

An Assessment of the Fibrogenic Potential of Very Short 4T30 Chrysotile by Intratracheal Instillation in Rats¹

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Three groups of five rats each received, respectively, a single intratracheal instillation of saline (control), 5 mg of UICC chrysotile B asbestos, and 5 mg of a preparation of very short chrysotile fibers (4T30, 100% < 8 μ m) isolated by a sedimentation procedure. At various intervals after the treatment (1 to 60 days), assessment of lung morphology was performed on each animal. Although the two types of chrysotile fibers have similar chemical composition, structure, and surface charge, the lung tissue reaction differed considerably. Lungs of animals exposed to UICC chrysotile B showed significant pathological alterations as early as 7 days following treatment. The lesions were localized in and around terminal bronchioles and consisted of inflammatory cells, fibroblasts and collagen deposition which distorted and obstructed small airways. Reaction to very short 4T30 chrysotile fibers was quite distinct. Seven days after treatment, lungs of these animals showed alveolar and interstitial accumulation of inflammatory cells. The alveolitis persisted 60 days after treatment and no fibrosis was apparent. It appears that very short 4T30 chrysotile fibers are much less fibrogenic than UICC chrysotile B and that intratracheal instillations in rats may represent a useful mean of rapidly assessing the fibrogenic potential of various dusts. These observations support the concept that fiber length is an important factor for fibrogenicity of asbestos. © 1985 Academic Press, Inc.

INTRODUCTION

In vitro models have been extensively used to study the biological activity of asbestos and different pollutant particles. In many instances, however, the observed *in vitro* effects of asbestos did not closely correlate with its fibrogenic and tumorigenic potential *in vivo* (Harrington *et al.*, 1975) mostly because important mechanisms of physiological regulation cannot be duplicated in culture. Whereas *in vitro* assays have given a number of clues concerning the mechanism of action of asbestos at the cellular level, *in vivo* experimentation is more likely to give definite answer about the fibrogenicity of any given material. Inhalation experiments have been used to examine the fibrogenic potential of dusts and this method provides a physiological model system allowing for the natural clearance of inhaled minerals. However, chronic exposure of animals as performed in inhalation studies often requires months of experimentation and necessitates elaborate and expensive facilities. For these reasons, this approach is less suitable for studying the molecular processes associated with the onset of fibrosis and for the com-

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Very Short 4T30 in Rats¹

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ison of large numbers of different particles. For these purposes, intratracheal
illation offers an interesting compromise since it is rapid, easily performed,
satisfactory in reproducing most of the main histopathological features of
rosis in various animal models (Burger and Engelbrecht, 1970; Wright and
schner, 1977; Bégin *et al.* 1983).

Previous work has suggested that the fibrogenic potential of asbestos is influ-
enced by the length of the fiber (Beck *et al.*, 1971, Wright and Kuschner, 1977).
In this regard, various investigators have reported that short fibers were either
fibrogenic (Holt and Mills, 1963; Holt *et al.*, 1964), less fibrogenic than long
fibers of the same chemical composition (Klosterkötter, 1968; Burger and En-
gelbrecht, 1970; Davis *et al.*, 1978), or not fibrogenic (Gross, 1974). Many of
these studies were performed with fibers of diverse origin or fibers prepared in
such a way as to modify their physicochemical properties (Langer *et al.*, 1978).
Recently, very short chrysotile fibers were isolated from bulk 4T30 chrysotile
asbestos by a sedimentation procedure that does not modify the structure of the
fiber fibers (Jolicœur *et al.*, 1981). The present study was undertaken in order
to determine the fibrogenic potential of this more homogeneous preparation of
very short chrysotile using Union International Contre le Cancer (UICC) standard
reference sample of chrysotile B as a control fibrogenic material. The second aim
of this investigation was to evaluate the usefulness of intratracheal instillations
in rats for reproducing rapidly the histopathological lesions caused by various
aspirable dusts and particles and for assessing their relative fibrogenicity.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 250–300 g were purchased from Charles
River Canada Inc. (St.-Constant, Québec).

Asbestos fibers. UICC standard sample of chrysotile B was obtained from the
National Research Institute for Occupational Diseases, Johannesburg, South Af-
rica. Very short 4T30 fibers were isolated from Quebec 4T30 chrysotile according
to the sedimentation procedure of Jolicœur *et al.* (1981). Each sample was au-
toclaved for 45 min and suspended at a concentration of 10 mg/ml in sterile
phosphate-buffered saline (PBS, pH 7.4) with a Dounce glass homogenizer prior
to instillation into the animals.

