 vitreous fibre and long amosite) the results suggest that the mechanisms of fibre toxicity and inflammation in the peritoneal cavity, at least in the short term, are different from those occurring in the alveolar space following inhalation;

3) the peritoneal inflammation assay described here may have some use as an assay of ability to cause injury to mesothelial cells and cause mesothelial pathology, if used in combination with some pre-treatment to mimic residence in the lung during transit to the pleura. It is unreasonable to expect any short-term bio-assay of a slowly bio-degradable material to be of any use without some such treatment. Short-term treatment with oxalic acid dramatically reduced the activity of Code100/475 microfiber in this assay. We believe that application of some form of dissolution treatment to a test fibre, and then its use in the peritoneal inflammation assay, would produce an *in vitro/in vivo* assay having the ability to discriminate fibres with the potential to injure the mesothelium.

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We would like to acknowledge the Thermal Insulation Manufacturers Association (TIMA) for supplying repository samples of respirable fibres.

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TABLE 1 Details of the fibres used in the study

Fibre	Source/reference	Fibre category	Mass in 8.2×10^7 fibres (mg)
Amosite	Donaldson *et al* 1989	Asbestos	0.50
Crocidolite	TIMA	Asbestos	0.19
JM Code 100/475	Commercial sample	Microfiber glass	0.67
Silicon carbide A	Commercial sample	Silicon carbide	1.16
Silicon carbide T	Commercial sample	Silicon carbide	2.98
RCF 1	TIMA	Refractory Ceramic	9.10
RCF 2	TIMA	Refractory Ceramic	15.48
RCF 3	TIMA	Refractory Ceramic	9.60
RCF 4	TIMA	Refractory Ceramic	7.42
MMVF 10	TIMA	Man-Made Vitreous	11.84
MMVF 11	TIMA	Man-Made Vitreous	5.72
MMVF 21	TIMA	Man-Made Vitreous	15.01
MMVF 22	TIMA	Man-Made Vitreous	10.63

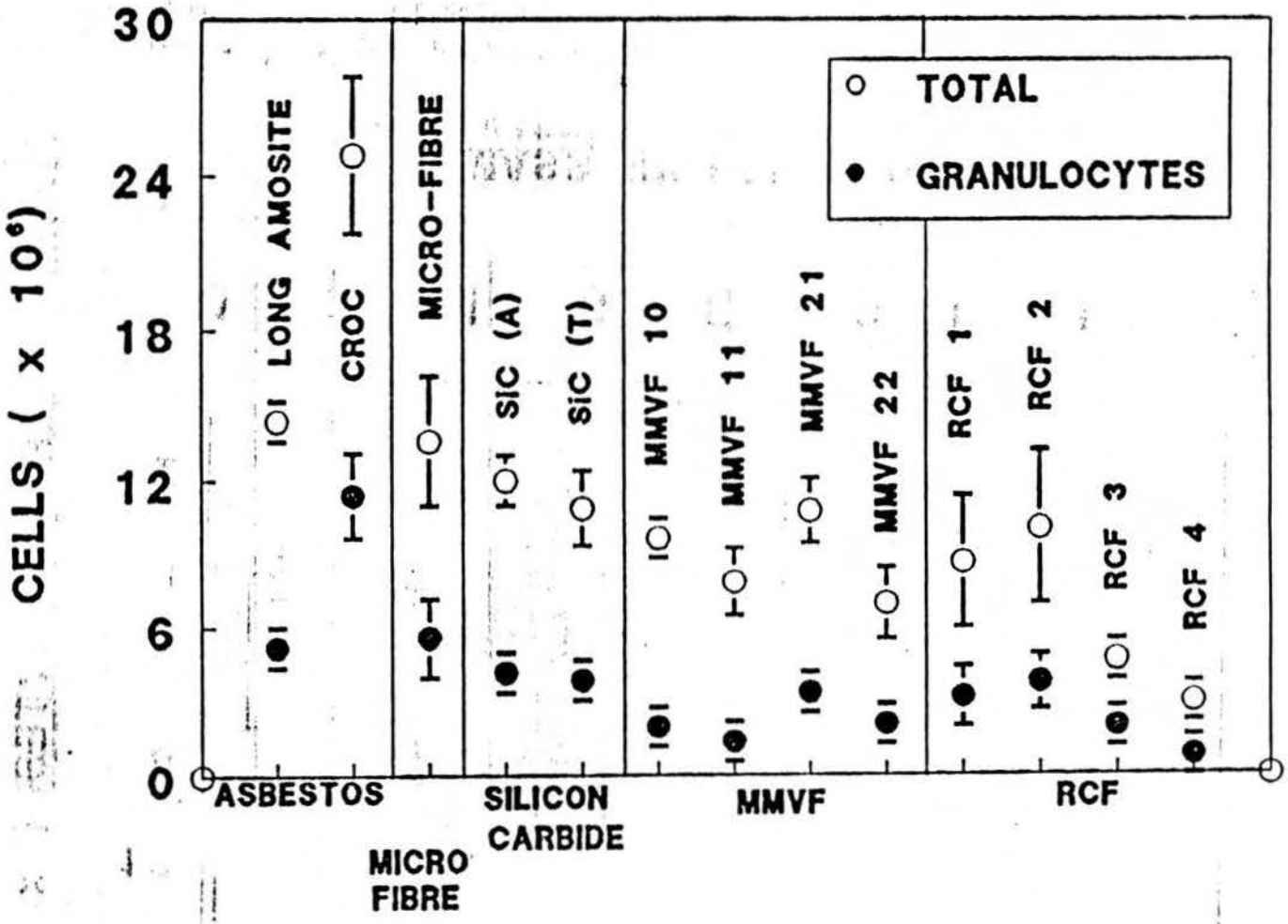


Figure 1. Numbers of macrophages and granulocytes recruited to the mouse peritoneal cavity following instillation of 8.2×10^7 fibres of the various fibre types. Data is mean and SEM of 3 or 4 separate animals.

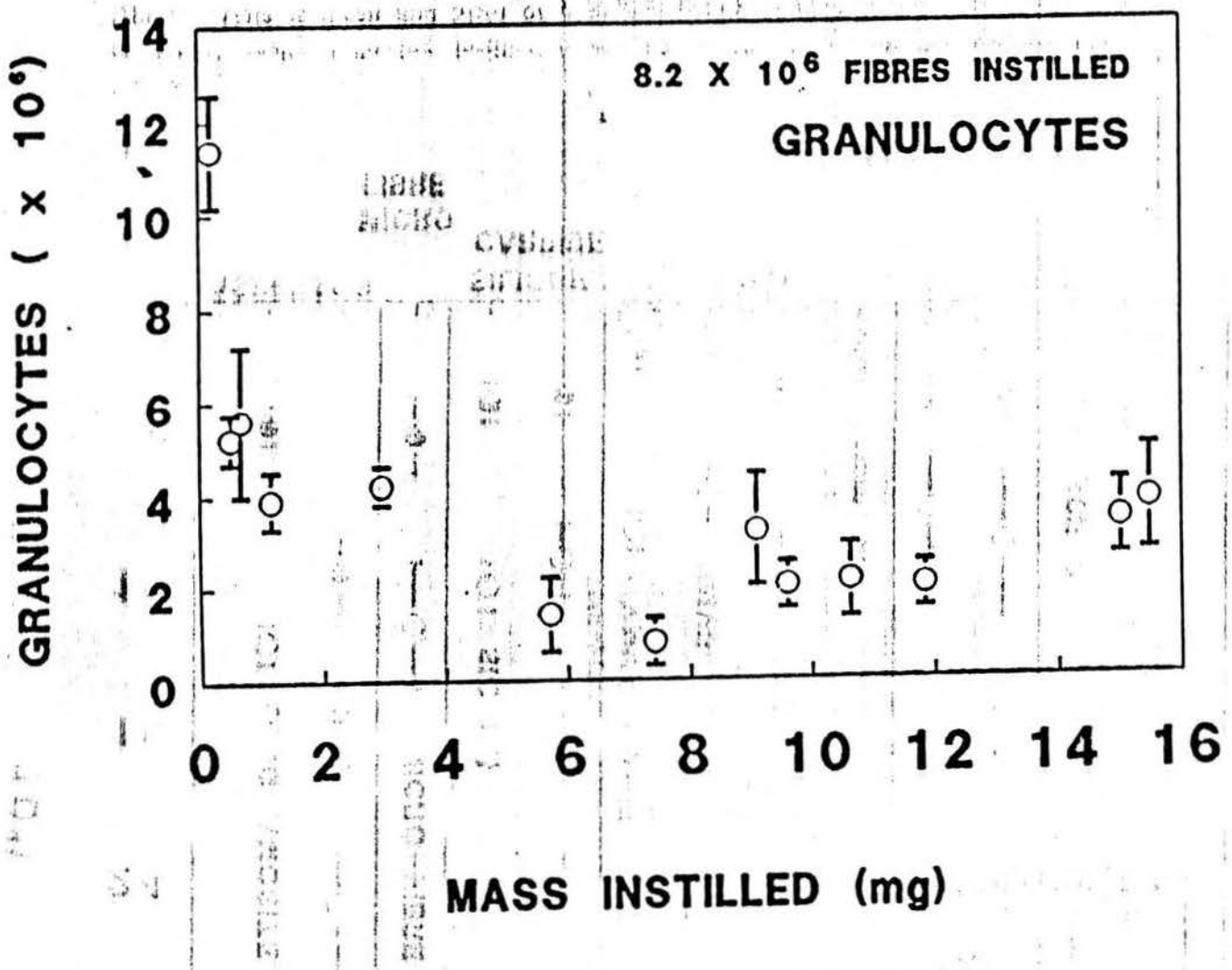


Figure 2. A plot of the mass of each fibre type instilled versus the granulocyte response. Data is the mean and SEM of 3–4 separate animals.

Figure 3. The ability of untreated and oxalic acid-treated Code 100/475 fibres to cause inflammation in the peritoneal cavity. Data represents the mean and the SEM of 4 mice.

Use of the Short-term Inflammatory Response in the Mouse Peritoneal Cavity to Assess the Biological Activity of Leached Vitreous Fibers

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Institute of Occupational Medicine, Edinburgh, Scotland

We used a special-purpose glass microfiber sample, Johns-Manville Code 100/475, to study the effects of various acid and alkali treatments on biological activity as assessed by inflammation in the mouse peritoneal cavity, the leaching of Si, and the phase contrast optical microscopy (PCOM) fiber number. We used mild and medium treatments with oxalic acid and Tris buffer and harsh treatment with concentrated HCl and NaOH. Mild oxalic acid and Tris treatment for 2 weeks had no effect on any of the end-points, but prolonging the mild oxalic acid treatment time to 2 months reduced the biological activity and the fiber number. Medium oxalic acid treatment reduced the biological activity and the fiber number and caused a loss of Si. Medium Tris alkali treatment reduced the PCOM-countable fibers and the biological activity but did not cause a substantial loss of Si. Harsh treatment with strong HCl did not affect the fiber number or cause leaching but the biological activity was reduced; strong NaOH reduced the fiber number and biological activity, and caused marked leaching of Si. The medium oxalic acid conditions (pH 1.4) were more acid than those found in lung cells but produced the same effects (reduction in fiber number and biological activity) as the more physiological mild treatment (pH 4.0), when prolonged. This study suggests that medium oxalic acid treatment can be used as a short-term assay to compare loss of Si, reduction in fiber number, and change in biological activity of vitreous fibers. Such a combination of *in vitro* and *in vivo* assays is likely to provide the best approach to assessing the complex factors involved in changes in the toxicity of vitreous fibers caused by residence in the lung. — Environ Health Perspect 102(Suppl 5):159–162 (1994)

Key words: vitreous fibers, dissolution, peritoneal cavity, inflammation

Introduction

The durability of fibers within the lung, following their deposition, is likely to be important for the subsequent development of pathological responses. In support of this contention, studies have shown leached fibers to have altered biological activity in terms of clearance (*1*) and carcinogenicity (*2*). However, most of these studies have been focused on asbestos fibers. There is a clear need for assays to assess and predict the persistence and biological activity of vitreous fibers in the various pulmonary milieus. Such assays should involve dissolution treatment followed by the measurement of a range of parameters including fiber number, fiber size, and aspects of fiber surface chemistry, any of which could be affected by the dissolution treatment. A further level of testing is needed to determine the biological activity of such leached fibers. Any dissolution-induced changes in the physicochemical parameters can then be matched against the biological activity to identify variable(s) responsible for any altered activity. The bioassay system ideally would be sensitive to the toxicity of fibers and would be a short-term test, such that it would quickly detect changes in fiber number, size, and surface reactivity. We believe that the short-term inflammatory response in the mouse peritoneal cavity fulfills these criteria for a biological test system. In this article we describe the characteristics of the assay system and its response to leached vitreous fibers.

The experiments described here are development work toward refinement of a protocol to be applied to a large range of different man-made fibers in the Colt Fiber Research Programme, for comparison with their biological activity in a range of assays.

This paper was presented at the Workshop on Biopersistence of Respirable Synthetic Fibers and Minerals held 7–9 September 1992 in Lyon, France.

This research was funded by the Colt Fiber Research Programme.

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Materials and Methods

Fibers

The fibers used were Johns Manville Code 100/475 Special Purpose Glass Microfiber. The sample was generated in a 1 m^3 perspex chamber by a glass-lined, glass-tipped, propeller-driven Timbrell Dust Generator. The fibers passed through a cyclone to increase the respirable fraction and the airborne fibers were collected on an open-faced filter in the chamber.

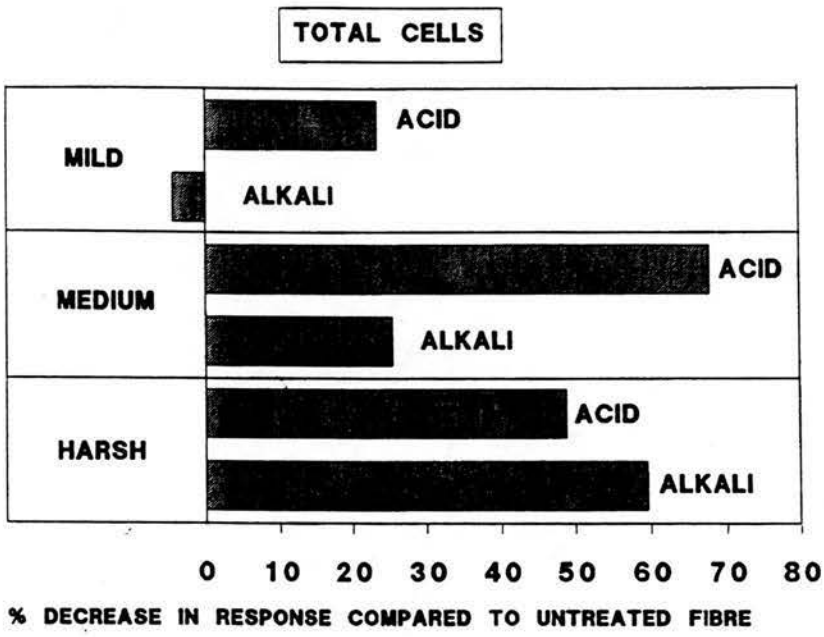
Fiber Dissolution Treatment

To induce some degree of dissolution, mild, medium, and harsh treatments were used.

Mild. Fibers at a concentration of 0.4 mg/ml were rotated for 14 days in either oxalic acid, pH 4.0 (10^{-4} M), or Tris (tris hydroxymethyl methylamine), pH 9.0 (1.5×10^{-4}M). At the end of the treatment period the fibers were spun down, washed to neutrality, and made up to 1 mg/ml (assuming the original mass) for injection.

Medium. Fibers were treated with oxalic acid, pH 1.4, or Tris, pH 10.6 (10^{-1} M in both cases) for 21 days. At the end of the treatment, the fibers were handled as described for mild treatment.

