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 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 review²) 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 fibres 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 inflamma-

tion in vivo⁹ 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

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 $>0.4\ \mu\text{m}$ 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 $0.6\ \mu\text{m}$ 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, Dudingon, 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 activation 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 previously¹²; 10^5 cells in 100 μl of RPMI 1640 + 10% fetal calf serum (Gibco; Paisley) were placed on $6 \times 22\ \text{mm}$ 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 $2.5\ \mu\text{m}$ 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 $>10\ \mu\text{m}$ 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 $>10\ \mu\text{m}$ 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 $6.76 \times 10^6 \pm 0.86$ macrophages and $0.06 \times 10^6 \pm 0.05$ neutrophils two days after injection.

Fig 1 Length distribution of long and short fibre amosite samples. (SEM sizing: $10\,000 \times$ magnification. Fibres longer than $0.4\ \mu\text{m}$ diameter less than $3\ \mu\text{m}$ 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.)

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

Fig 3 Numbers of macrophages lavaged from peritoneal cavity of mice injected with short amosite, long amosite, UICC amosite, or titanium dioxide at indicated doses two or four days previously.

comparisons; long, however, showed the most consistent, highly significant, increases in recruitment over TiO_2 , being $p > 0.001$ 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 TiO₂ in ability to cause macrophage recruitment. Significant differences relate to increases in the response with dust (a) compared with dust (b)

		Macrophages					
Dust (a)	(b)	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 TiO₂. Significant differences relate to increase in the response with dust (a) compared with dust (b)

		Neutrophils					
Dust (a)	(b)	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

Treatment	Cells $\times 10^6$	
	Macrophages	Neutrophils
Long amosite	23.25 (3.56)*	13.28 (3.71)
Short amosite	4.65 (0.56)	0.20 (0.02)

* $\times$ (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

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)

* $\times$ (SD).

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.

The results described here are complementary to those published³ 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 potential¹⁶; 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 macrophages²⁰ 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 asbestos²¹ combined with a generalised "promoting" effect during the proliferative phase of inflammation could favour neoplastic change.²²

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