Experimental protocol. All injections were made by the transtracheal route.
Rats were lightly anesthetized with a mixture of ketamine/xylazine (85/15, 100
mg/kg). The trachea was exposed surgically and the asbestos suspension or saline
was briskly injected through a 20-gauge needle in a final volume of 0.5 ml. Three
groups of animals received, respectively, a single intratracheal injection of saline
(control), UICC chrysotile B (5 mg), and very short 4T30 chrysotile (5 mg).
Animals in each group were sacrificed at 1, 7, 14, 21, and 60 days after instillation.
Because of the number of animals which could be handled experimentally in any
day, five sets of experiments were done, each corresponding to a given time
point.

Lung morphology. A median longitudinal section of the upper left lobe (1 mm
thick) was fixed by immersion in a phosphate-buffered 4% formaldehyde–1%
glutaraldehyde solution for histologic examination. After fixation, lung tissue was

embedded in paraffin and representative sections 5 μm thick were cut and stained with hematoxylin-eosin.

RESULTS

Properties of injected materials. The preparation of 4T30 chrysotile used in our study did not contain fibers longer than 8 μm and 98% were smaller than 3 μm . The surface charge of these fibers was +40 mV as represented by the potential at pH 7.4 and their specific surface area equal to 38 m^2/g (Table 1). The chemical and structural properties of very short 4T30 chrysotile fibers (Jolicoeur *et al.*, 1981) were not significantly different from those reported for UICC chrysotile B (Timbrell, 1970). Thus, the major differences between these two types of chrysotile asbestos reside in their fiber length and specific surface area and this is best illustrated in Fig. 1. Very short 4T30 asbestos fibers are well dissociated and give homogeneous and stable suspensions which are more easily instilled into rats than UICC chrysotile B suspensions.

Survival and incidence of histopathological lesions. There was no significant difference in survival between animals treated with the two asbestos samples and all animals survived the exposure period. When the average weights of animals in the different groups were considered, however, some differences were noticeable at Days 14 and 21 following exposure. Rats exposed to chrysotile B asbestos averaged lower body weight (284–288 g) than animals injected with saline (324–329 g) or very short 4T30 chrysotile fibers (308–311 g). This difference was no longer apparent at 60 days after exposure. A summary of the histological findings is given in Table 2. Histological changes which could be related to treatment were seen at the 7-day interim and thereafter. Descriptions of typical lesions are given below and almost all animals in a particular group were affected.

Saline instillation. Rats injected with saline showed normal lung morphology.

TABLE I
PROPERTIES OF INJECTED MATERIALS

	Short 4T30 chrysotile	UICC chrysotile B
Length	<0.5 μm , 50% <3 μm , 98% <8 μm , 100%	>2 μm , 100% >5 μm , 42% >10 μm , 21%
Specific surface area (m^2/g)	38	26.8 ^b
Surface charge (mV, pH 7.4)	+40	+36
Fe	2.5% ^a	2.6% ^b
MgO	40.8% ^a	32.0% ^b
X-ray diffraction (d)	7.39 ($\text{\AA}$) ^a 4.56 3.65 2.44 1.53	7.36 ($\text{\AA}$) ^b 4.55 3.66 2.46 1.54

^a As reported by Jolicoeur *et al.* (1981).

^b As described by Timbrell (1970).

1. Electron micrograph of chrysotile dispersed with Do

k were cut and stained.

4T30 chrysotile used in the study were smaller than 3 μm , as represented by the BET surface area of 38 m^2/g (Table 1). The UICC chrysotile fibers (Jolicoeur) reported for UICC chrysotile B are well dispersed between these two types of fibers, which are more easily

There was no significant difference in the body weights of animals between the two groups. Differences were noticed in the body weights of animals injected with saline (324 g) and those injected with chrysotile B (324 g). This difference was not significant. The histological findings related to treatment were similar. Typical lesions are given in Table 2. Normal lung morphology