Harsh. Fibers were treated with 1 M HCl and 1 M NaOH for 7 days and then washed and made up for injection assuming a fiber concentration equivalent to that present prior to dissolution.



ure 1. The effect of various treatments on the ability of Code 100/475 fibers to cause inflammation in the peritoneal ity as measured by the total cells recruited. The scale represents the percentage decrease in response compared to reated Code 100/475. Although mild acid caused a decrease, this did not attain statistical significance. The other reases were significant ($p < 0.01$). Percent decrease obtained as follows:

$$\frac{\text{(Mean cells recruited in response to native fiber} - \text{Mean cells recruited in response to treated fibers)}}{\text{Mean cells recruited in response to native fiber}} \times 100$$

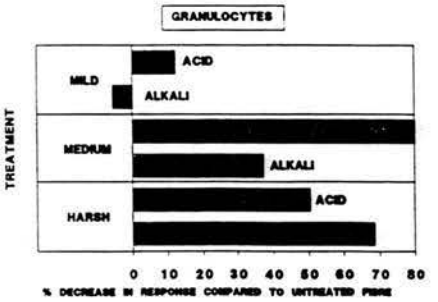


Figure 2. The effect of various treatments on the ability of Code 100/475 fibers to cause inflammation in the peritoneal cavity as measured by total granulocytes recruited. The scale represents the percentage decrease in response compared to untreated Code 100/475. Although mild acid caused a decrease, it did not attain statistical significance. Percent decrease calculated as for total cells but using the total granulocyte data.

sessment of Fiber Number

ber number was determined by Phase ntrast Optical Microscopy (PCOM) cording to World Health Organization HO) rules for regulated fibers (all fibers µm in length, <3 µm in diameter and h an aspect ratio of >3:1).

flammation in the Mouse Peritoneal vity

is was assessed as described previously . Briefly, groups of four C57/B16 mice d >6 weeks were instilled with 0.5 ml saline containing 0.5 mg of fiber that d been treated in the ways described. At lays the peritoneal cavity was washed out th 4 × 2 ml volumes of saline and the al and differential cell count was deter- ned. All data were expressed as millions either total cells or granulocytes.

easurement of Silica Dissolution om Fibers

e supernatants from treated fibers were alyzed for the presence of silica, using me atomic absorption with a nitrous ide acetylene flame (Thermo-electron deo 22; Warrington). Calibration solu- ns were prepared, using a Si standard, in e same solutions as the dissolution regimen under test (i.e., Tris buffer, oxalic acid, HCl, etc.).

Statistical Analysis

Effects of treatment were analyzed with the Minitab Statistical Package utilizing either two factor or three factor analysis of variance using the General Linear Model. The response variables were total cells from the lavage or total granulocytes, expressed in millions. In some cases the data were logarithmically ($\log_e$ or $\log_{10}$) transformed to obtain a normal distribution prior to analysis. The classifying variables in the analyses are as described below.

Results

Effect of Treatments on Inflammation in the Mouse Peritoneal Cavity

The results are presented as the mean percent decrease in total cells (Figure 1) or granulocytes (Figure 2) recruited to the mouse peritoneal cavity following instillation of treated fibers, compared to instillation of untreated fibers in the same experimental sequence. The total number of cells recruited in response to untreated fibers ranged from 6 to 12×10^6, while total neutrophils ranged from 1 to 6×10^6.

Mild. With untreated, mild oxalic acid and mild Tris alkali treatment as the classifying variables, analysis of variance showed no significant effect on the total number of cells recruited to the mouse peritoneal cavity ($F = 0.825$; $p > 0.25$) nor in the total granulocytes ($F = 1.094$; $p > 0.25$). This is shown in the top panels of Figures 1 and 2 as a slight decrease in activity with the acid treatment and a slight increase with alkali treatment.

When mild oxalic acid treatment (normally 2 weeks) was prolonged to 2 months, there was a decrease in the total fiber count (Table 1) and a decrease in the inflammatory activity.

Medium. In analysis of variance using untreated fibers or fibers following medium acid, or medium alkali treatments as classifying variables, there was a significant variance ratio of $F = 11.43$ ($p < 0.01$) for total cells and $F = 18.28$ ($p < 0.01$) for granulocytes. Individual comparisons of each leaching treatment with the controls revealed significant ($p < 0.01$) decreases in total cells recruited with acid treatment, while alkaline treatment just failed to attain statistical significance ($p > 0.05 < 0.1$; see

Table 1. Effect of increasing the treatment time with mild oxalic acid, pH 4.0, on the fiber number and inflammogenicity.

Oxalic acid treatment	Number of fibers/µg	Total granulocytes
Mild	164,239	1.51 (0.29)
Prolonged mild (2 months)	77,046	0.81 (0.10)

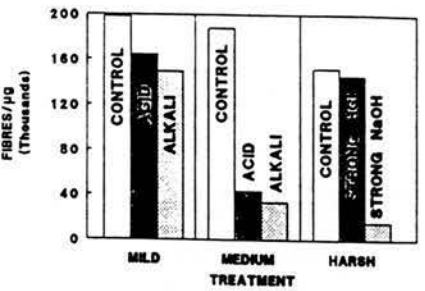


Figure 3. The total regulated fibers (>5 µm in length, <3 µm in diameter and with an aspect ratio >3:1, assessed by PCOM), following treatment with the various acid and alkali solutions; data derived as a single measurement.

Figure 1, middle panel). In the case of granulocytes, both acid ($p < 0.001$) and alkaline ($p < 0.05$) treatments significantly reduced the number of cells recruited (Figure 2, middle panel).

Harsh. Using untreated fibers, or fibers following harsh acid (HCl) or harsh alkali (NaOH) treatments as classifying variables, there was a significant effect on both total cells ($F = 6.24$; $p < 0.01$) and granulocytes ($F = 10.25$; $p < 0.01$). Individual *t*-tests confirmed that there were significant decreases compared to controls with both treatments. Significance with acid treatments for total cells and for granulocytes, $p < 0.05$; with alkali treatment for total cells, $p < 0.01$, and for granulocytes, $p < 0.001$. In general, there was a greater effect from the alkali than from the acid treatment (Figures 1, 2, bottom panels).

Effect of the Various Treatments on the Leaching of Si

All of the data from these experiments are shown in Figure 3. It is clear that only two of the treatments caused a substantial loss of Si into solution; the harsh alkali treatment caused the greatest effect, while medium acid was showing a modestly increased leaching effect at 21 days. Neither strong HCl nor medium Tris alkali was effective in causing loss of Si.

Effect of the Treatments on the Number of Fibers Countable by PCOM

Although both of the mild treatments caused a reduction of PCOM-countable fibers, this was not substantial (Figure 4). By contrast, both the medium acid and the medium alkali treatments caused a dramatic reduction in the number of fibers countable by PCOM. Fibers given harsh treatment, surprisingly, showed no effect from the acid treatment while the alkali treatment did cause marked reduction in the countable fibers (Figure 4).

Table 2. Summary of the effects of the various treatments on the inflammogenicity, loss of Si and number of PCOM countable fibers of Code 100/475 special purpose glass fiber.

Treatment	Inflammation	Si leaching	Fibers
Mild oxalic acid	No effect[a]	+	No effect
Mild tris alkali	No effect	+	No effect
Medium oxalic acid	Reduced[b]	+++	Loss
Medium tris alkali	Reduced	+	Loss
Harsh HCl	Reduced	+	No effect
Harsh NaOH	Reduced	++++	Loss

[a] Prolonging the mild oxalic acid treatment resulted in decreases in fiber number and inflammation.
[b] The effect of medium acid was greater than the effect of medium alkalai.

Summary of Results

The results from the study are summarized in Table 2.

Discussion

The main conclusions to be drawn from the effects of the acid and alkaline treatments of special purpose glass microfiber Code 100/475 are as follows:

Neither mild Tris alkali (pH 9.0; 1.5×10^{-4} M) nor mild oxalic acid (pH 4.0; 1×10^{-4} M) treatment affected fiber size distribution, number, or biological activity; and no significant leaching of Si was detected. However, increasing the duration of the oxalic acid treatment to two months did result in a decrease both in the fiber number and in biological activity. Unfortunately there were no data available on the leaching of Si during this prolonged mild acid treatment.

Medium oxalic acid (pH 1.4; 0.1 M) and medium alkali (pH 9.6; 0.1 M) treatment for 21 days both caused a reduction in inflammatory potential, although this was greatest with the acid treatment. The effect of alkali treatment on the granulocytes was significant; it was not significant, however, on the total cell count, confirming the lesser effect of the alkali treatment on biological activity. However, both treatments substantially reduced the fibers countable by PCOM in all length categories. The Si leaching data tended to parallel the biological activity data, since oxalic acid treatment caused a loss of Si, while the leaching from Tris alkali-treated fibers was around control levels. The main discrepancy in these data relates to the alkali treatment-related reduction in PCOM-countable fibers of the same order as that seen with the acid treatment, in the absence of loss of Si and with less reduction in the biological activity than that seen with acid treatment. One explanation

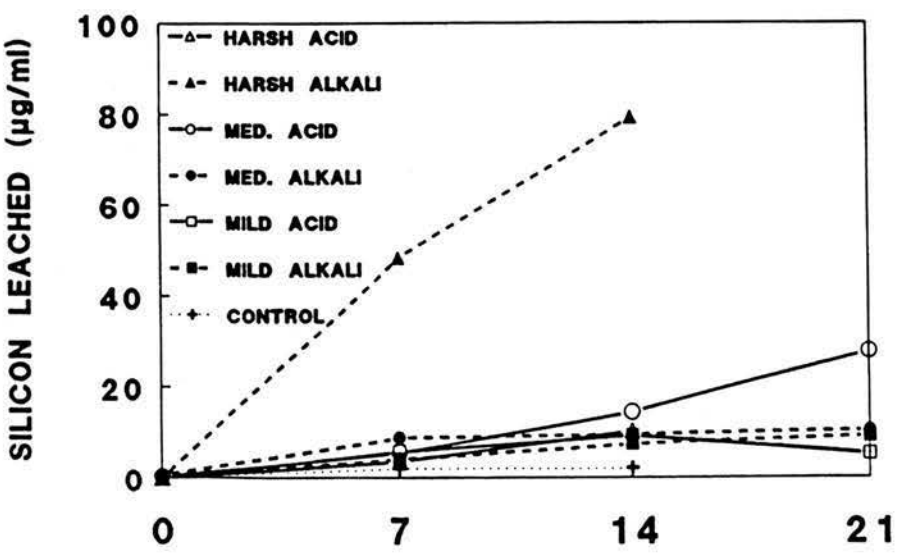


Figure 4. Leaching of silicon from fibers into the various acid and alkali solutions over time; data derived as a single measurement.

ld be that alkali treatment, through loss lements other than Si, renders the fibers visible by PCOM, although they retain logical activity. This was, indeed, evi- t in the bulk solution of treated fibers, ere the fibers themselves were invisible he naked eye following alkali treatment. ditional evidence that this could be true nes from scanning electron microscope ges of alkali-treated fibers, which are ch reduced in their electron density.

With both harsh acid (HCl) (pH 0.1;1 and alkaline (NaOH) (pH 13.9;1 M) tment for 7 days there was a reduction biological activity. However, surpris- y, only the alkali solution caused loss of and loss of fibers in all size categories, ereas the strong acid (HCl) treatment sed neither leaching of Si nor a change the PCOM-countable fiber number. is could be explained by some effects of ng HCl at the fiber surface, which pre- ted the process of leaching, perhaps by ming an insoluble layer that also uced the biological activity. More work eeded to explain this phenomenon.

These results show that, for the conditions and the vitreous fiber used here, neither measuring the loss of Si into solution nor assessing the fiber size (by PCOM, at least) is a perfect descriptor of a change in biological activity, as measured by inflammation in the peritoneal cavity. For instance, strong HCl treatment caused neither leaching of Si nor a reduction in the fiber number, but the biological activity was reduced. In contrast, medium alkali treatment did not cause substantial leaching of Si from the fiber, but both the PCOM fiber counts and the biological activity were reduced.

The aim of this study was to establish the conditions suitable for a benchtop test of vitreous fiber durability, not necessarily to mimic the exact conditions in the lung. Both alkaline and acid conditions were chosen (although probably only acid conditions exist in the lung) since it has been reported that glass may be more chemically degradable at alkaline pH. In fact, the particular glass under study was degradable under both acid and alkaline conditions. The harsh treatment was included despite its extreme unphysiological nature, as a first attempt to induce rapid dissolution, used in previous studies (*2*). This yielded an interesting result, namely the failure of strong HCl to produce Si dissolution while at the same time causing a decrease in biological activity.

We demonstrated that treating the vitreous fibers in pH conditions that could obtain in the lung (mild oxalic acid for 2 weeks) produced no change in the biological activity of the fibers. However, extending that same treatment to 2 months both changed the biological activity and reduced the fiber number, an effect similar to that produced by a short-term treatment in medium oxalic acid. Medium oxalic acid treatment for 2 weeks caused differential release of Si from a range of fibers (data not shown) and this could form the basis of an *in vitro* dissolution assay. However, further research would be necessary, comparing prolonged treatment in mild oxalic acid with short-term treatment in stronger oxalic acid on a range of different fibers, before there could be confidence in the short-term assay.

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Biological activity of respirable industrial fibres treated to mimic residence in the lung

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Abstract

The durability of fibres in the lung environment after deposition could be a key factor in determining whether they accumulate to a sufficient tissue dose to cause pathological change. There is a shortage of information on the relative durabilities of respirable industrial fibres of various types. We describe a strategy for assessing the ability of different fibre types to persist in the lung milieu and to retain their biological activity. This is particularly important for the development of mesothelioma, where the long latent time that characterises this disease would be expected to exclude, from culpability, fibres that are not durable. We have combined a pre-treatment step in pH 5.0 or 7.0 with an assay that relies on the ability of fibres to damage the mesothelium. The long-term aim is to assess the impact that treatment in various pH solutions has on (a) fibre size/number, (b) loss of key elements, (c) the ability to damage the mesothelium. Such information should enable us to better predict the potential of fibres to cause mesothelioma.

Key words: Fibres; Mesothelioma; Inflammation

1. Introduction

The toxicity of asbestos has been well documented but the risks associated with inhalation of other non-asbestos industrial fibres has yet to be elucidated. It has been suggested that the relative solubility of these fibres in the lung could be a factor in modifying their ability to accumulate in lung tissue to a pathogenic dose [1]. To try and determine the susceptibility of different fibre types to dissolution in the lung a

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number of approaches have been utilised including in vivo retention studies following inhalation and instillation, and in vitro studies using cells and 'simulated lung fluid'. Previously we have used a 2-handed approach: (1) in vitro treatment of fibres with strong and mild organic acids to induce some degree of dissolution consistent with residence in the lung milieu; (2) followed by injection into the mouse peritoneal cavity and assessment of the elicited inflammatory response as a measure of the biological activity [2,3]. The latter assay may be a measure of the ability of fibres to damage the mesothelium and so may be predictive of the potential of fibres to cause mesothelioma.

In addition to determining the effect that different pH treatments have on the biological activity of fibres, it is necessary to document the changes that occur in the fibres. Dissolution could cause chemical changes, shape changes or both, and only by documenting biological activity, fibre dimension and change in chemistry will it be possible to fully understand the interplay of these factors. In the present paper we confirm the responsivity of the mouse peritoneal cavity inflammation assay to fibres and describe the effect of dissolution on the biological activity of asbestos, vitreous fibres and refractory ceramic fibres.

2. Materials and methods

Fibres and compact dusts used to demonstrate the sensitivity of the assay to fibres were the following compact dusts: respirable samples of Fly-ash, dust collected from the air of a coal mine, DQ 12 quartz and rutile titanium dioxide (Tioxide Ltd, UK); and the following fibrous dusts: fibrous erionite, long fibre amosite, UICC chrysotile and a sample of Kevlar fibres/fibrils.

