

# The Advantages and Limitations of an *in Vivo* Test System for Investigating the Cytotoxicity and Fibrogenicity of Fibrous Dusts

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The acute response of the rat lung to a range of fibrous materials has been investigated by bronchopulmonary lavage, at dose levels of 0.5 and 1.0 mg, 1 and 7 days after their administration by intratracheal instillation. The materials chosen for study included UICC chrysotile A, amosite, crocidolite and anthophyllite, and samples of S. African "long" amosite and glass fiber. In addition, the subacute response to 1, 2 and 3 mg of chrysotile and amosite has been studied at 50 and 100 days after instillation. In the acute phase at 1 day after instillation, the response to chrysotile was greater than that to any of the other materials, but by 7 days there was no gradation in the response to different dusts. In the subacute phase, cell recoveries were low, and it was not possible to assess the long-term cytotoxic or fibrogenic effects of amosite and chrysotile by analyses of lung washes, even though biochemical and histological methods indicated gross changes in lung pathology.

## Introduction

Many test systems have now been developed which attempt to predict the fibrogenicity and tumorigenicity of mineral dusts. In particular, the hemolytic activity of these materials has been correlated with their ability to cause fibrosis *in vivo* (1) and more recently, the activity of a range of materials against V79-4 cells has been correlated with their tumorigenic potential (2). While these test systems can certainly tell us more about the mechanisms of interaction between the dusts and biological membranes (3), their validity as long-term predictive tests is still open to question.

In this laboratory, we have concentrated on the development of an *in vivo* system for investigating mineral dust cytotoxicity. The test materials are administered to rats by intratracheal instillation and the response of the lung assessed by bronchopulmonary lavage. Following the instillation of quartz and other mineral dusts into the lung, we have observed a marked influx of polymorphonuclear leukocytes

(PMN) into the alveolar spaces which reached a maximum at approximately 24 hr after dust administration. For cytotoxic materials such as bentonite and noncytotoxic materials (such as titanium dioxide) which are not fibrogenic, the acute inflammatory response of the lung was short-lived, and the free cell population of the lung returned to normal within a few weeks. For quartz, however, which is both cytotoxic and fibrogenic, the influx of PMN was followed by a subacute phase in which the inflammatory response persisted and was even exacerbated over a period of at least three months (4, 5). This prolonged inflammatory response is now known to occur both in experimentally induced fibrosis in animal models (6) and in patients with idiopathic pulmonary fibrosis (7).

In this study, the application of the test system to work with fibrous dusts has been investigated. First the acute response to a range of materials was assessed in an attempt to determine the relative cytotoxicities of the different dusts *in vivo* and, second, the subacute response to amosite and chrysotile was studied with a view to assessing the usefulness of intratracheal instillation and bronchopulmonary lavage for long-term toxicity testing of fibrous dusts.

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## Materials and Methods

### Instillation and Lavage

The animals were an inbred strain of rats from the MRC Radiobiological Unit, Harwell.

The materials chosen for study of the acute response were the UICC standard reference samples of chrysotile A, amosite, crocidolite and anthophyllite and a sample of Cape S. African amosite ("long" amosite), in which the average length of the fibers was 117  $\mu\text{m}$  and at least 63% fell within the range 50-150  $\mu\text{m}$ . In addition, a sample of glass fiber was included (TIMA) of nominal diameter 1.5  $\mu\text{m}$  and varying in length from 10  $\mu\text{m}$  to > 100  $\mu\text{m}$ . UICC chrysotile, UICC amosite and "long" amosite were also included in the study of the subacute response.

The method of preparation of the dusts and instillation were as described previously (8). For the acute response, each of the UICC materials was administered in a single instillation at doses of 0.5 mg and 1.0 mg. Long amosite and glass fiber were administered at a single dose only (0.5 mg and 1.0 mg, respectively). For the subacute response, chrysotile and amosite were instilled at doses of 1, 2 and 3 mg. These quantities were fractionated so that one-third of the dose was given in each of three instillations over a period of one week. Long amosite was instilled at the two lower doses, but at the top dose of 3 mg, there was significant mortality and the UICC sample was preferred.

Animals were sacrificed 1 and 7 days after instillation for studies of the acute response and 30, 50 and 100 days after instillation for studies of the subacute response. The method of bronchopulmonary lavage was as described by Morgan et al. (8). Lungs were lavaged four times with 8 mL of balanced salt solution (BBS, Gibco-Biocult) and ten times with 8 mL of physiological saline (PS).