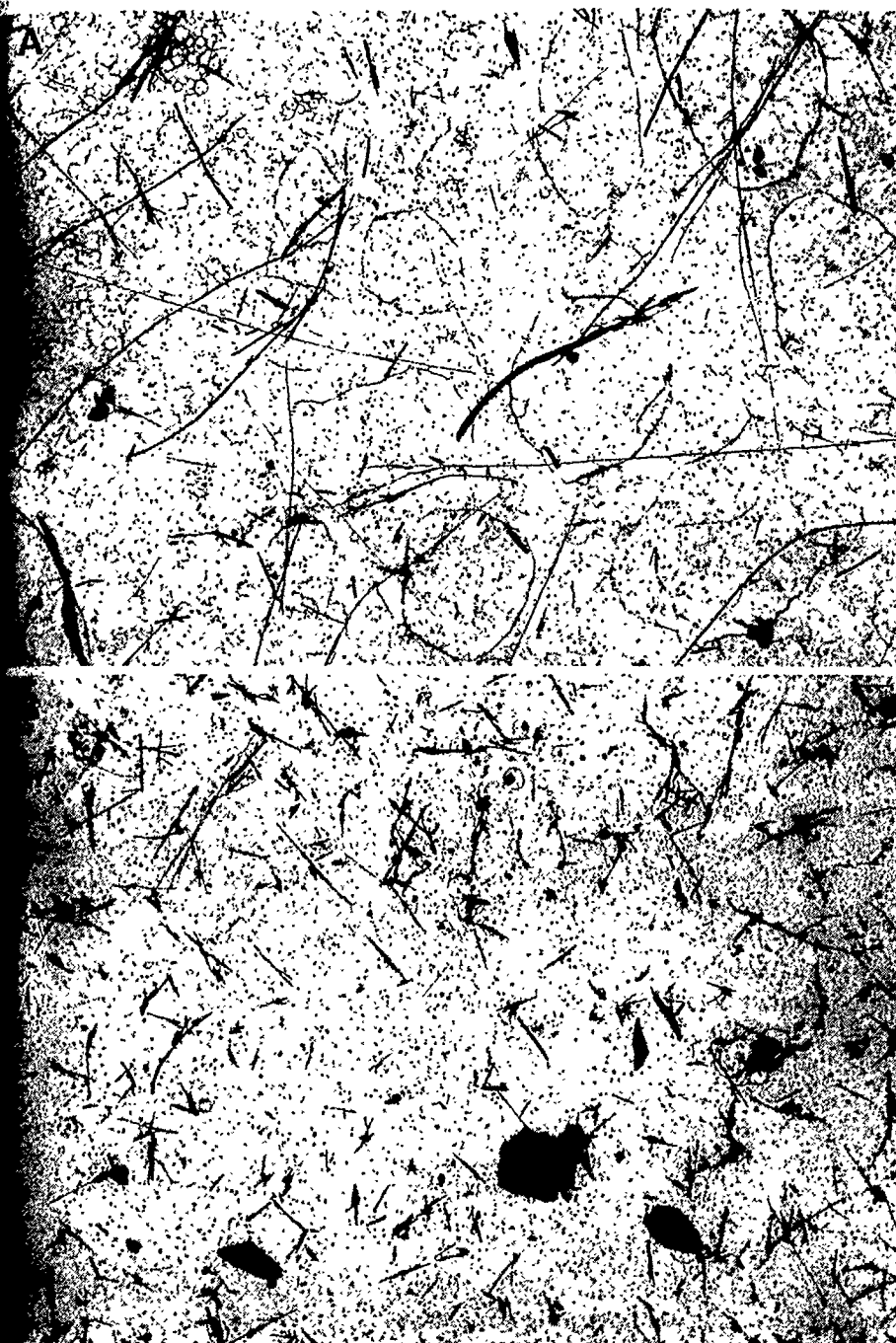


Fig. 1. Electron micrographs of suspensions of (A) UICC chrysotile B and (B) very short 4T30 chrysotile dispersed with Dounce homogenizer ($\times 2100$).

UICC chrysotile B
>2 μm , 100%
>5 μm , 42%
>10 μm , 21%
26.8 ^b
+36
2.6% ^b
32.0% ^b
7.36 ($\text{\AA}$) ^b
4.55
3.66
2.46
1.54

TABLE 2
SUMMARY OF NUMBERS OF ANIMALS WITH HISTOLOGICAL LESIONS

Treatment	Days postinjection	Number of animals examined	Mean body wt (g)	Number of animals with histopathological lesions	
				Alveolitis	Bronchiolitis obliterans
Saline	1	5	279 ± 14	0	0
	7	6	278 ± 14	0	0
	14	5	324 ± 5	0	0
	21	6	329 ± 11	0	0
	60	2	397 ± 12	0	0
UICC chrysotile B	1	5	275 ± 8	0	0
	7	5	250 ± 21	0	4
	14	6	288 ± 5*	0	6
	21	6	284 ± 14*	0	5
	60	5	401 ± 15	0	5
Very short 4T30	1	5	281 ± 7	0	0
	7	6	278 ± 3	6	0
	14	5	308 ± 5	5	0
	21	6	311 ± 9	5	0
	60	4	416 ± 13	4	0

* Significantly different from control ($P < 0.05$).

at each time point studied. Typical lung histopathology of these animals with normal terminal bronchioles, alveolar ducts, and alveoli is shown in Fig. 2.