The fibres used in the dissolution studies were: *(1) Johns Manville Code 100/475 Special Purpose Glass Microfiber.* The sample was collected by generation in a 1-m^3 perspex chamber by a glass-lined, glass propeller-tipped Timbrell Dust Generator. The fibres passed first through a cyclone to increase the respirable fraction and the airborne fibres were collected on an open-faced filter in the chamber. *(2) Long fibre amosite asbestos.* This fibre sample was prepared as described by Donaldson et al. [4]. *(3) MMVF 21.* This was a sample of Rock Wool supplied by the TIMA fibre repository and size-selected to be respirable. *(4) RCF 1.* This was a sample of a titanium-containing refractory ceramic fibre supplied by the TIMA fibre repository and size-selected to be respirable.

Fibre dissolution treatment. In order to induce some degree of dissolution 3 treatments were used: (1) Fibres at a concentration of 0.4 mg/ml were treated for 14 days in oxalic acid, pH 4.0 (10^{-4} M). (2) Fibres at 0.4 mg/ml were treated with 1 M HCl for 7 days. (3) Fibres were placed in 1 mM acetate buffer, pH 5.0 and pH 7.0 for 1 month. In all cases, at the end of the treatment period the fibres were spun down, washed to neutrality and then made up to 1 mg/ml (assuming the original mass) for injection.

Measurement of silica dissolution from fibres. Selected supernatants from fibres treated as described above were analysed for the presence of silica. This was carried out using a flame atomic absorption with a nitrous oxide acetylene flame. Inflammation in the mouse peritoneal cavity. This was assessed as described previously [5].

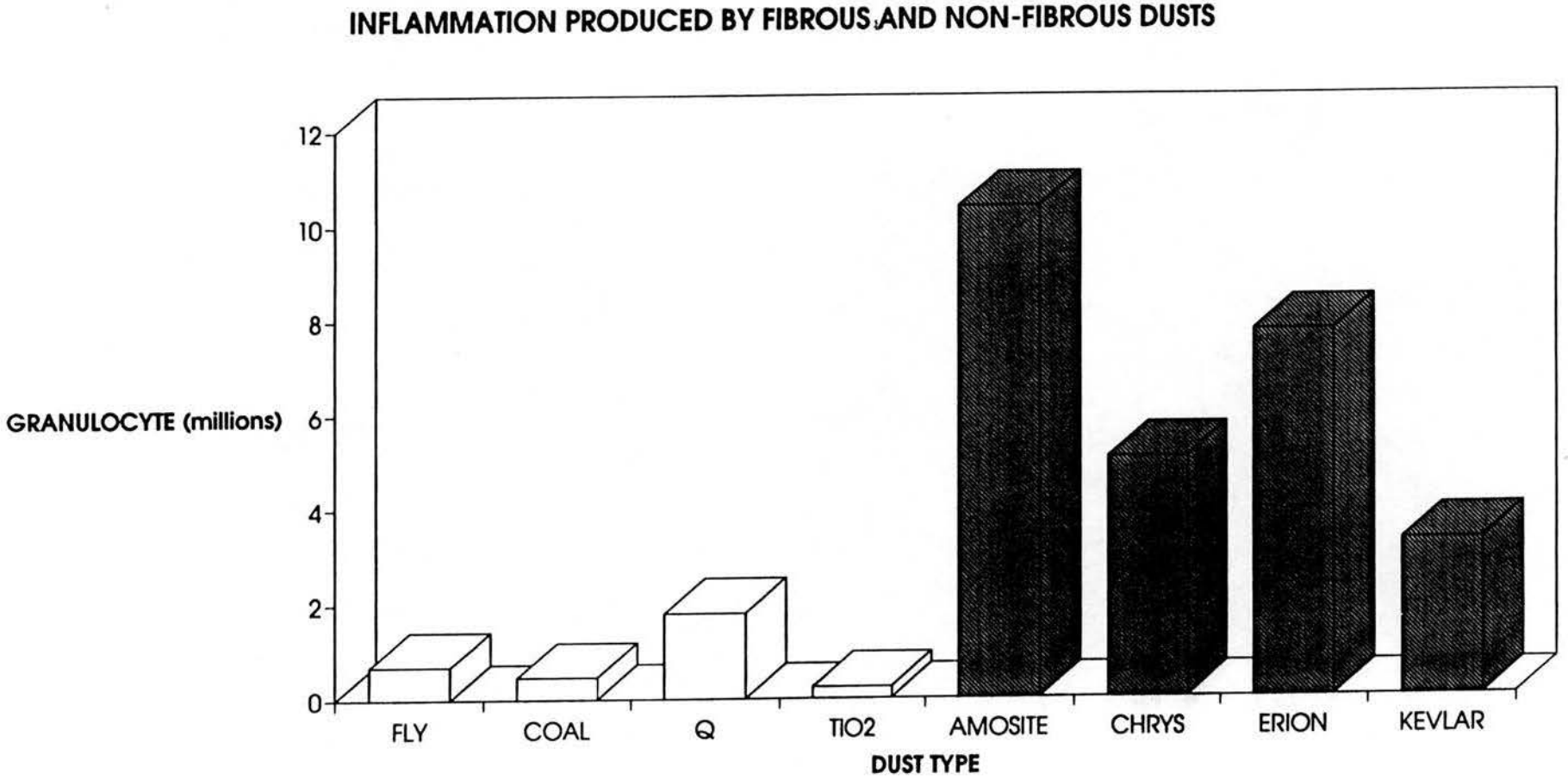


Fig. 1. Inflammation caused by an equal mass of various non-fibrous and fibrous dusts. Data is the mean number of granulocytes from lavage of 3–4 mice.

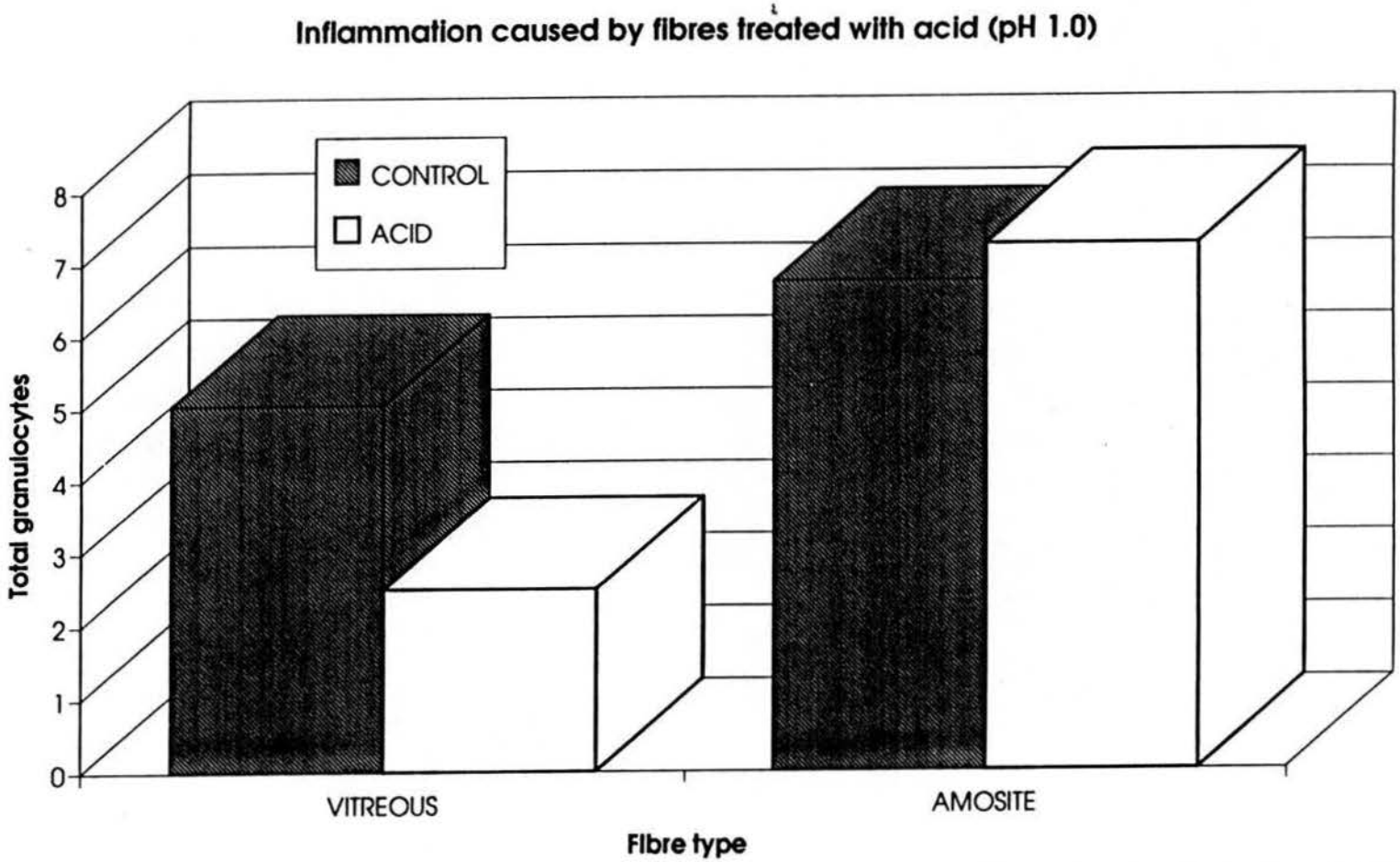


Fig. 2. Effect of treatment with strong acid (pH 1) on the biological activity of Code 100/475 vitreous fibre and amosite asbestos. Data is the mean number of granulocytes from lavage of 3–4 mice.

Briefly, groups of 3 or 4 C57 BI6 mice aged > 6 weeks were instilled with 0.5 ml of saline containing 0.5 mg of particles or fibres treated in various ways. At 4 days the peritoneal cavity was lavaged with 3 × 2 ml volumes of saline and the total and differential cell count determined. All data were expressed as millions of total cells or granulocytes per mouse.

3. Results

(1) Sensitivity of the mouse peritoneal inflammation assay to fibres. When a range of fibrous and non-fibrous particles of differing type were instilled into the peritoneal cavity at equal mass (0.5 mg) the response to the fibres greatly exceeded that of the non-fibrous particles (Fig. 1).

(2) Failure of strong acid conditions to alter the biological activity of amosite asbestos. Treatment with strong acid conditions reduced the biological activity of vitreous fibre but failed to alter the activity of amosite asbestos (Fig. 2).

(3) Comparison of the solubility and biological activity of MMVF 21 and RCF 1 at pH 7.0 and pH 5.0. When MMVF 21 (Rock Wool) and RCF 1 (titanium refractory ceramic fibre) were treated with strong acid, the MMVF 21 showed a greater propensity to dissolve and lose its silicon than did the RCF 1: percentage loss of silicon

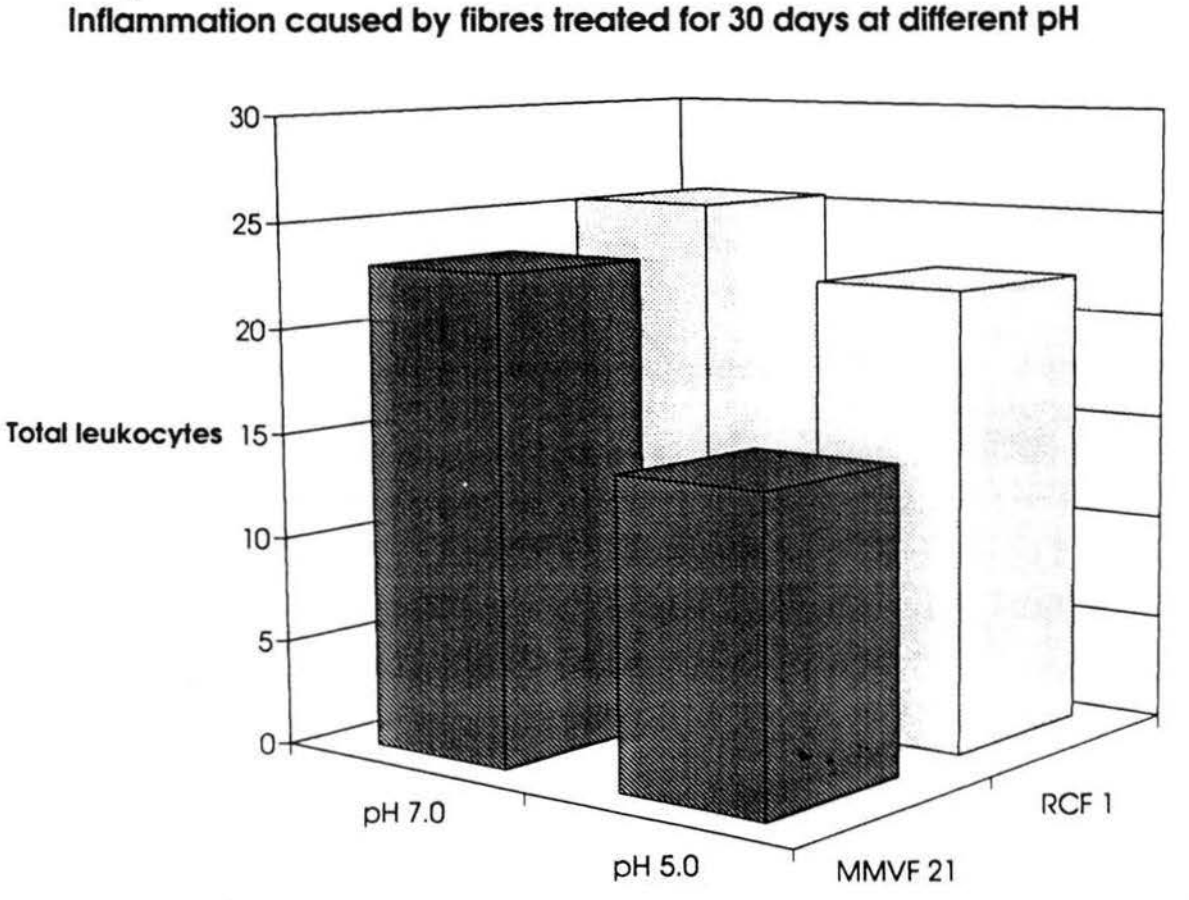


Fig. 3. Inflammation caused by MMVF 21 and RCF following treatment at pH 5.0 and pH 7.0. Data is the mean number of granulocytes from lavage of 3 mice.

after 1 week at pH 1.0 — RCF 1 = 12%, MMVF 21 = 92%. When the two fibre types were placed for 1 month at pH 5.0 or 7.0, there was a reduction in the biological activity in the case of MMVF 21 at pH 7.0 and much less with RCF 1 (Fig. 3).

4. Discussion

We show here that the peritoneal cavity of the mouse is particularly prone to the inflammation-generating effects of fibres compared to compact particles as shown by the much greater inflammation caused by fibres than by compact particles when they were all injected at equal mass. The particle number per unit mass varied greatly with different materials depending on density and there was no tendency for either the compact or the fibrous materials to have the greater number of particles per unit mass. The persistent increase in response to the fibres is therefore very impressive. The case of quartz is particularly noteworthy, since it is so inflammogenic to the lung parenchyma [6]. This supports our previous findings that the peritoneal cavity is not sensitive to quartz nor to compact particles in general [5]. We believe that this is a consequence of direct toxic effects on the mesothelial lining cells, which are very sensitive to the damaging effects of fibres [7]. We previously reported a marked similarity in the inflammatory response of the mouse peritoneal cavity to a range of fibres of very different chemical composition, suggesting that the unifying factor in the toxicity was the fibrous habit [3].

The present study was part of a programme of work aimed at developing a short-term assay of the ability of fibres to cause mesothelioma. The central overall strategy of the assay is to overcome the problem inherent in using a short-term assay to determine the changes in fibres that take place over a protracted timescale. Since dissolution is likely to be a relatively slow process, an assay of a few days would not be expected to be useful. We therefore suggest that an initial dissolution step, of varying length, be imposed on the fibre in order to mimic some degree of dissolution in the lung; this is then followed by testing in the short-term assay (Fig. 4).