### Analytical Methods

The methods of cell counting and differentiation were as described by Morgan et al. (8). Lactate dehydrogenase (LDH) activities and protein concentrations were assessed in the supernatants of the BSS washes only. LDH was assayed as described by Moores et al. (9). Protein concentrations were determined by the method of Lowry et al. (10).

### Lung Biochemistry and Histology

At 50 and 100 days after the instillation of chrysotile and amosite, lungs were assessed for signs of pathological changes by both biochemical and histological methods. The lungs were excised and lobes A, C, D and E removed and weighed. They were homog-

enized in 10 mM Tris-HCl (pH 7.6) and freeze dried. The dry weights were recorded and the freeze-dried extracts taken for measurements of total protein and hydroxyproline content. Hydroxyproline was assayed as described by Moores et al. (11).

For histological studies, lobe B of each lung was fixed by inflation with buffered formalin and embedded in paraffin wax. Sections 5  $\mu\text{m}$  thick were cut and stained with hematoxylin and eosin (H & E), reticulin and Van Gieson.

## Results

### Acute Response

The effects of the fibrous dusts on lung weights at 1 and 7 days after instillation are shown in Figure 1. To overcome the influence of variations in animal size, lung weights were calculated as percent of body weights. At both time points, the response to chrysotile was greater than that to the other dusts and showed some dependence on dose. The effects of anthophyllite and glass fiber were not significant when compared to saline controls, and by 7 days, only chrysotile- and amosite-treated animals showed any persisting response. There were no apparent differences in the effects of the two samples of amosite.

In Figure 2, the numbers of PMN recovered and the activity of LDH in the lavage fluids are shown at 1 day after instillation, and it is clear that there was some correlation between these two parameters. In both cases, the largest response was seen with chrysotile, but there was no evidence of any dose-dependence.

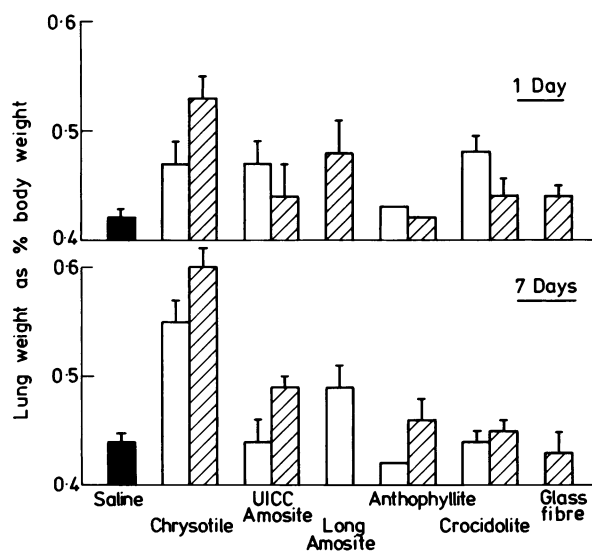


FIGURE 1. Changes in lung weights at 1 and 7 days after the instillation of 0.5 (□) or 1.0 mg (▨) of fibrous dusts. Values are means  $\pm$  SEM.

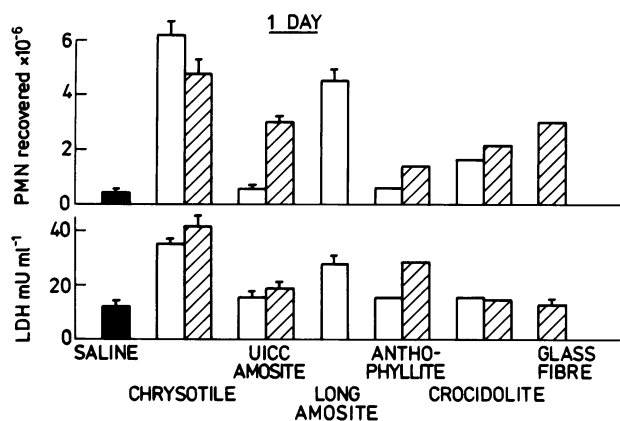


FIGURE 2. Numbers of PMN recovered and the activity of LDH in lavage fluids at 1 day after the instillation of 0.5 (□) or 1.0 mg (▨) of fibrous dusts. Values are means  $\pm$  SEM.