UICC chrysotile B instillation. The rat lung reacted rapidly to chrysotile fibers and significant lesions appeared within 7 days in lungs of animals exposed to 5 mg of UICC chrysotile B (Fig. 3B). Lesions found at 21 days (Fig. 3C) and 60 days (Fig. 3D) following instillation were not significantly different from those observed earlier (7 and 14 days). No attempt was made to quantify the number or extent of fibrotic lesions and their distribution at various intervals following exposure although lung sections of animals sacrificed at 60 days showed, upon examination, a higher incidence of fibrotic foci (an average of 10 compared to 4 at Day 7 and none at Day 1). Distal lung structures were more affected and the predominant lesions were located in and around terminal bronchioles. These were characterized by focal fibroblastic proliferation in peribronchiolar tissues which distorted and obstructed small airways (Fig. 4A). These features of peribronchiolitis and bronchiolitis obliterans were seen at 7 days and thereafter in all animals exposed to UICC chrysotile B (Fig. 3A-D). Some lesions were located at bifurcations of the distal airways (Fig. 4B) and consisted of granulation tissue with fibroblastic proliferation (Fig. 4C).

Very short 4T30 chrysotile instillation. Animals treated with very short 4T30 asbestos fibers had the following pattern of lung histological changes: Within 7 days lesions appeared as focal areas of interstitial inflammatory reactions consisting of mononuclear cells (Fig. 5B). Intraalveolar accumulation of mononuclear cells was seen (Fig. 6A) and small sheets of packed mononuclear cells began to obliterate alveolar lumena, resulting in multifocal septal thickening and alveolar

Fig. 2. Lung histopa
airways and alve
s and sacs (B, ×11

LUNG LESIONS

Number of animals with
histopathological lesions

Alveolitis	Bronchiolitis obliterans
0	0
0	0
0	0
0	0
0	0
0	0
0	4
0	6
0	5
0	5
0	0
6	0
5	0
5	0
4	0