The results showed that short-term treatment of a durable fibre such as amosite asbestos was without influence in terms of affecting the biological activity. By contrast this, admittedly extreme, treatment reduced the biological activity of the vitreous fibre. The fibre number and length of the vitreous fibre were also reduced [2]. This suggests that the assay may indeed be able to discriminate between high and

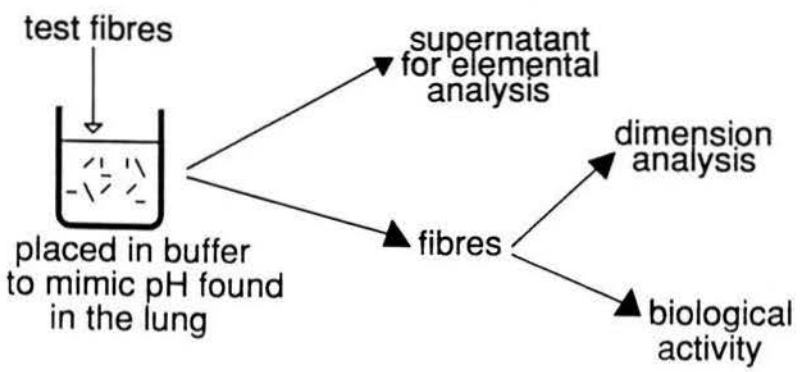


Fig. 4. An approach towards evaluating the effect of the lung environment on the biological activity of fibres.

low durability fibres. In order to better test the hypothesis that mimicking the pH milieu of the lung could reduce the biological activity of non-durable fibres we chose a pH of 5.0, which can be found in the macrophage phagolysosome, and extended the exposure to 1 month. A pH of 7.0 was also used since this is the pH of the alveolar lining fluid. Only pH 5.0 treatment reduced the biological activity of the man-made vitreous fibre sample (MMVF 21, Rock Wool) but had no effect on the more durable refractory ceramic fibre (RCF 1). Based on this assay we would therefore predict that, compared to MMVF 21, RCF 1 would be more likely to retain its biological activity during transport to the pleura where it would be toxic to mesothelial cells and so would be more likely to cause mesothelioma. There is evidence that RCFs can indeed cause mesothelioma in hamsters [8], although it is not clear how representative of a general ability to cause mesotheliomas this finding is. More validation of our own assay is clearly required.

At time of writing there is no data available on the effect of pH 5.0 treatment on the loss of silicon or the fibre number of the 2 samples treated at pH 5.0 and this data is necessary in order to untangle the effects of chemical change in the fibres from those of changes in the fibre length and diameter. As shown in Fig. 4 it is necessary to examine all 3 aspects of fibres during dissolution in order to fully understand the events that occur as a fibre is changed by the environment of the lung.

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Inhaled Particles VII

PRODUCTION OF TUMOUR NECROSIS FACTOR BY PLEURAL LEUKOCYTES FOLLOWING AIRSPACE DEPOSITION OF ASBESTOS, QUARTZ AND TiO_2, ALONE AND IN COMBINATION*

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Abstract—Since asbestos exposure may commonly be accompanied by exposure to other non-fibrous particles, we investigated the effects of combinations of crocidolite asbestos with quartz or titanium dioxide (TiO_2) in causing bronchoalveolar and pleural leukocyte responses. One month following intratracheal instillation, quartz alone caused a stimulation of leukocytes recruitment to the bronchoalveolar space whilst crocidolite or TiO_2 alone did not. A combination of crocidolite and quartz showed a synergistic effect in recruiting leukocytes into the lungs. Pleural leukocytes showed a different response compared to bronchoalveolar leukocytes. When any dust was instilled alone, there was recruitment of pleural leukocytes. The effect of crocidolite plus TiO_2 was not different to either dust alone, but crocidolite plus quartz produced an additive effect with respect to cell population. With instillation of dusts alone the pleural leukocytes produced reduced levels of the proinflammatory cytokine, tumour necrosis factor (TNF), compared to controls. However, when the dusts were given in combination, this resulted in a marked increase in TNF production for crocidolite plus TiO_2 and an even greater increase with crocidolite plus quartz. *In vitro* studies showed that alveolar macrophages responded to quartz exposure with an increased secretion of TNF. The combination of asbestos with quartz or TiO_2 resulted in raised TNF production by the macrophages. These results indicate that there are different responses in the lungs and the pleura to fibres and fibres plus other dusts. Mixed exposure to asbestos and other mineral dusts result in a dramatic alteration in terms of cell types and TNF secretory activity and this may relate to the increased pleural pathology previously reported for such exposure in the experimental situation.

INTRODUCTION

ASBESTOS exposure causes both lung and pleural pathological changes. The effect of asbestos fibres on bronchoalveolar lavage leukocytes has been investigated extensively (DONALDSON *et al.*, 1988; DRISCOLL *et al.*, 1990), but only limited studies have been carried out on pleural leukocytes in this respect.

Asbestos exposure may occur in combination with other types of particles. Such a mixed exposure could cause increased pathological responses in the lungs and the pleura (FINKELSTEIN, 1983; DAVIS *et al.*, 1988, 1993).

Tumour necrosis factor (TNF) is a cytokine which possesses an extensive array of biological activities including enhancement of inflammation (SCHOLLMEIER, 1990). Asbestos exposure increases the secretion of TNF by alveolar macrophages (ROSENTHAL *et al.*, 1989; SCHOLLMEIER, 1990). The question therefore arises as to whether a mixed exposure to asbestos with other mineral dusts will modify the response, particularly, with regard to the pleural leukocytes. The present study was carried out to address this question.

*This paper was included in Poster Session 6 and the discussion included in the summary presented in Section 12.

TABLE 1. BRONCHOALVEOLAR LEUKOCYTE COMPONENTS 1 MONTH AFTER I.T DUSTS INDIVIDUALLY OR IN COMBINATION

Dust	Total No. ($\times 10^6$)	Differential per cent Macrophages	PMN	Lymphocytes
Control	5.0 (2.7)	99 (0.7)	0	1 (0.8)
TiO_2	3.8 (0.6)	98 (1.2)	1 (1.2)	1 (0)
Quartz	26.3 (3.4)	58 (26.2)	41 (26.2)	1 (0)
Cro	2.5 (0.7)	98 (1.5)	1 (1.5)	1 (0)
Cro + TiO_2	3.6 (0.5)	97 (1.2)	3 (1.2)	0
Cro + quartz	43.3 (11.2)	72 (5.3)	27 (5.3)	1 (0)

$N = 9$ rats for controls and three rats for all other treatment groups.

Results are presented as means (SD). PMN = crocidolite. There is statistically significant difference between control vs quartz ($P < 0.001$), control vs Cro + quartz ($P < 0.001$) and quartz vs Cro + quartz ($P < 0.05$).

MATERIALS AND METHODS

Syngeneic, Wistar-derived rats of the HAN strain purchased from Western General Hospital Animal Centre, Edinburgh, U.K., aged 12 weeks, were used throughout. The dusts used were: (1) UICC crocidolite; (2) titanium dioxide (TiO_2 rutile, Stockton-on-Tees, U.K.); and (3) DQ_{12} standard quartz.

Intratracheal instillation (I/T) of 2.5 mg of asbestos and/or other dusts, bronchoalveolar and pleural lavage 1 month following I/T as well as leukocyte supernatant preparation, were carried out as previously described (LI *et al.*, 1991; DONALDSON *et al.*, 1988). In *in vitro* systems, alveolar macrophages at a concentration of 1×10 particles per ml were exposed to 50 $\mu g\ ml^{-1}$ fibres and/or particles for 24 hr.

TNF activity in these supernatants was determined using the L929 cell line (a gift from Dr J. Symonds, Northern General Hospital, Edinburgh, U.K.) lysis assay, modified according to WARNER and LIBBY (1989), by comparison with a TNF alpha standard (a gift from Dr J. Symonds) dilution curve. The TNF activity in the supernatants was confirmed by a rabbit anti-TNF serum (from Drs J. Fantone and S. Kunkel, University of Michigan, U.S.A.).

The contaminating endotoxin levels in all media used in the study was detected using a kit (Coatest, KABI Diagnostica, Middlesex, U.K.). Polymyxin columns (Detoxi-Gel Affinitypak Columns, Pierce Chemical Company, Illinois, U.S.A.) were used to remove endotoxin from medium. Results were subjected to analysis of variance. When there was a significant F value for the effect of treatment, individual means were compared for significance using t-tests.

RESULTS

Cellular changes in bronchoalveolar and pleural spaces after I/T dusts

Table 1 shows the components of bronchoalveolar leukocytes 1 month after 2.5 mg dust intratracheal instillation (I/T). Individually, TiO_2 and crocidolite did not cause significant changes in leukocyte components, whereas quartz resulted in massive recruitment of leukocytes with a high percentage of PMN. A synergistic effect on total number of leukocytes was observed with crocidolite plus quartz. The slight decrease in total number in the asbestos-treated groups is considered to be due to lavage difficulties caused by airway blockage by asbestos fibres.

TABLE 2. PLEURAL LEUKOCYTE COMPONENTS 1 MONTH AFTER I/T DUSTS INDIVIDUALLY OR IN COMBINATION

Dust	Total No. ($\times 10^6$)	Differential per cent				
		Macrophages	PMN	Lymphocytes	Mast cells	Eosinophils
Control	4.5 (1.0)	82 (5.6)	0	1 (1.2)	11 (5.4)	6 (2.8)
TiO_2	10.8 (2.3)	84 (2.3)	0	1 (1.0)	8 (1.2)	7 (3.0)
Quartz	9.6 (1.7)	89 (0.7)	0	0	5 (1.4)	6 (0.7)
Cro	11.6 (2.5)	82 (1.2)	0	1 (1.0)	10 (1.2)	7 (2.1)
Cro + TiO_2	12.5 (2.3)	77 (7.2)	0	1 (0.6)	10 (6.2)	12 (5.2)
Cro + quartz	19.6 (2.3)	79 (2.1)	0	2 (1.5)	6 (2.0)	13 (2.7)

$N = 14$ rats for control and three rats for all other treatment groups.

Results are presented as mean (SD), PMN = neutrophils, Cro = crocidolite. All dust groups are significantly higher than control group ($P < 0.001$). Crocidolite vs crocidolite + quartz is also significantly different ($P < 0.01$).

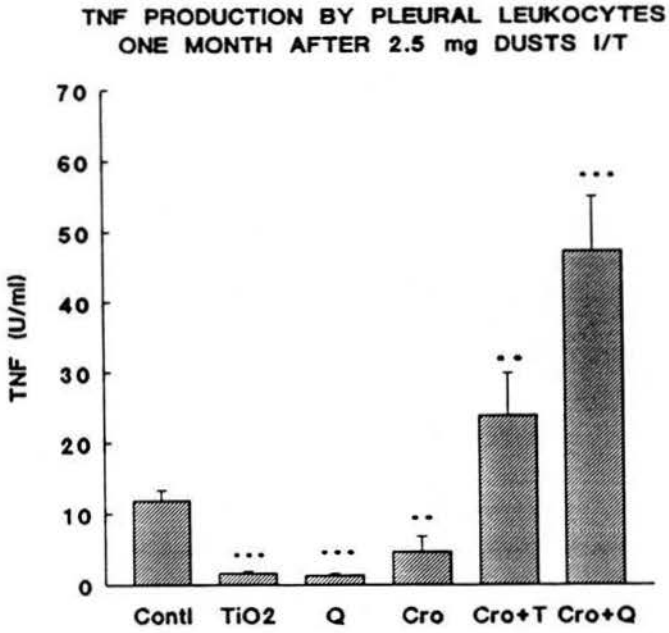


FIG. 1. TNF production by pleural leukocytes from rats 1 month after 2.5 mg dust intratracheal instillation (I/T). Each bar stands for the mean and SEM of three separate experiments performed in triplicate. The dilution of supernatant was 1:64. Asterisk denotes a significant difference from control: $^{**}P < 0.01$; $^{***}P < 0.001$. Contl = control; cro = crocidolite; T = TiO_2; and Q = quartz.

The components of pleural leukocytes after I/T asbestos and/or combined dusts are presented in Table 2. Instillation of any dust caused an increase in total numbers. In the crocidolite plus quartz group, however, the total number increased markedly.

TNF production by pleural leukocytes after I/T dusts

As Fig. 1 illustrates, I/T asbestos and other dusts alone resulted in decreased TNF secretion by pleural leukocytes. In contrast, a considerable synergistic effect in stimulating TNF production by the leukocytes was observed with asbestos in combination with quartz. A similar but less marked effect was found with crocidolite plus TiO_2.

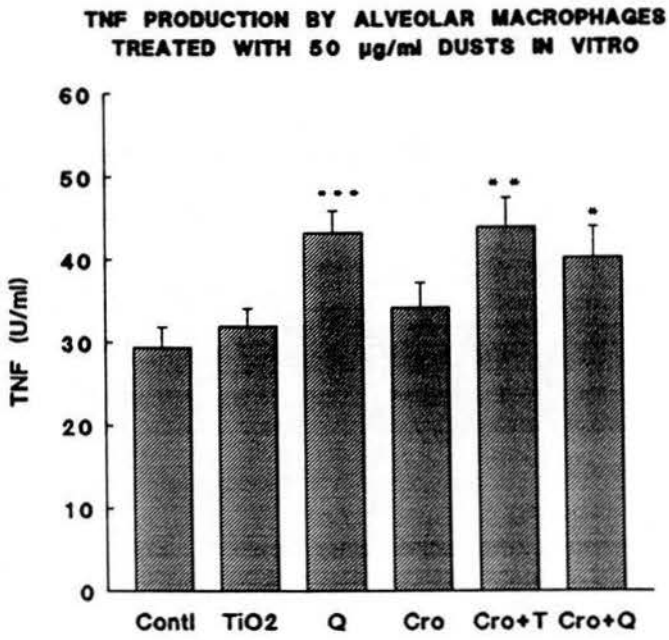


FIG. 2. TNF production by alveolar macrophages treated with 50 g ml^{-1} dust *in vitro*. Each bar stands for the mean and SEM of four separate experiments performed in triplicate. The dilution of supernatant was 1:64. Asterisk denotes a significant difference from control: $*P=0.05$; $**P<0.01$; $***P<0.001$. Abbreviations as for Fig. 1.

TNF production by alveolar macrophages treated with dusts in vitro

Normal bronchoalveolar leukocytes (macrophage >98%) were treated with 50 g ml^{-1} crocidolite, TiO_2 and DQ_{12} individually or in combination *in vitro*. Figure 2 shows that TiO_2 or crocidolite alone did not stimulate TNF production by alveolar macrophages, whereas quartz did. The combination effect of crocidolite and TiO_2 was an increase in TNF release compared to either dust alone. A combination of crocidolite and quartz produced no greater response than quartz alone.

Low endotoxin levels, from 0 to 106 pg ml^{-1}, in media and serum used in the assay were determined, but the removal of endotoxin by polymyxin columns did not influence TNF secretion.

DISCUSSION

The present study reveals that quartz is a strong inflammogen in the bronchoalveolar region, and this is consistent with our previous findings (DONALDSON *et al.*, 1988). In addition, crocidolite plus quartz caused the highest recruitment of leukocytes, indicating a synergistic effect between these two dusts. This increased inflammation may underlie the initiation of greater fibrosis observed by DAVIS *et al.* (in press) with quartz–asbestos exposure.