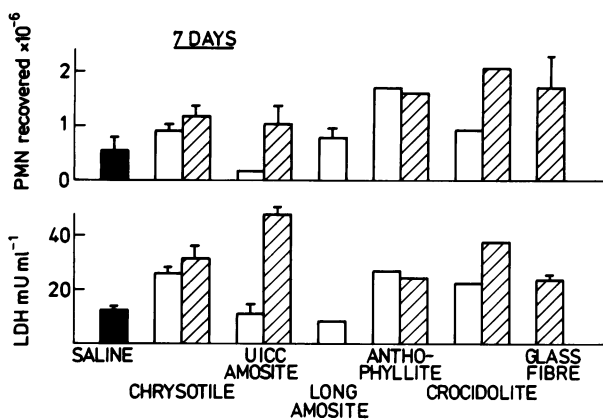


FIGURE 3. Numbers of PMN recovered and the activity of LDH in lavage fluids at 7 days after the instillation of 0.5 (□) or 1.0 mg (▨) of fibrous dusts. Values are means  $\pm$  SEM.

dence. At the 0.5 mg dose level, long amosite gave rise to a much larger influx of PMN than the same dose of the UICC material and, correspondingly, higher levels of LDH. Very little response to anthophyllite, crocidolite or glass fiber was detected.

Similar data at 7 days after instillation are presented in Figure 3. The numbers of PMN recovered from chrysotile- and amosite-treated animals had fallen to around  $1 \times 10^6$  cells, whereas numbers of PMN from animals given anthophyllite, crocidolite or glass fiber were approximately the same as at 1 day. Similar trends were seen in the results of the LDH measurements with the exception of 1 mg of UICC amosite, where LDH levels were around 44 milliunit (mU)/mL, compared to only 19 mU/mL at 1 day after instillation.

The numbers of macrophages recovered from the same animals are given in Table 1. There was no significant increase in macrophage numbers for any ma-

Table 1. Recoveries of alveolar macrophages (AM) at 1 and 7 days after the instillation of 0.5 mg or 1.0 mg of fibrous dusts (means  $\pm$  SEM).

Material	Numbers of AM recovered $\times 10^6$			
	1 day		7 days	
	0.5 mg	1.0 mg	0.5 mg	1.0 mg
Saline	9.5 $\pm$ 2.1	—	12.9 $\pm$ 2.6	—
Chrysotile	8.7 $\pm$ 0.8	5.4 $\pm$ 0.6	8.7 $\pm$ 1.5	7.5 $\pm$ 0.7
UICC amosite	10.7 $\pm$ 2.0	10.6 $\pm$ 0.6	10.5 $\pm$ 0.9	8.3 $\pm$ 0.1
Long amosite	12.9 $\pm$ 2.9	—	13.4 $\pm$ 1.3	—
Anthophyllite	11.9	8.4	16.1	12.2
Crocidolite	8.5 $\pm$ 0.1	8.2 $\pm$ 3.3	12.5 $\pm$ 1.9	14.4 $\pm$ 1.6
Glass fiber	—	10.3 $\pm$ 1.3	—	11.4 $\pm$ 1.1

terial at either time point. With one exception (1 mg crocidolite at 7 days) fewer cells were recovered from animals given a dose of 1 mg of each dust compared to those given only 0.5 mg.

### Subacute Response

The effects of chrysotile and amosite on lung weights are shown in Figure 4. For chrysotile, lung weights were elevated at all times compared to control values, and at the 3 mg dose level, this effect increased with time after instillation. For amosite, the results are complicated by the inability to use long amosite at the top dose. At the 1 mg dose level, lungs were heavier than those from saline controls but there was no change with time after instillation. At 2 mg, however, there was a dramatic increase in lung weights between 50 and 100 days after dust administration. Unfortunately, a similar increase using 3 mg of UICC amosite was not observed.

The recoveries of PMN and AM in the subacute response are shown in Figure 5. At all time points and all three dose levels, the numbers of PMN were very similar at about  $1-2 \times 10^6$  cells compared to values of  $<0.1 \times 10^6$  for saline controls. There was no evidence of any time or dose dependence in this response. The numbers of macrophages recovered were generally depressed compared to saline controls. For amosite, at 50 days, this depression increased with increasing amounts of dust instilled. For chrysotile, at doses of 1 and 2 mg, the recoveries of AM were not significantly lower than control values, but at the top dose, only about  $5 \times 10^6$  cells were washed out of the lung.