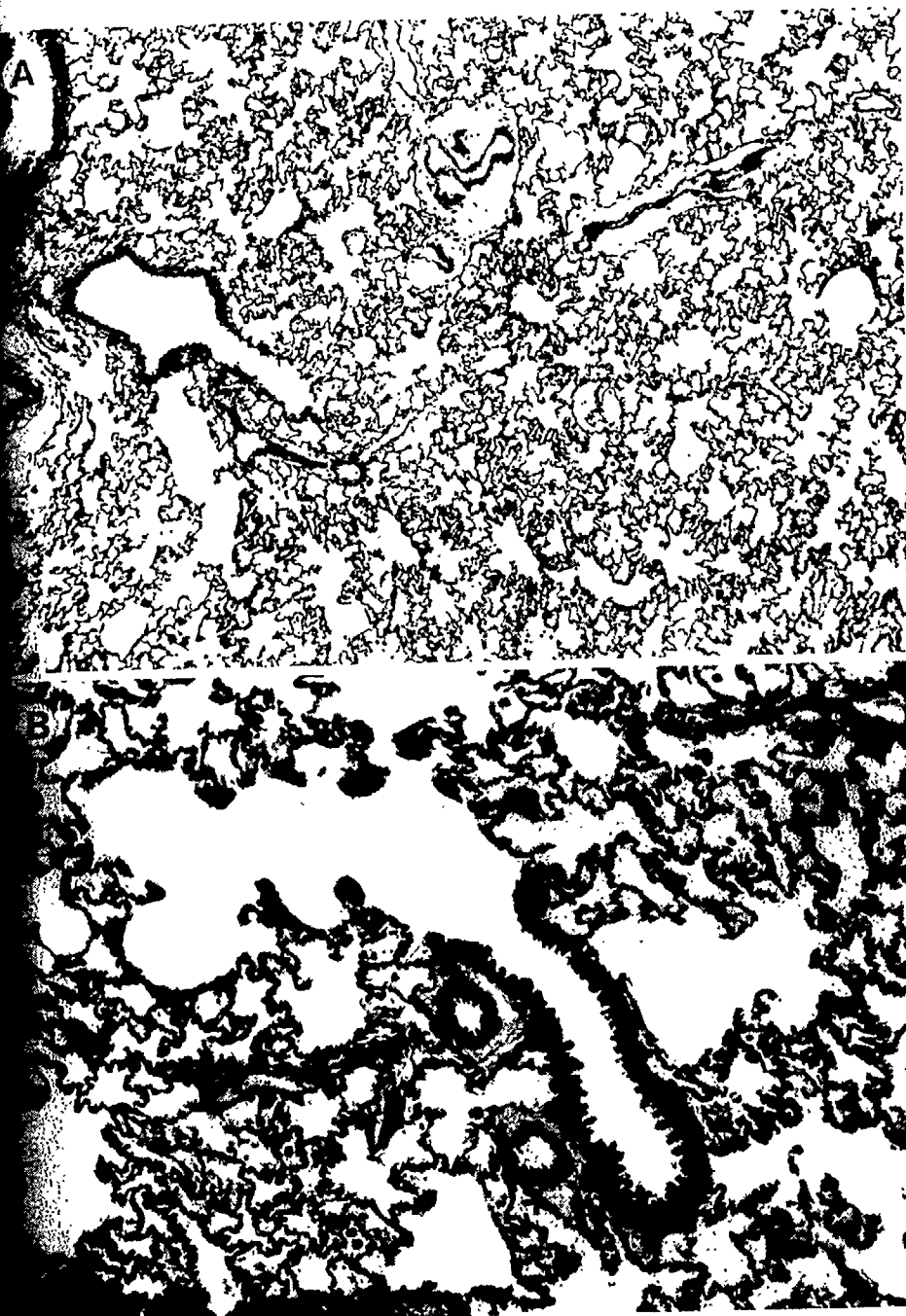


FIG. 2. Lung histopathology of saline-treated rats showing only perivascular edema with normal airways and alveoli (A, $\times 46$) and normal terminal and respiratory bronchioles with alveolar ducts and sacs (B, $\times 117$).

gy of these animals with li is shown in Fig. 2. apidly to chrysotile fibers, s of animals exposed to 5 21 days (Fig. 3C) and 60 ntly different from those le to quantify the number; arious intervals following at 60 days showed, upon erage of 10 compared to 4 ere more affected and the l bronchioles. These were r bronchiolar tissues which eatures of peribronchiol- d thereafter in all animals ions were located at bifur- of granulation tissue with

ated with very short 4T30 ological changes: Within 7 lammatory reactions con- umulation of mononuclear nononuclear cells began to tal thickening and alveolar