All dust treatment caused an increase in number of pleural leukocytes compared to the control. However, crocidolite and quartz in combination produced a significantly greater effect than any other treatment. In the case of pleural leukocyte TNF, intratracheal administration of asbestos or particle alone had an inhibitory effect on cytokine release. The local release of a TNF inhibitor may play a part in this effect. The combination of crocidolite with either TiO_2 or quartz, by contrast, resulted in an

increase in the release of TNF by the pleural leukocytes although the stimulation was greater with crocidolite plus quartz. In experimental studies in rats, DAVIS *et al.* (in press) demonstrated that both quartz and TiO_2, when given in combination with asbestos, caused an increase in the numbers of mesotheliomas and that the tumours tended to occur earlier than tumours with asbestos alone. This may be related to the report of FINKELSTEIN (1983) who described high mesothelioma incidence (10 out of 328) in pipe makers who had mixed exposure to crocidolite and 〈silica' in cement. It was suggested by DAVIS *et al.* (in press) that particulate dusts may have a much greater ability to penetrate through the pleural barrier than asbestos fibres and that, once particle penetration is occurring, fibre penetration is made easier. In animals inhaling mixtures of asbestos and quartz in the study of DAVIS *et al.* (in press) there was an increased transport of fibres across the visceral pleura. From the present study, significant increases in TNF production by pleural leukocytes after I/T asbestos coupled with both TiO_2 or quartz, indicates that these mixed exposures lead to increased pleural leukocyte response. These are likely to be important in subsequent pleural pathology and may be related to increased transport of fibres into the pleural space, although we have not seen fibres within pleural leukocytes.

It was notable that the greatest pleural leukocyte recruitment and TNF production was with crocidolite plus quartz. Crocidolite plus TiO_2 stimulated TNF production but to a much less extent than that caused by crocidolite plus quartz. The leukocyte response to crocidolite plus TiO_2 was increased compared to crocidolite or TiO_2 alone, but not significantly. This suggests that crocidolite plus TiO_2 is also stimulatory to pleural leukocyte response but less than crocidolite plus quartz and this is an agreement with the results of DAVIS *et al.* (in press).

The results of the study *in vitro* showed that quartz stimulates TNF production by normal alveolar macrophages whilst TiO_2 has only limited effects; this is consistent with previous findings on IL-1 secretion (KUSAKA *et al.*, 1990) from our laboratory. The combination of crocidolite and TiO_2 was also stimulatory whilst each of them on their own was not, suggesting that there may be a synergism in the action of compact particles and fibres in this respect. It might be anticipated that there would be a relationship between alveolar macrophage production of TNF after response to dusts *in vivo* and alveolar inflammation caused by the same dusts. The discrepancy between them, with regard to crocidolite plus TiO_2 in particular, which caused no inflammation but did cause TNF release, may be related to a time difference. There is likely to be acute inflammation immediately following instillation possibly mediated by TNF but this does not persist up to 1 month. Reliable data on TNF production by bronchoalveolar leukocytes at 1 month would help eliminate this question.

The present study indicates that different leukocyte responses occur in the pleural and bronchoalveolar space after exposure to asbestos and/or mineral dusts. The interaction between asbestos and other dusts in leading to stimulation of leukocyte secretion in the pleural space may underlie the previously demonstrated enhancement of the pathogenic effect of exposure to particle–fibre combinations.

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A comparison of alveolar macrophage cytotoxicity and ability to cause inflammation in the mouse peritoneal cavity for a range of different fibre types at equal fibre number.

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INTRODUCTION

We have previously reported on the ability of fibres to damage epithelial cells and fibroblasts and described a relationship between increasing toxicity and increasing fibre length for a range of asbestos samples (Brown et al 1986). We have also utilised the mouse peritoneal cavity as site to study the inflammogenic potential of fibres (Donaldson et al 1989). As part of a programme aimed at examining the relationship between the biological activity of a range of non-asbestos fibres and aspects of their structure, we examined the ability of these vitreous and composite fibres to damage alveolar macrophages and to cause peritoneal inflammation. The study was carried out at equal fibre number in both studies so that a direct comparison could be made for fibres of varying density where matching by mass would have resulted in dramatic differences in fibre number.

MATERIALS AND METHODS

Fibres. The fibres used in the study are shown in the table below. All fibres were sized for length by counting 400 fibres by phase contrast optical microscopy according to WHO rules and the % of fibres longer than 10 and 20μm are shown in Figure . It shows that except for Short fibre amosite, all of the samples had a large proportion of fibres in the Stanton range (i.e. > 8μm) but there was variation in the proportions of longer (>20μm) fibres.

Cytotoxicity assay. This assay of cytotoxicity was as described in Donaldson et al (1988). In brief normal rat alveolar macrophages were labelled overnight with ^{51}Cr and then incubated with equal numbers (8.2 x 10^6 fibres/ml) of the various fibre types. Cytotoxicity was expressed as the

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amount of free radioactive label in the supernatant after 24 hours of incubation. Experiments were carried out on 3 separate occasions, in triplicate, and the data were log transformed and averaged.

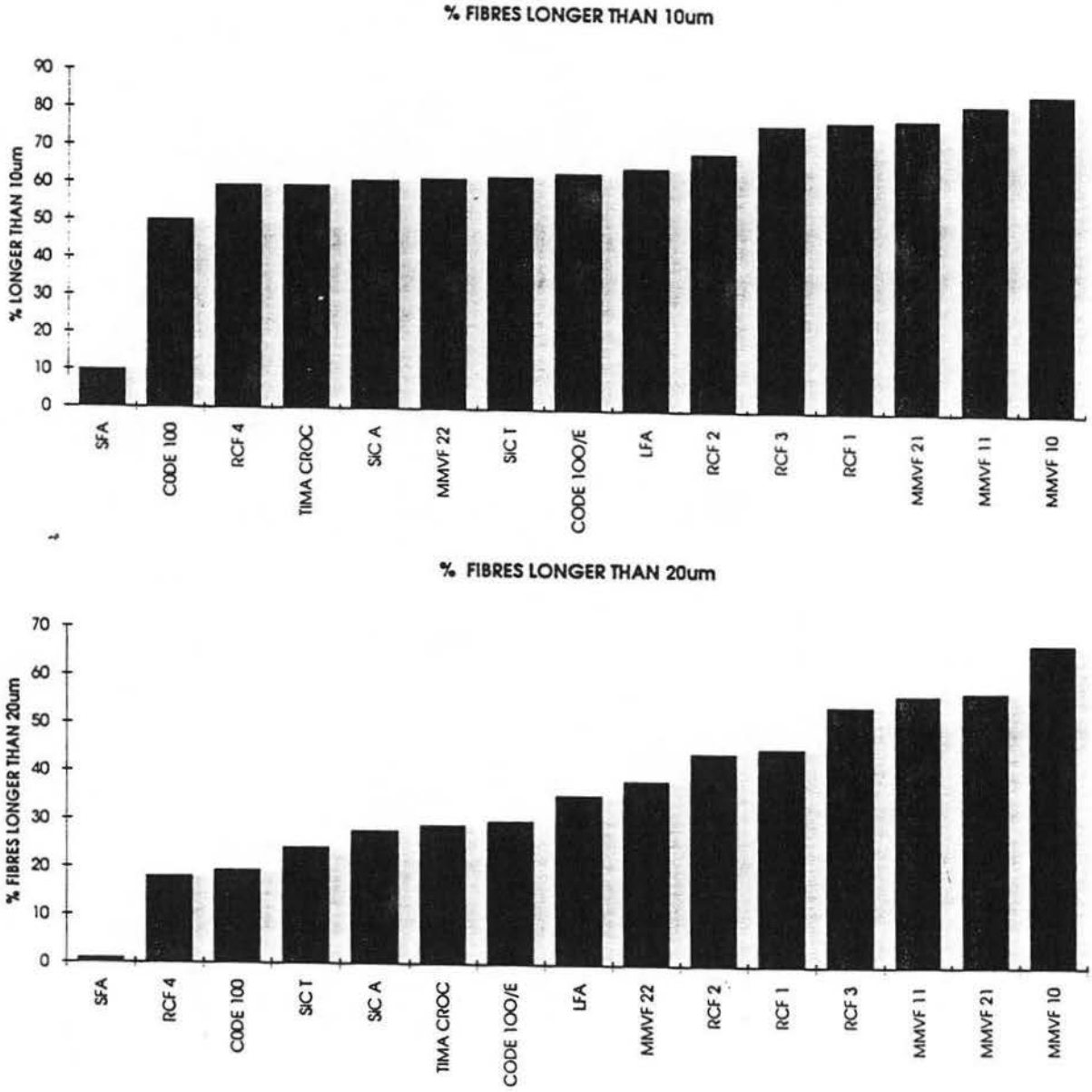


Figure 1 Percentage of fibres of each type used in the study that were longer than 10μm (upper panel) and 20μm (lower panel).

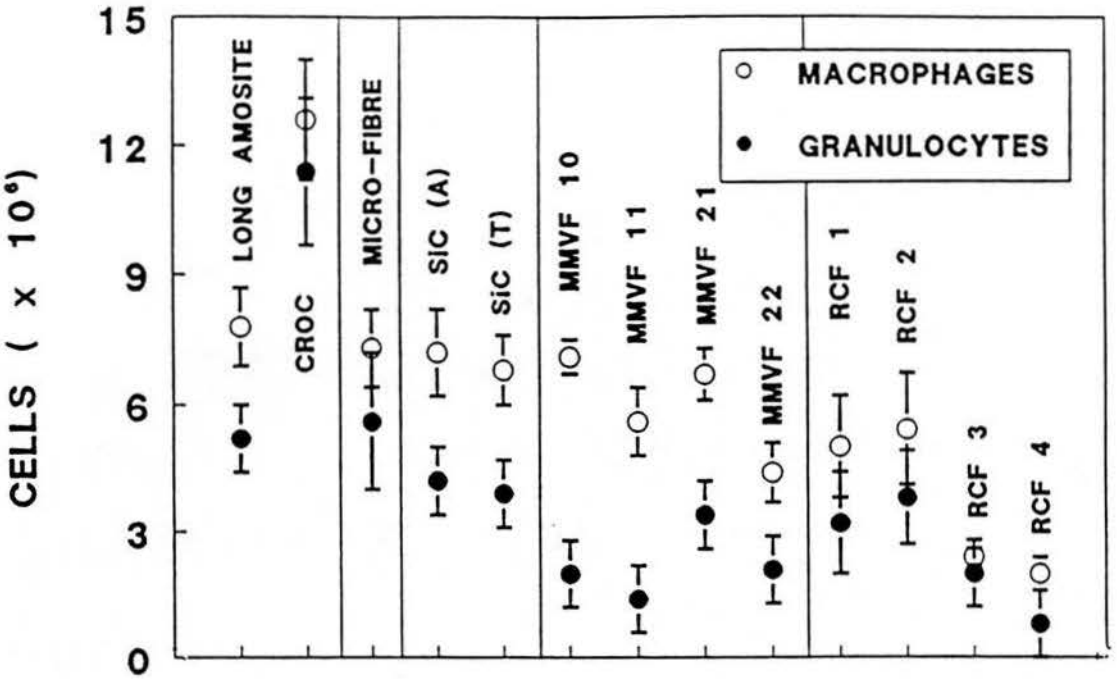


Figure 2 Number of macrophages (open circles) and granulocytes (closed circles) lavaged from the peritoneal cavity of mice instilled with equal numbers of the indicated fibre types. Data given as mean ± SD millions.

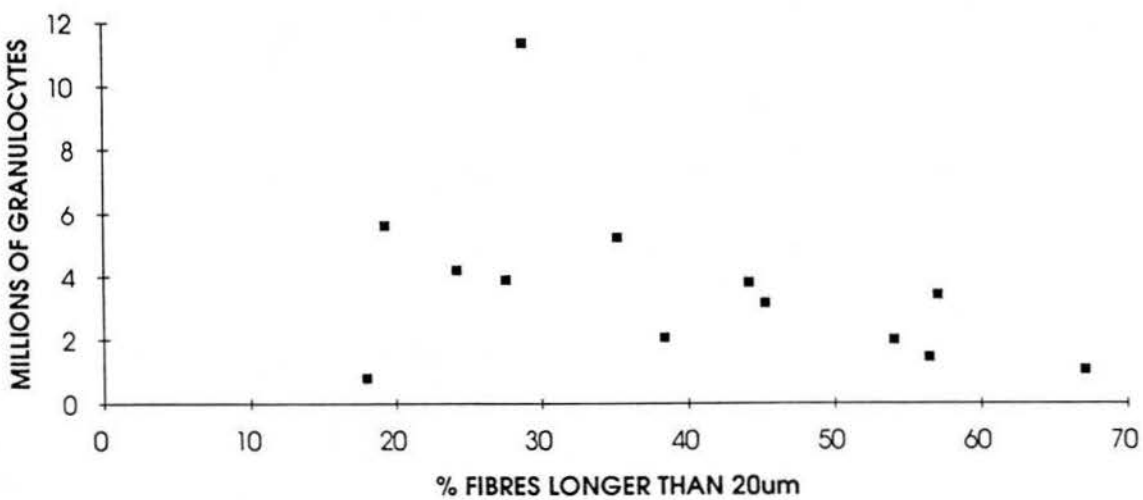


Figure 3 Peritoneal inflammation data of mean millions of granulocytes plotted against percentage of fibres longer than 20μm.

Inflammation in the mouse peritoneal cavity. This was assessed as described previously (Donaldson et al 1989). Briefly groups of 3 or 4 C57 Bl6 mice aged > 6 weeks were instilled with 0.5 ml of saline containing a mass of fibres that yielded 8.2 x 10^7 fibres. At 4 days the peritoneal cavity was lavaged with 3 x 2ml volumes of saline and the total and differential cell count determined. All data were expressed as mean millions of macrophages or granulocytes for 3-4 mice.

TABLE The fibre samples used in the study

Fibre	Abbreviation	Source/reference
Long fibre amosite	LFA	Davis et al 1988
Short fibre amosite	SFA	Davis et al 1988
Crocidolite	TIMA Croc	TIMA repository
Code 100/475 vitreous fibre	Code 100/475	Shuller (Manville)
Code 104/E vitreous fibre	Cide 100/E	ditto
Silicon carbide A	SiC A	Industrial sample
Silicon carbide T	SiC T	ditto
Refractory ceramic fibre 1	RCF 1	TIMA repository
Refractory ceramic fibre 2	RCF 2	ditto
Refractory ceramic fibre 3	RCF 3	ditto
Refractory ceramic fibre 4	RCF 4	ditto
Man-made vitreous fibre 10	MMVF 10	ditto
Man-made vitreous fibre 11	MMVF 11	ditto
Man-made vitreous fibre 21	MMVF 21	ditto
Man-made vitreous fibre 22	MMVF 22	ditto

RESULTS

Peritoneal inflammation assay.

Despite differences in chemical composition, at equal fibre number the different fibre types produced inflammation that was essentially similar (Figure 2). There were exceptions, principally RCF 3 and RCF 4. RCF 4 was, in general much shorter than any of the other samples and so the low response it caused was not surprising but there was no obvious reason for the reduced inflammogenicity of RCF 3. When millions of granulocytes, taken as a general measure of inflammation was plotted against % of fibres longer than 20μm for all of the fibres types, there was no obvious relationship (Figure 3).

Macrophage cytotoxicity assay. There were differences in the ability of different fibre populations to cause cytotoxic effects to alveolar macrophages at equal fibre number (Figure 4). When toxicity to macrophages was plotted against the % of fibres longer than 20μm in each fibre sample no relationship was found (data not shown) .