The results of measurements of LDH activities and protein concentrations in lavage fluid supernatants are given in Table 2. In all cases, levels of LDH were elevated compared to saline controls, but values were very similar for both dusts at all time points and all doses. The response to amosite was consistently greater than that to chrysotile. Similarly, protein levels were raised compared to con-

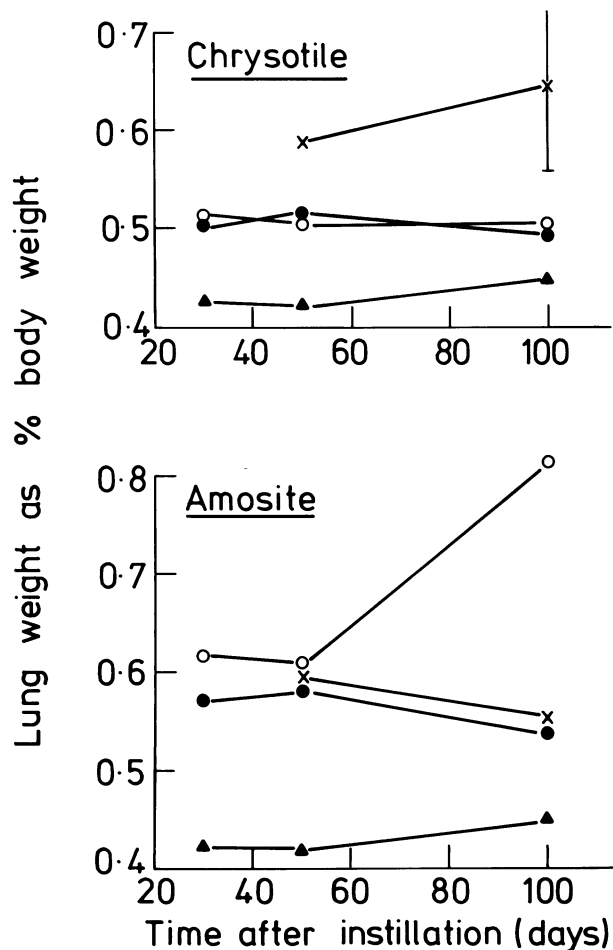


FIGURE 4. Effects of chrysotile and amosite on lung weights up to 100 days after instillation: (●) 1 mg; (○) 2 mg; (×) 3 mg; (▲) data for saline-instilled controls.

controls, but there was no evidence of dose dependence in this response or any significant change with time after instillation.

### Measurements on Lung Homogenates

The results of these measurements are shown in Figure 6. At both time points, the dry weights of the lungs from all experimental animals were increased compared to saline controls. This effect was more pronounced for chrysotile than for amosite, but for both dusts, there was no progressive response with time after instillation.

Levels of lung hydroxyproline were raised in experimental animals compared to controls, and in this case the response did increase with time after instillation. By 100 days, the hydroxyproline content of lungs from animals treated with either of the fibrous dusts had more than doubled compared to control values.

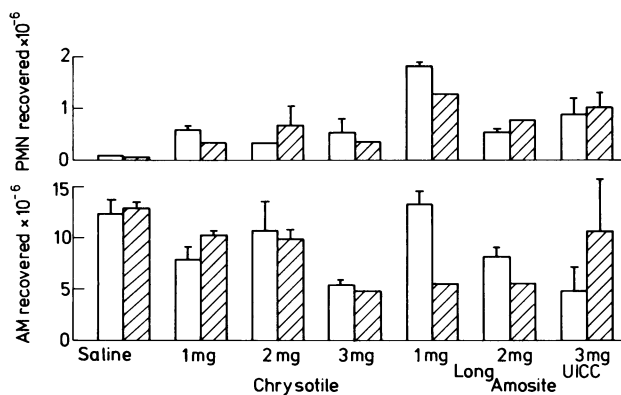


FIGURE 5. Numbers of PMN and AM recovered at 50 (□) and 100 (▨) days after the instillation of 1, 2 or 3 mg of chrysotile or amosite, compared to saline controls. Values are means ± SEM.

Table 2. LDH activities and protein concentrations in lavage fluid at 50 and 100 days after the instillation of 1, 2 and 3 mg of chrysotile and amosite (means ± SEM).