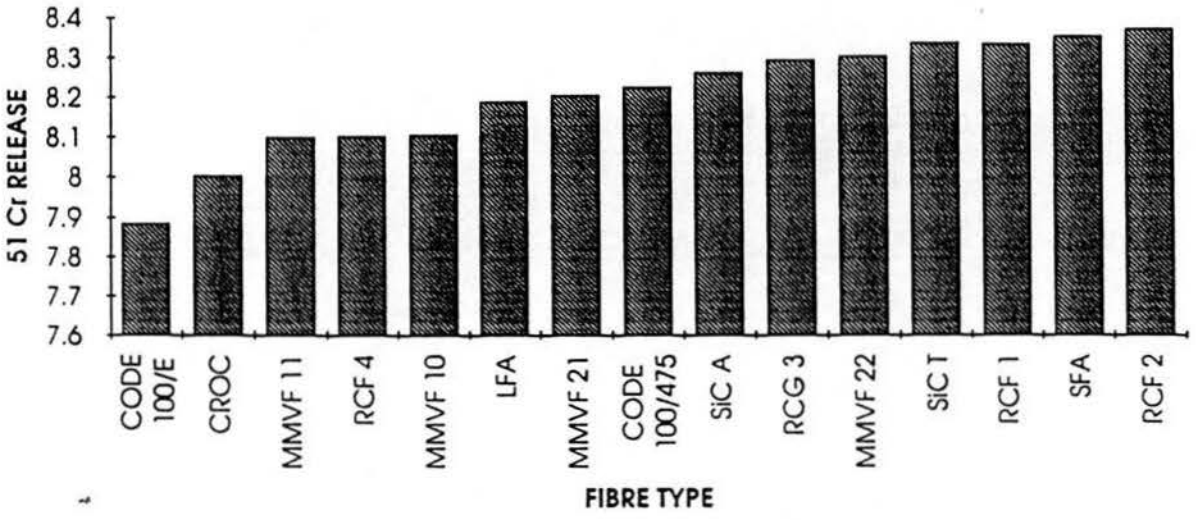


Figure 4 Cytotoxicity of different fibres at equal fibre number; cytotoxicity data log transformed and expressed as the average of triplicate wells in 3 separate experiments.

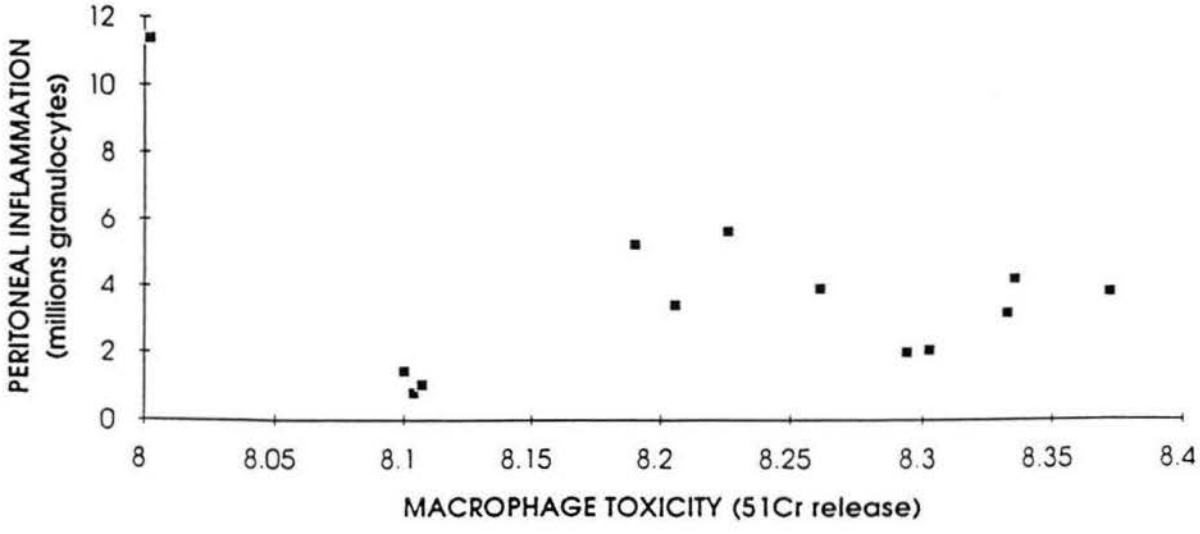


Figure 5 Macrophage toxicity versus peritoneal inflammation for the different fibres. Data expressed as in the above figures.

Comparing peritoneal inflammation with cytotoxicity to alveolar macrophages. When ability to cause inflammation was compared with ability to damage alveolar macrophages, no relationship was found (Figure 5).

DISCUSSION

In this paper we have utilised two short-term assays of fibre toxicity and compared their responses for a large range of different fibre types with broadly similar size ranges. There was no relationship between the response of the two assays to the fibre samples. In general, as previously described (Donaldson et al 1993a) all of the samples gave a broadly similar response in the peritoneal inflammation assay; in the macrophage cytotoxicity assay there were differences between samples but they showed no relationship to known toxicities of the fibres.

This exercise points out problems inherent in utilising short-term assays of toxicity for fibres but the shortcomings of the two assays are explicable in different ways. The peritoneal inflammation assay was found to respond more or less equally to all of the samples. This was not surprising since all of the fibre preparations contained a large proportion of fibres that were longer than 8-10μm. This assay responds to 'Stanton' fibres (Donaldson et al 1989) but does not apparently discriminate between fibres longer than 20μm as shown by the lack of correlation.

The likely importance of durability in determining the ultimate pathogenicity of fibres has led us to suggest that the peritoneal inflammation assay will be of value only if the dissolution potential of fibres is taken into consideration (Donaldson, in press). We suggest that if a fibre is pre-treated to mimic residence in the lung (eg immersion in mild acid for a time) before it is tested in this assay there will be more likelihood that the true toxicity of the fibre sample will be demonstrated. When this pre-treatment was carried out for a soluble vitreous fibre and a more durable ceramic fibre we demonstrated loss of inflammogenic potential in the case of the soluble fibres but little change in the case of the ceramic fibre (Donaldson, in press).

With the macrophage toxicity assay there were differences between fibre samples but these bore no relationship to the known pathogenIcities of the samples. For instance the two least cytotoxic samples were Code 100/E glass and TIMA crocidolite; whilst the glass fibre could be low in toxicity the crocidolite sample is known to be highly pathogenic. Also, long fibre amosite,

known to be highly pathogenic, was around the middle of the ranking, being similar to Code 100/475. We have previously reported the Code 100/475 sample to be substantially less toxic than long amosite (Donaldson et al 1993b).

Cytotoxicity to alveolar macrophages was considered at one time to be a major descriptor of the pathogenenicity of dusts, as exemplified by quartz. However, it is now clear that to be fibrogenic in the lung the ability of any dust to stimulate the release of key macrophage products is just as likely to be a determining factor as the ability to kill macrophages. These products, which tend to enhance inflammation and promote pathological change include chemotactic factors, histotoxic agents such as proteases and oxidants, and also cytokines. Thus more subtle measures of 'toxicity' such as activation of cell secretion are becoming the markers of choice for predicting/explaining the toxicity of respirable dusts and these may bear no relation to ability to cause cell death. In addition the same arguments that are used above for the peritoneal inflammation assay are applicable to the toxicity assay and fibres pre-treated with mild acid would be more likely to reflect the long term toxicity of these samples in the lung.

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INFLAMMATORY RESPONSES IN THE LUNGS OF RATS EXPOSED TO AMOSITE ASBESTOS*

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Abstract—Studies with a long fibre sample of amosite asbestos demonstrated its deposition in the terminal bronchi and alveolar ducts and its phagocytosis by macrophages. Rats inhaling UICC amosite asbestos showed accumulation of inflammatory leukocytes and evidence of toxicity, indicated by increased levels of the cytoplasmic enzyme lactate dehydrogenase, in the bronchoalveolar region. We believe this inflammatory response to be due to long fibres present in the UICC amosite. We investigated possible consequences of these events for the inflammatory response by assessing the ability of two amosite samples, one long fibre and one short fibre, to induce release of the proinflammatory cytokine, tumour necrosis factor (TNF) from alveolar macrophages *in vitro*. The long fibre preparation was much more active in causing the release of TNF. Furthermore, 1 mg of the long fibre sample, instilled into rat lungs, produced marked cell proliferation leading to severe fibrosis at 1 month. The short fibre sample, by contrast, produced lung inflammation but little cell proliferation or fibrosis.

INTRODUCTION

INHALATION of asbestos fibres is associated with the accumulation of inflammatory leukocytes in the bronchoalveolar region in man and experimental animals (BEGIN *et al.*, 1986; DONALDSON *et al.*, 1988) and precedes the development of fibrosis and cancer in some individuals. Secretion of toxic products and growth factors by inflammatory cells has been implicated in lung injury (HUNNINGHAKE *et al.*, 1984) and may contribute to subsequent pathology.

In rats exposed to chrysotile asbestos, the primary site of particle deposition is the first alveolar duct bifurcation and particles deposited at this site are rapidly phagocytosed by macrophages (BRODY et al., 1981). Activation of macrophages in response to such asbestos exposure may lead to release of the proinflammatory cytokine, tumour necrosis factor (TNF). The multifunctional activity of TNF in recruiting and activating neutrophils (PMN) and in stimulating the proliferation and secretory activity of mesenchymal cells (LARRICK and KUNKEL, 1988) indicates a potentially important role for this cytokine in the pathological process.

MATERIALS AND METHODS

Dusts

The UICC amosite, long fibre amosite (LFA) and short fibre amosite (SFA) used in this study have previously been described in detail (DAVIS, 1986). Briefly, while the

*This paper was included in Poster Session 4 and the discussion included in the summary presented in Section 12.

diameter of all three samples was similar, the percentage of fibres > 10 μm in length was 0.1% for SFA, 10% for LFA and 2% for UICC amosite. Titanium dioxide (TiO_2) was used as an inert particulate control as previously described (DONALDSON *et al.*, 1988).

Dust exposure and assessment of the tissue response

For the deposition experiments, male HAN rats were exposed to 1000 fibres ml^{-1} of long fibre amosite or TiO_2 for 7 h (approximately 5 mg m^{-3}) as detailed in DONALDSON *et al.* (1988). In further experiments rats were exposed to 50 mg m^{-3} UICC amosite for up to 52 days; bronchoalveolar leukocytes and fluid were obtained by lavage at each time point and cell counts and LDH estimations were carried out as previously described (DONALDSON *et al.*, 1988). Secretion of tumour necrosis factor by alveolar macrophages was assessed, using the L929 bioassay, according to the method of FLICK and GIFFORD (1984) following *in vitro* exposure to 50 g ml^{-1} of TiO_2, LFA and SFA.

Intratracheal instillation of 1 mg LFA and SFA was carried out according to standard procedures (DONALDSON *et al.*, 1988). Cellular proliferation was assessed 2 days after injection by measuring uptake of the DNA precursor bromodeoxyuridine (BRDU). BRDU was injected intraperitoneally 24 h before the rats were killed; the lungs were then obtained and prepared for routine histology. Cells containing BRDU were visualized using anti-BRDU antibody and peroxidase staining. Lung pathology was assessed 1 month after IT injection. The site of deposition of LFA was assessed by scanning electron microscopy of lung sections according to the method of BRODY *et al.* (1981).

Statistical analysis

Data were analysed by analysis of variance and differences between means tested using the Student's *t*-test. In the UICC amosite study differences between TiO_2 and amosite were assessed using logged data.

RESULTS AND DISCUSSION

We have shown here that, as previously described for chrysotile asbestos in rats (BRODY *et al.*, 1981), long fibres of amosite asbestos deposit in the terminal bronchioles and alveolar ducts (Figs 1 and 2) and that these fibres are phagocytosed by macrophages. Sustained exposure to UICC amosite, which contains many long fibres, caused inflammation as assessed by PMN recruitment (Fig. 3) and tissue injury measured by increased levels of LDH in the BAL (Fig. 4). The inflammation caused by this UICC amosite sample, which contains both long and short fibres, is very likely due in the main to the long fibres. This was supported by the finding of an increase in secretion of the proinflammatory cytokine, TNF, by alveolar macrophages exposed to LFA *in vitro*, compared with those exposed to SFA (Fig. 5). TNF may also be involved in the fibrogenic process since, following instillation of 1 mg of LFA or SFA, there was a florid proliferative response only to LFA [Fig. 6(a)] and this developed into severe fibrosis at 1 month [Fig. 7(a)]. TNF has previously been demonstrated to be required in silica-induced pulmonary fibrosis (PIGUET *et al.*, 1990). SFA, which did not elicit TNF secretion by macrophages *in vitro*, caused more persistent PMN inflammation

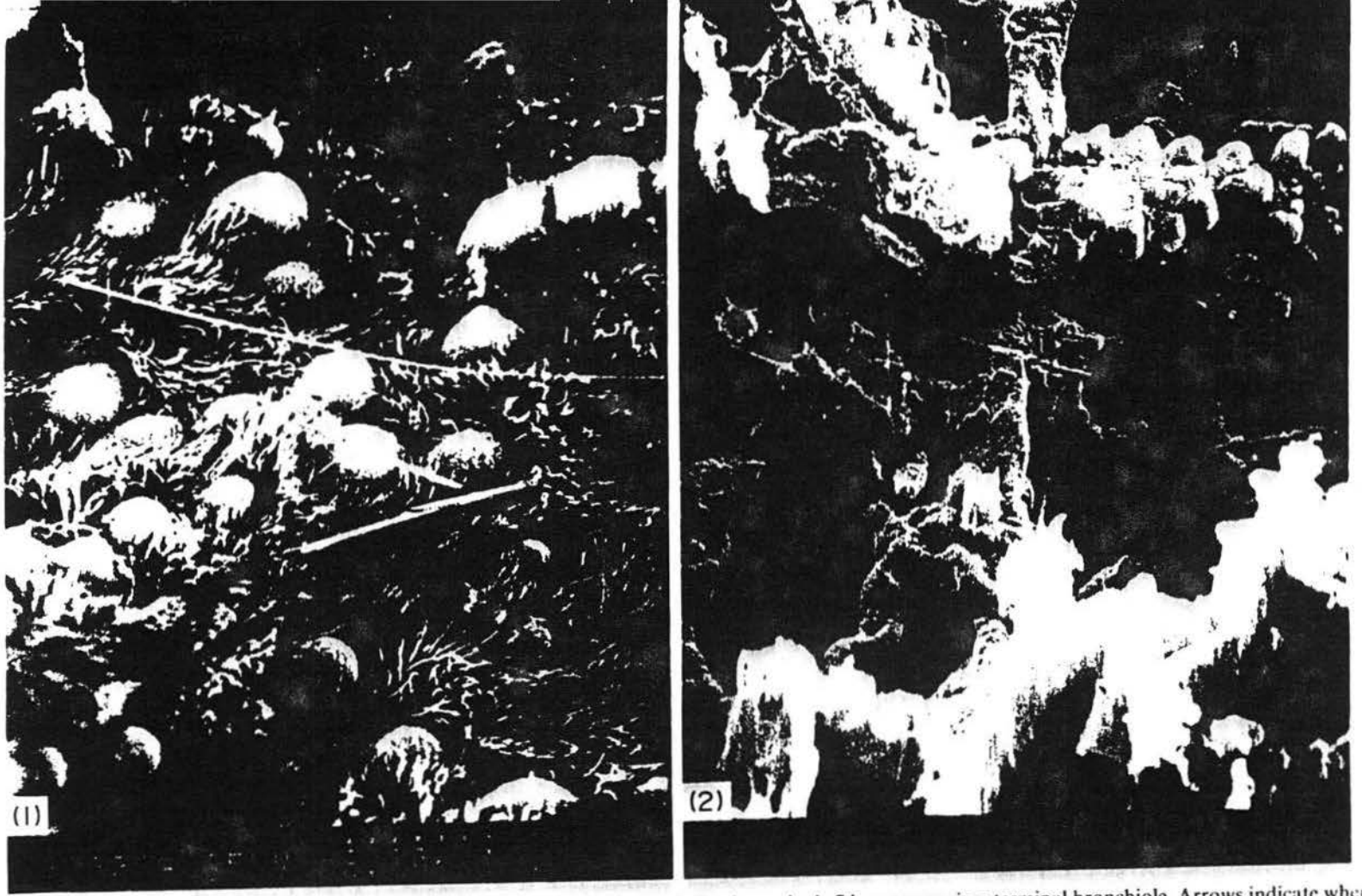


FIG. 1. Scanning electron micrograph showing deposition of long fibres of amosite, following a single 7 h exposure, in a terminal bronchiole. Arrows indicate where the fibres have been phagocytosed by macrophages.

FIG. 2. Scanning electron micrograph showing deposition of long fibres of amosite, following a single 7 h exposure, in an alveolar duct. Arrows indicate where the fibres have been phagocytosed by macrophages.

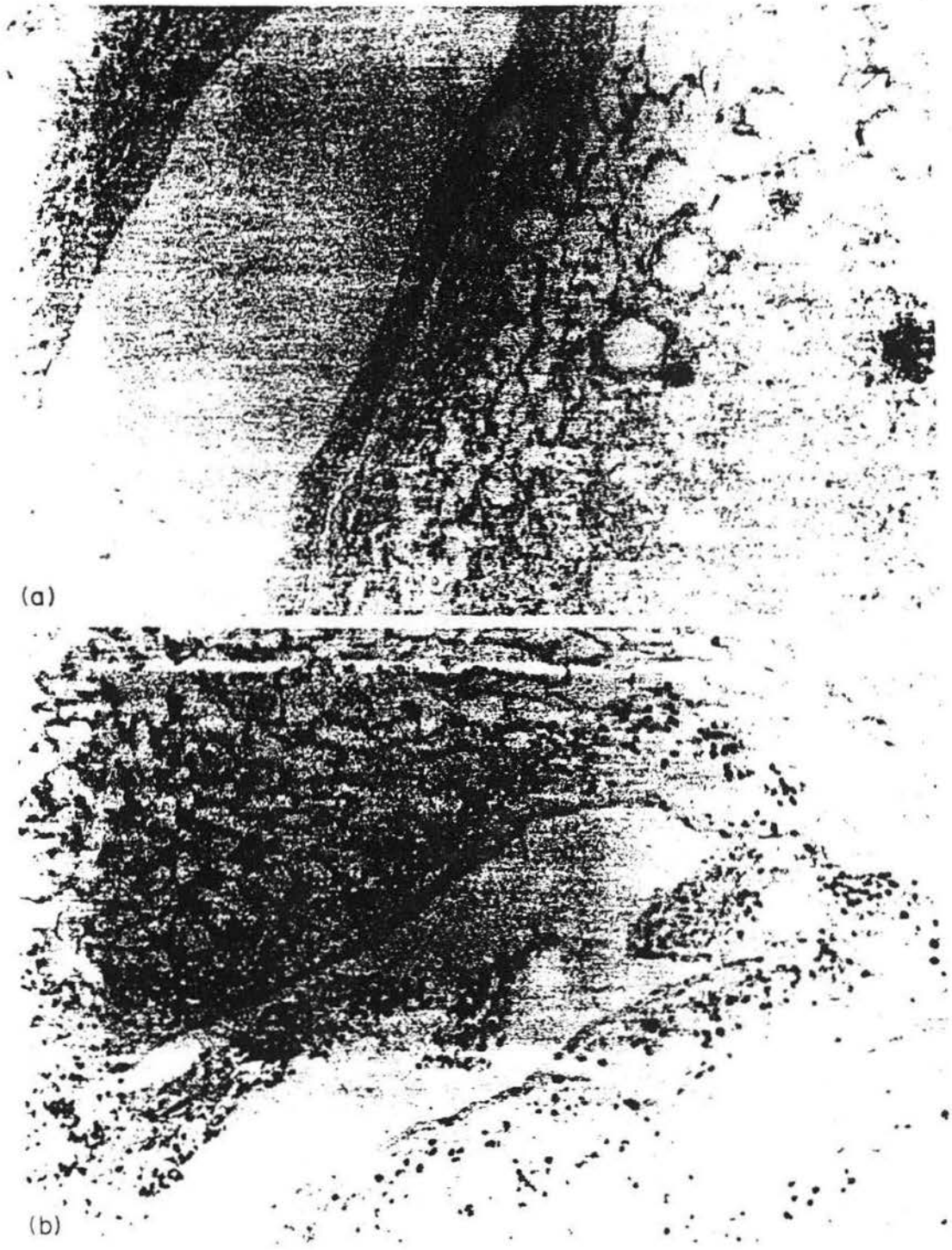


FIG. 6. Cell proliferation in rats 1 day following intratracheal injection of (a) 1 mg LFA or (b) SFA. Darkly stained cells are those which have incorporated BUDR into the DNA.

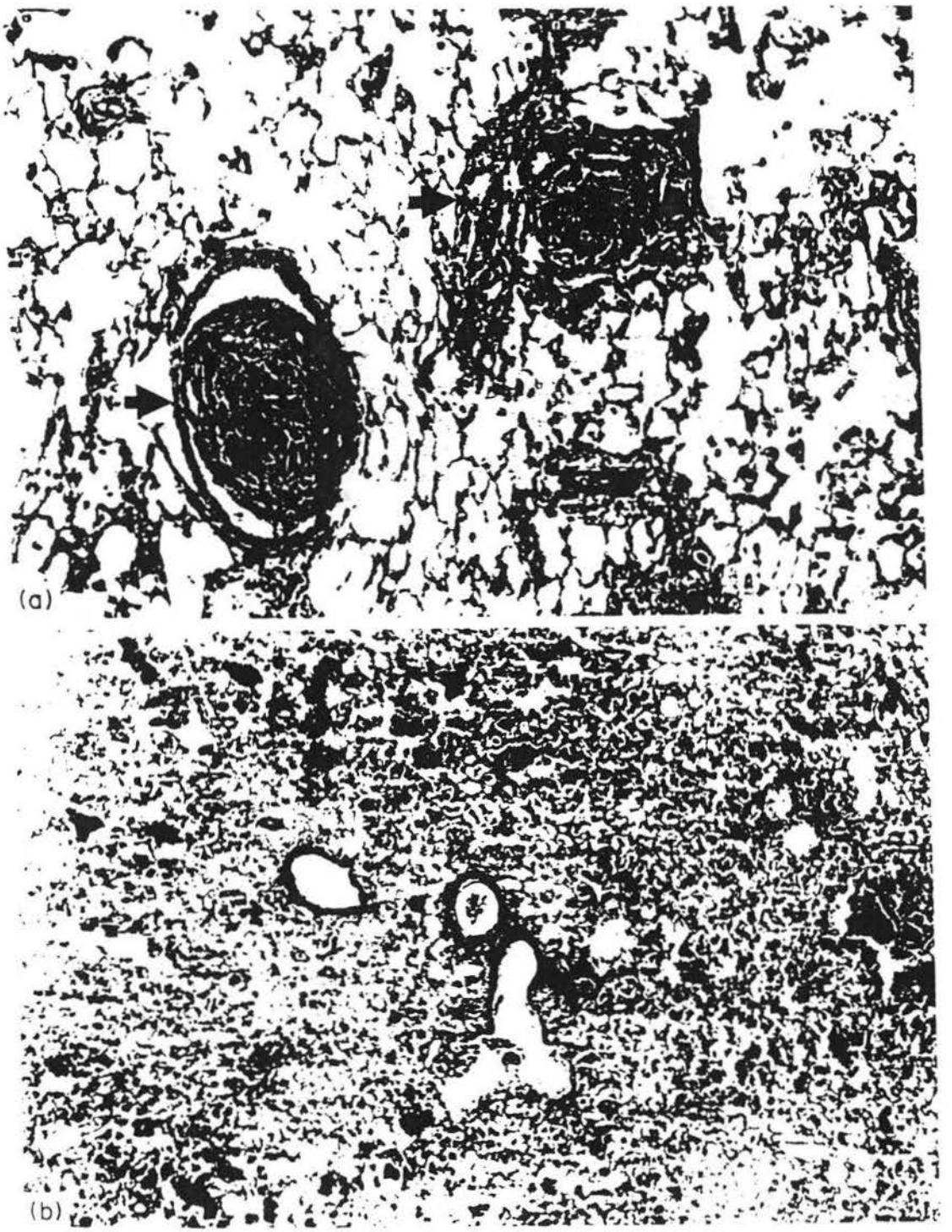
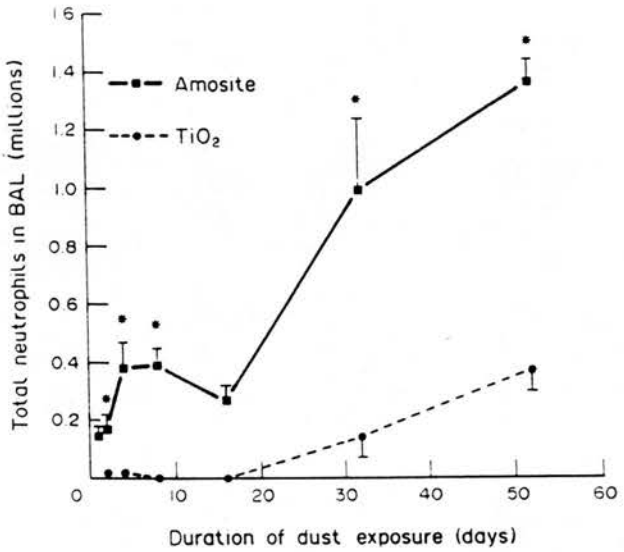
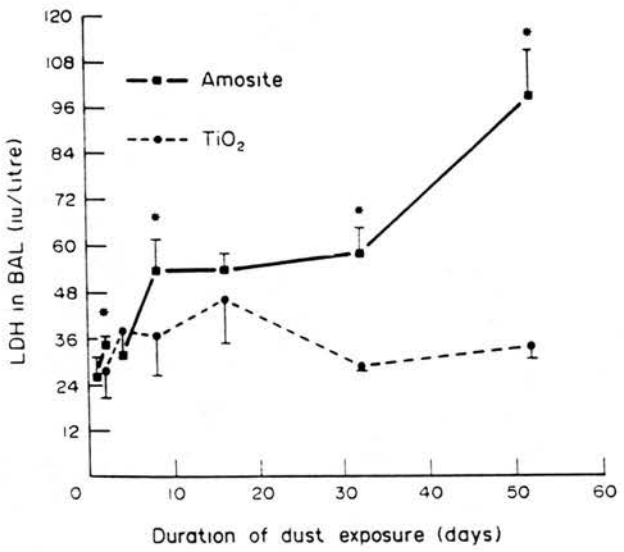


FIG. 7. Tissue response 1 month after injection of (a) LFA or (b) SFA. Arrows indicate areas of fibrosis.



IG. 3. Accumulation of PMN in the BAL with duration of exposure to UICC amosite asbestos in omparison with TiO_2 (each dust at 50 mg m^{-3}). Results are the mean of data from four rats per group at ach time point. Asterisks indicate a significant increase, the 5% level, in amosite compared with TiO_2.



IG. 4. Accumulation of LDH in the BAL with duration of exposure to UICC amosite asbestos in comparison with TiO_2 (each dust at 50 mg m^{-3}). Results are the mean of data from four rats per group at each time point. Asterisks indicate a significant increase, the 5% level, in amosite compared with TiO_2.

than the LFA but virtually no persistent fibrosis [Figs 6(b) and 7(b)]. Despite reports that TNF is chemotactic for PMN (MING *et al.*, 1987) the increased TNF release presumed to occur with LFA *in vivo*, based on the *in vitro* results, was associated with less PMN recruitment (Figs 6 and 7). Differences in the observed inflammation with LFA and SFA may be due to difficulties in instilling LFA which are likely to have

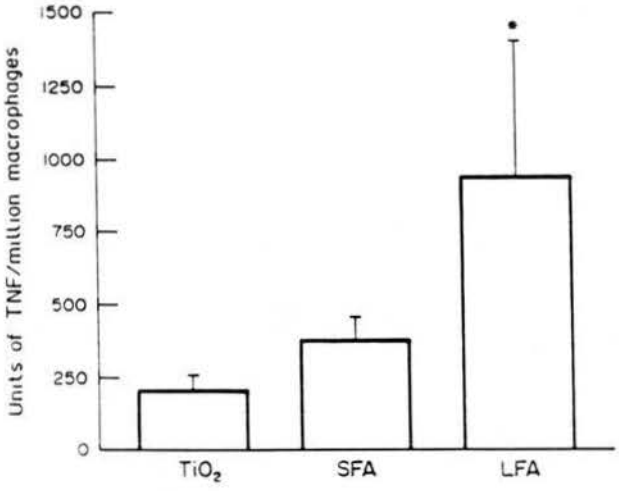


FIG. 5. Secretion of TNF by normal rat alveolar macrophages following *in vitro* exposure to LFA. LFA exposure caused significantly greater secretion of TNF than either SFA or TiO_2 ($P<0.05$).

produced a lower tissue dose of LFA than SFA. By contrast 1 mg of SFA, when administered by intratracheal instillation, may be close to a tissue dose at which overload of the clearance mechanisms could result in inflammation even with an innocuous dust as reported by us for other dusts elsewhere (LI and DONALDSON, 1994; CULLEN and LI, 1994).

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Inhaled Particles VII

ACTIVITY OF WOOL MILL DUST *IN VITRO* AND *IN VIVO*; CYTOTOXICITY, CYTOKINE PRODUCTION, LYMPH NODE STIMULATION AND HISTOPATHOLOGY*

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Abstract—Dust collected from the air of wool mills in the North of England showed no significant toxic effect to rat alveolar macrophages or a human epithelial cell line *in vitro*. In contrast, macrophages stimulated *in vitro* with the dust released significant amounts of the pro-inflammatory cytokines Tumour Necrosis Factor (TNF) and Interleukin-1 (IL-1). Examination of the lung lymph nodes from rats exposed to wool dust showed an increased proliferative response. Possibly, alveolar macrophages, which had phagocytosed the dust and already had increased secretion of immunostimulatory cytokines, could be migrating to the lymph nodes and causing stimulation. However, transfer of a soluble stimulatory factor present in the dust, such as endotoxin, to the lymph node could also be responsible. Histological examination of the lung showed evidence of granuloma formation and fibrosis in airway walls. Our results suggest that symptoms of airway irritation described in wool mill workers, could not be explained by direct toxic effects on airway lining cells. These symptoms are more likely to be caused by stimulation of airway wall leukocytes in rats, leading to chronic inflammation.

INTRODUCTION

EARLIER studies carried out by the Institute of Occupational Medicine reported symptoms of airway irritation in wool textile mill workers in the North of England (LOVE *et al.*, 1988). Inhalation of other organic dusts has been shown to produce inflammation as measured by bronchoalveolar lavage profile (SEMENZATO, 1988; LECOURS *et al.*, 1986). Activation of alveolar or airway macrophages after phagocytosis of wool dust, or via contaminating endotoxin, could result in the secretion of cytokines which cause inflammation (VUK-PAVLOVIC and ROHRBACH, 1990; DUBOIS *et al.*, 1989). Activated alveolar macrophages could also migrate to lymph nodes or bronchial lymphoid tissue causing activation of the immune system. Prolonged immune responses–inflammation in the airways and lung parenchyma could lead to pathological changes and, with organic dust exposure, granuloma formation has been described (PIMENTEL, 1970). In the present study, a rat model was used to examine the possible mechanisms whereby wool dust could cause inflammation and airway irritation.

MATERIALS AND METHODS

Rats

Male HAN rats were used throughout the study.

*This paper was included in Poster Session 7 and the discussion included in the summary presented in Section 12.