Material	Time, days	Dose, mg	LDH, mU/mL	Protein, μg/mL
Chrysotile	50	1	18.4 ± 2.3	105 ± 2
		2	12.8 ± 1.2	68 ± 2
		3	24.0 ± 0.1	122 ± 10
	100	1	15.8 ± 2.4	96 ± 4
		2	22.8 ± 1.2	87 ± 6
		3	27.4 ± 11.1	158 ± 36
Amosite	50	1	27.4 ± 2.6	96 ± 4
		2	22.5 ± 2.7	93 ± 3
		3	25.9 ± 5.9	115 ± 15
	100	1	36.6 ± 16	144 ± 26
		2	22.7 ± 1	109 ± 9
		3	34.4 ± 0.2	165 ± 47
Saline	50, 100	—	10.6 ± 2.1	73 ± 5

### Histology

At 50 days after the instillation of all three doses of chrysotile, there was some infiltration of macrophages into the alveolar spaces and a few PMN were present, mostly in the interstitium. At 100 days, no marked cellular infiltration was apparent. At both time points, and again for all doses of chrysotile, the most notable feature was the occurrence of nodules, mainly in the bronchioles and alveolar ducts, which appeared to have a full covering of epithelium around them. In some cases, they formed protrusions into the bronchioles and, in the terminal bronchioles, appeared to occlude the airways. At the higher doses, the nodules were larger and more obtrusive but did not appear to be more frequent. Within the epithelial layer, these nodules were also encapsulated by a layer of collagenlike fibrous material, enclosing mononuclear cells, some fibroblasts and a few

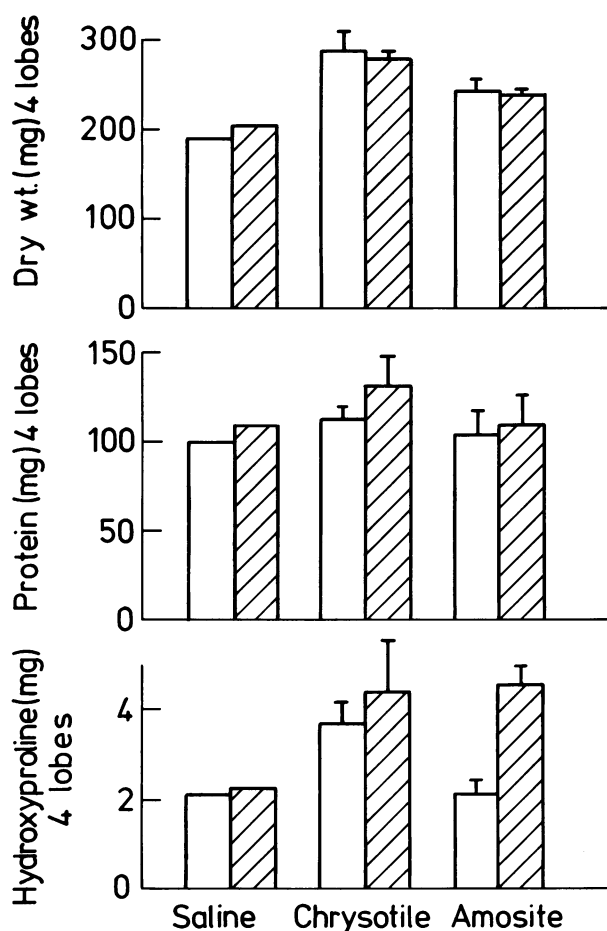


FIGURE 6. Effects of 3 mg chrysotile or amosite on lung dry weights and on protein and hydroxyproline contents at 50 (□) and 100 (▨) days after instillation. Values are means  $\pm$  SEM.

PMN. For chrysotile, it was not possible to detect the location of the fibers themselves.

For amosite-treated lungs, again there was some cellular infiltration at all doses and at 50 and 100 days after instillation, but this was not extensive. Similar nodules were observed as those described for chrysotile and in this case the amosite fibers were clearly visible within the nodules. A few fibers were also seen in the alveolar spaces. For animals given 2 mg of long amosite, these nodules were very large, and there was massive reticulin staining. In comparison, the effects of 3 mg of UICC amosite were qualitatively similar but less extensive.