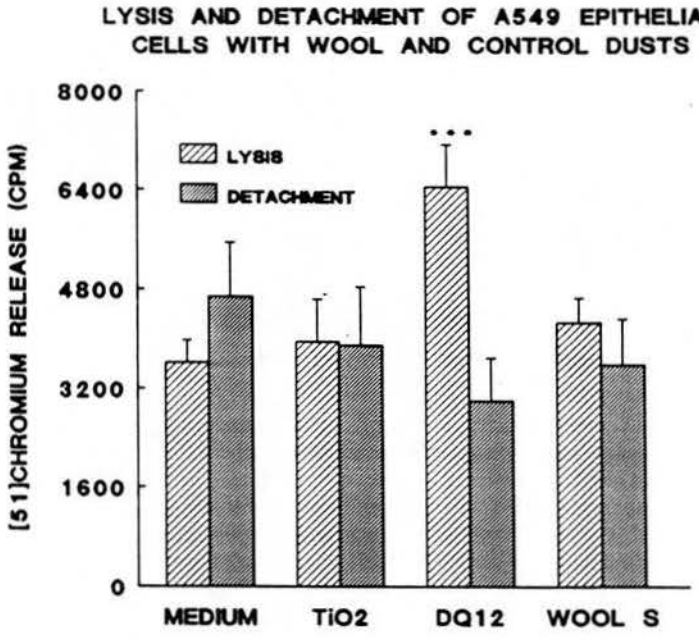


FIG. 1. The injurious effects of wool dust S, and the control dusts TiO_2 and quartz, on ^{51}Cr-labelled A549 epithelial cells. The graph shows the lytic and detaching ability of the dusts, at a concentration of 100 μg dust per well. The data are expressed as the mean and standard error of three separate experiments with triplicate measurements per treatment. Significant effects ($***P<0.001$) were seen only in the lytic activity of quartz compared with the medium control.

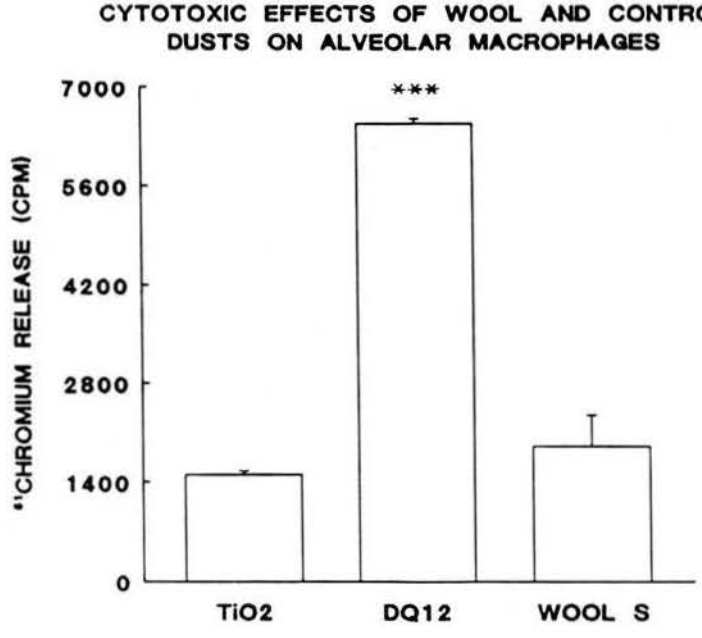


FIG. 2. The cytotoxic effects of wool dust S, and control dusts TiO_2 and quartz, on ^{51}Cr-labelled alveolar macrophages. The data are expressed as the mean and standard error of three separate experiments with triplicate measurements per treatment. A significant difference ($***P<0.001$) was only noted for quartz compared with the medium control.

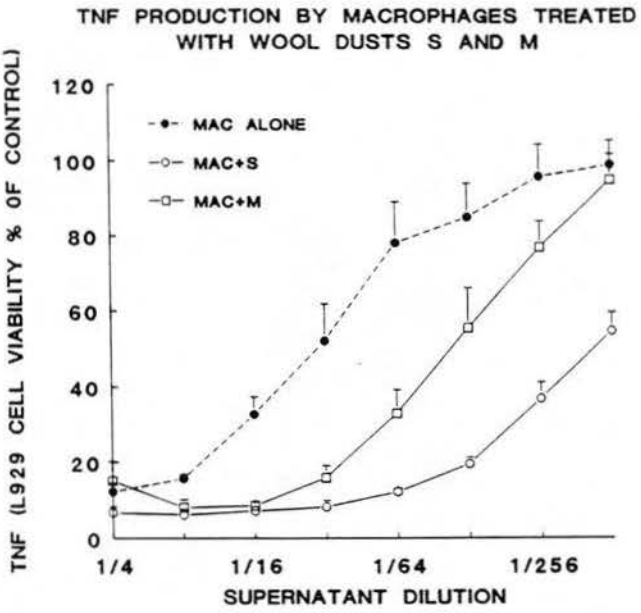


FIG. 3. TNF produced by alveolar macrophages treated *in vitro* with wool dusts S and M. The data are expressed as the mean and standard error of three separate experiments, with triplicate measurements per treatment. Increased TNF production evident as decreased viability of L929 cells, was observed in supernatants from macrophages treated with wool dusts, compared with the control group.

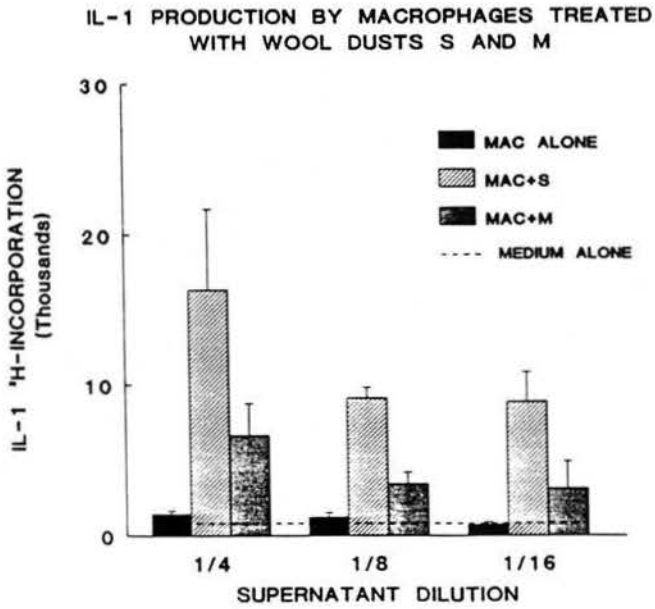


FIG. 4. IL-1 produced by alveolar macrophages treated *in vitro* with wool dusts S and M. The data is expressed as the mean and standard error of two separate experiments with triplicate measurements per dilution. Significantly increased IL-1 production ($P<0.001$) was noted for both dusts at each dilution compared with the control supernatant.

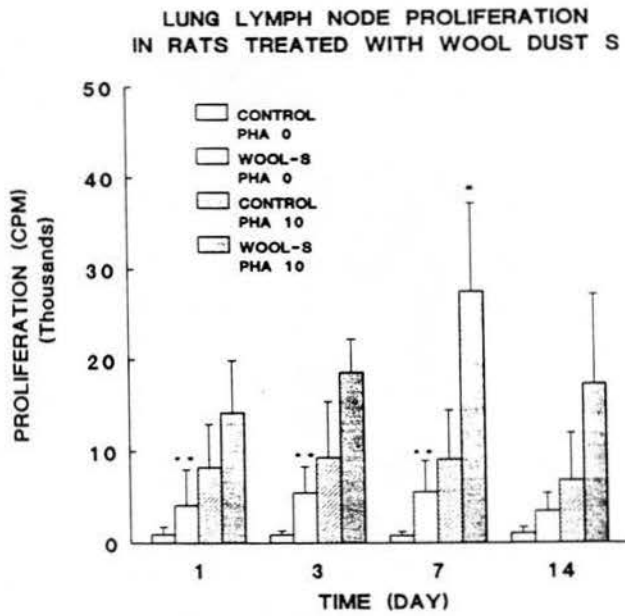


FIG. 5. Proliferation of lung lymph node cells after *in vivo* treatment with wool dust S. The data are expressed as the mean and standard error of three separate experiments, each consisting of three animals per treatment with triplicate measurements per animal. Significant differences (**$P<0.01$) were seen between control and wool dust treated animals at 1, 3 and 7 days post-exposure with no PHA treatment. At day 7, a significant effect (*$P<0.05$) was observed between wool dust treated and control animals with 10 μg ml^{-1} PHA.

Dust collection

Dusts were collected from two wool mills in the North of England, designated S (start) and M (middle), reflecting the opening–blending and carding processes, respectively, using IOM static inspirable dust samplers.

Epithelial and alveolar macrophage injury assays

Suspensions of wool dust in Minimal Essential Medium were added to ^{51}Cr-labelled A549 epithelial cells or rat alveolar macrophages, according to the method of DONALDSON et al. (1988).

Intratracheal instillation of wool dust

Wool dust suspended in PBS (0.5 ml, 2 mg ml^{-1}) was instilled via a blunt-ended needle into anaesthetized, tracheotomized rats.

Lymph node proliferation

Lung lymph node cells were obtained from disaggregated nodes of control and wool-exposed rats. Proliferative response in the presence and absence of phytohaemagglutinin (PHA) mitogenesis was assessed over 48 h by uptake of 3H-thymidine and liquid scintillometry.

Cytokine assays

Twenty-four hour supernatants of control alveolar macrophages were obtained after incubation with wool mill dust at a concentration of 50 μg ml^{-1}. TNF in the supernatant was assayed using the L929 cell line, as described previously (FLICK and

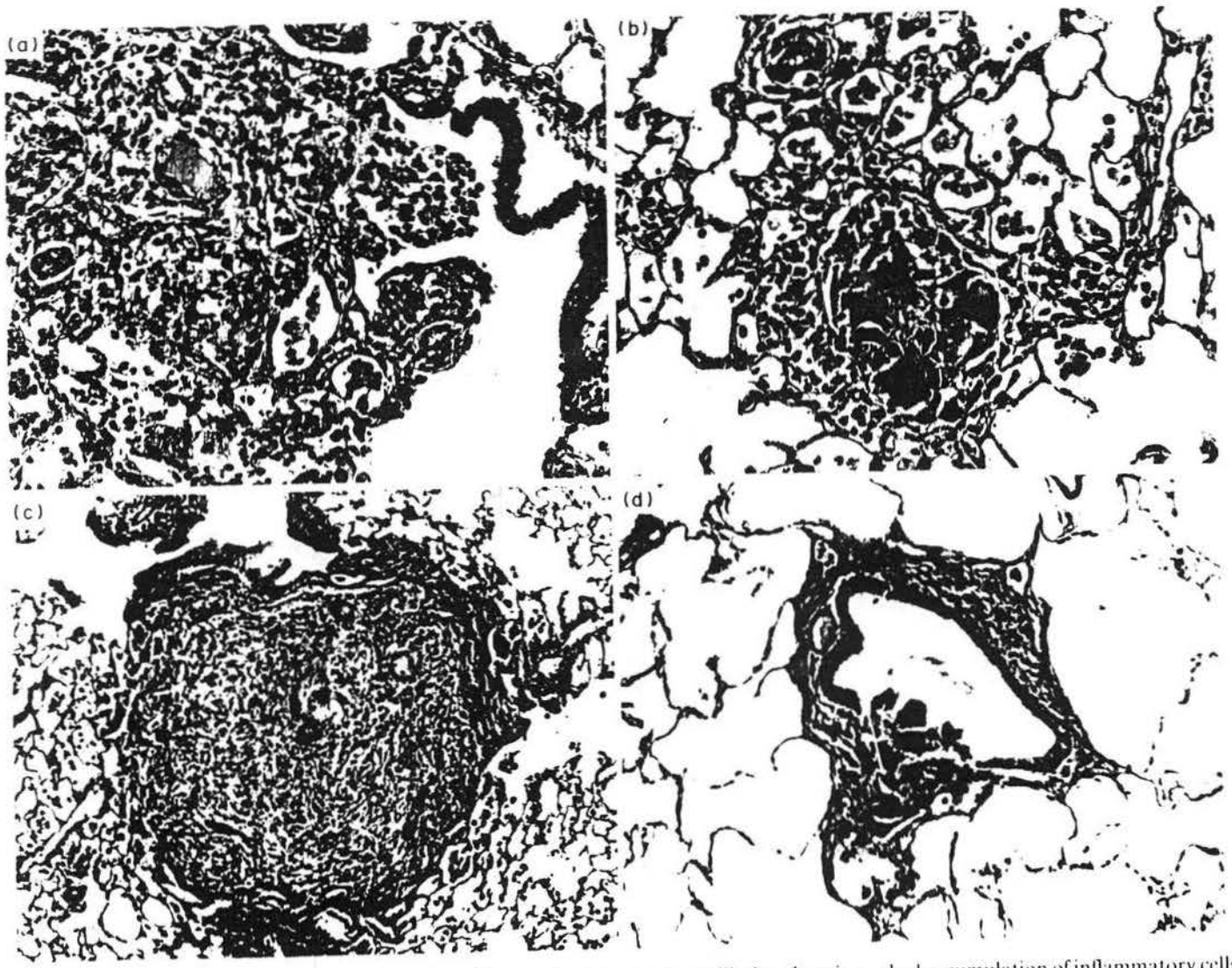


FIG. 6. Appearance of lungs from rats instilled with 1 mg of wool mill dust: < (a) 3 days post-instillation there is marked accumulation of inflammatory cells with epithelial detachment (× 20); (b) and (c) 7 days post-instillation. Giant cells are present with loose connective tissue forming a granuloma. (b) × 20; (c) × 10; and (d) 6 months post-instillation showing severe airway fibrosis (× 10).

GIFFORD, 1984), and IL-1 was measured using suboptimally stimulated thymocytes obtained from C3H mice (KUSAKA and DONALDSON, 1990).

Histology

Lungs from exposed animals were fixed in 10% formol saline, and processed for paraffin embedding. Sections were cut and stained with haematoxylin and eosin.

Statistical analysis

All experiments were repeated at least three times. Where indicated data were logarithmically transformed and analysed by two-way analysis of variance, experiments vs treatments, using the Minitab Statistical computer package. If there was a significant treatment effect detected by the *F*-test, individual differences were tested for significance using a *t*-test with a pooled estimate of standard error.

RESULTS AND DISCUSSION

The present study concentrated on the pro-inflammatory effects of dust collected from the air of wool mills, on the cells of the lung. The first cells that wool dust is likely to encounter after deposition are epithelial cells and macrophages. We first asked the question—is the wool mill dust toxic to these cells? There was no significant toxicity, although the positive control dust quartz was active in this respect toward both cell types (Figs 1 and 2).

In the absence of a toxic effect, the dust could cause inflammation by stimulating the alveolar macrophages to release pro-inflammatory cytokines, such as TNF or Interleukin 1. The two wool dusts were very active in causing release of TNF, as shown by a decrease in the viabiliy of the L929 cells, compared to macrophages incubated without wool mill dust (Fig. 3). Similar to TNF, the wool dust M was more active in causing macrophages to release IL-1 but it was also stimulatory (Fig. 4).

Since macrophages which have phagocytosed wool dust particles may migrate to the pulmonary lymph nodes, we investigated the activational state of the nodes themselves by measuring the responses of disaggregated lymph node lymphocytes to the mitogen PHA. The lymph node lymphocytes from rats which had been exposed to the wool dusts by instillation showed an enhanced proliferative respose to PHA (Fig. 5). This suggests that the alveolar macrophages, with increased secretion of immunostimulatory cytokines, could be migrating to the lymph node, stimulating the local lymphocytes in the node and giving rise to increased responsiveness to the mitogen. It is also possible that endotoxin or some other soluble mitogen was leaching from the wool mill dust and passing, in the lymphatic flow, to the lymph nodes and stimulating immunocompetent cells resident there.

Histological examination of tissue from animals instilled with the dusts revealed the potential of the wool mill dust to cause inflammation in the short term and, in the long term, to cause stimulation of fibroblasts leading to granuloma formation and fibrosis of airway walls (Fig. 6).

These results suggest that inflammation in the airways of workers exposed to wool mill dust is not likely to be caused by a direct toxic effect of the dust on the alveolar macrophages or epithelial cells lining the airspaces. It most likely arises as a result of macrophage activation, possibly involving endotoxin, and non-specific activation of

immune cells in the local lympohoid tissue. This could set up and sustain localized low-level chronic inflammation in airway walls.

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