## Discussion

At 1 day following instillation, the acute response to the fibrous dusts was greatest for chrysotile, but was very similar for amosite, anthophyllite, crocidi-

lite and glass fiber. Compared to quartz (5), the response to chrysotile was similar in terms of effects on lung weights and LDH levels, but the numbers of PMN recovered were less than 50% of those recovered from quartz-treated animals. There was no advantage in the use of doses of 1 mg rather than 0.5 mg of the dusts, agreeing with previous data (5), in which it was shown that it is still possible to detect and quantify changes in cell recoveries and enzyme activities at low doses, thus avoiding the dangers of saturation of the lung response. In the same report, it was suggested that the size of the acute response to mineral dusts *in vivo* may reflect the extent of interaction between each material and cell membranes. Since the hemolytic activities of mineral dusts are also reliant on these kinds of interactions, then there should be some correlations in the results of the *in vivo* and *in vitro* test systems. On using measurements of "free" LDH in lavage fluids as an index of *in vivo* cytotoxicity, from this study and results reported previously (5) the cytotoxicity of the mineral dusts decreases in the order: bentonite > chrysotile and quartz > amphiboles and glass fiber. The corresponding order for hemolytic activities (12) is bentonite > chrysotile > quartz > anthophyllite > amosite, and crocidolite, with there being very little difference between values for amphiboles. More recently, in a study by Woodworth et al. (3), the cytotoxicities of different dusts were shown to decrease in the order montmorillonite > chrysotile > quartz. The only apparent difference therefore is the equivalence of quartz and chrysotile in *in vivo* testing and the greater cytotoxicity of chrysotile than quartz in *in vitro* systems. This may relate to the differences in surface charge between these two dusts (13), resulting in the modification of their properties *in vivo* to different extents.

Finally, the results of the acute study show that the correlations between *in vivo* and *in vitro* cytotoxicity tests are closer when LDH measurements are used as the *in vivo* parameter, rather than numbers of PMN recovered. This suggests that the properties determining *in vivo* cytotoxicity may not be the same as those which influence the extent of chemotactic attraction for PMN into the lung.

In the study of the subacute response to chrysotile and amosite, the results from bronchopulmonary lavage suggest that only limited areas of the lung are being sampled successfully and that no real assessment of the processes going on in the lung is being made. This is important, since it questions the validity of using intratracheal instillation and lavage for monitoring the long-term damage to the lung from fibrous dusts. In other studies, using similar techniques, Schoenberger et al. (14) have looked at the effects of 25 mg of chrysotile in the guinea pig lung,

and Pickrell et al. (15) have looked at the response of the hamster lung to crocidolite and glass fiber. In both cases, the use of bronchopulmonary lavage to monitor the lung responses appeared satisfactory, although Pickrell et al. did comment on the limitations of the instillation technique and the possibilities of local overdosing.

In addition, a detailed study of the advantages and limitations of intratracheal instillation has recently been published from this laboratory (16). The main recommendations of this report included the use of sonicated dust suspensions to ensure complete delivery, the use of multiple instillations to achieve optimum dust distribution and the limitations on the doses administered to a maximum of 0.6 mg/g fresh weight of lung and in a volume not exceeding 0.3 mL/g to avoid excess stress to the animals or even fatalities.

However, even following these precautions, the results of histological analyses in the present study have shown that there are significant accumulations of fibers and cellular material both in the bronchioles and alveolar duct regions. These may arise either as a result of poor distribution of the dust when instilled or as a result of the attempts of the lung clearance mechanisms to remove the insult from the alveolar regions. The result of these accumulations is the blockage of certain areas of the lung so that they are not effectively sampled by lavage. In addition, AM and PMN have been observed concentrated around long fibers in lung washings, and since the recovery of these fibers from the lung is low, they may exaggerate the retention of free cells within the lung spaces during lavage. It is possible therefore that the shortcomings of the methodology may lie mainly with the use of bronchopulmonary lavage as a sampling technique rather than with the use of intratracheal instillation for dust administration.

The biochemical and histological measurements that we have reported indicate that the fibrogenicity of the materials chosen decreases in the order long amosite > UICC chrysotile < UICC amosite. This is in agreement with the findings of other workers who have commented on the increased fibrogenicity of asbestos dusts with increasing fiber length. Recently, Brown et al. (2) emphasized the importance of fiber length in a study of the effects of amphiboles on V79-4 cells. They proposed a threshold length of 6.5  $\mu\text{m}$ , below which the cytotoxicity of the fibers falls abruptly, and on this basis they estimated that approximately 30% of fibers in the UICC sample of amosite would be active. Whether a similar threshold is operative *in vivo* is not known.

In conclusion, although the techniques of instillation and lavage have proved useful for investigating the immediate cytotoxicities of fibrous dusts *in vivo*,

they do not appear to be adequate in assessing long-term effects. Many of the difficulties may arise from the limitations of the lavage technique rather than those of instillation, and it is difficult to visualize a successful method of sampling the affected lungs where fibrotic changes are localized in such obstructive and discrete nodules as are found in association with fibrous dusts.

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