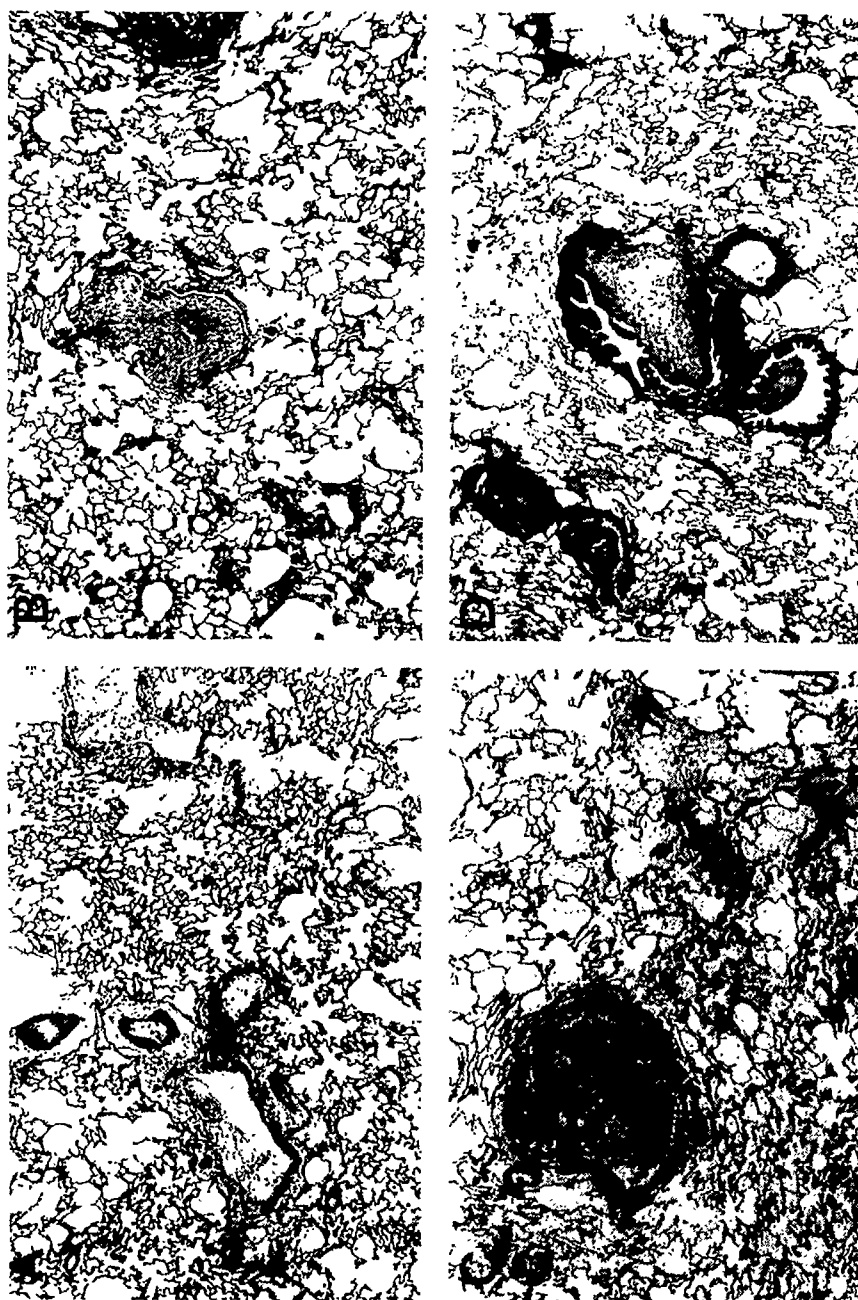


FIG. 3. Time course of lung histopathological changes following intratracheal instillation of UICC chrysotile B. No significant change was seen 1 day after exposure (A). Severe small airway distortion and stenosis by granulation tissue and fibroblastic proliferation were observed at 7 (B), 21(C), and 60 days (D) following instillation. (A-B, $\times 44$; C, $\times 73$).

FIG. 4. Fibrotic lesion in lung tissue ($\times 91$). Resulting stenosis.

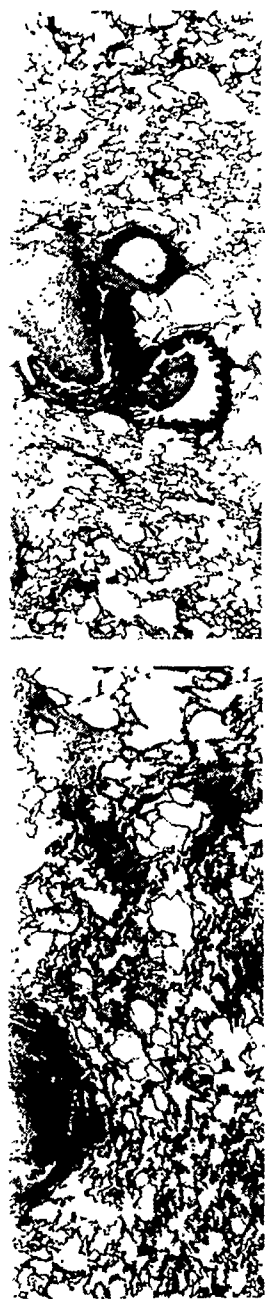


FIG. 3. Time course of lung histopathological changes following intratracheal instillation of UICC chrysotile B. No significant change was seen 1 day after exposure (A). Severe small airway distortion and stenosis by granulation tissue and fibroblastic proliferation were observed at 7 (B), 21 (C), and 60 days (D) following instillation. (A, B, C, $\times 44$; D, $\times 28$).



FIG. 4. Fibrotic lesions caused by UICC chrysotile B in rat lungs. (A) Bronchiolitis obliterans by fibrous tissue ($\times 91$). (B) A small airway bifurcation reveals thickening and fibroblastic proliferation resulting in stenosis ($\times 44$). (C) High-power view of Fig. 4B ($\times 112$).

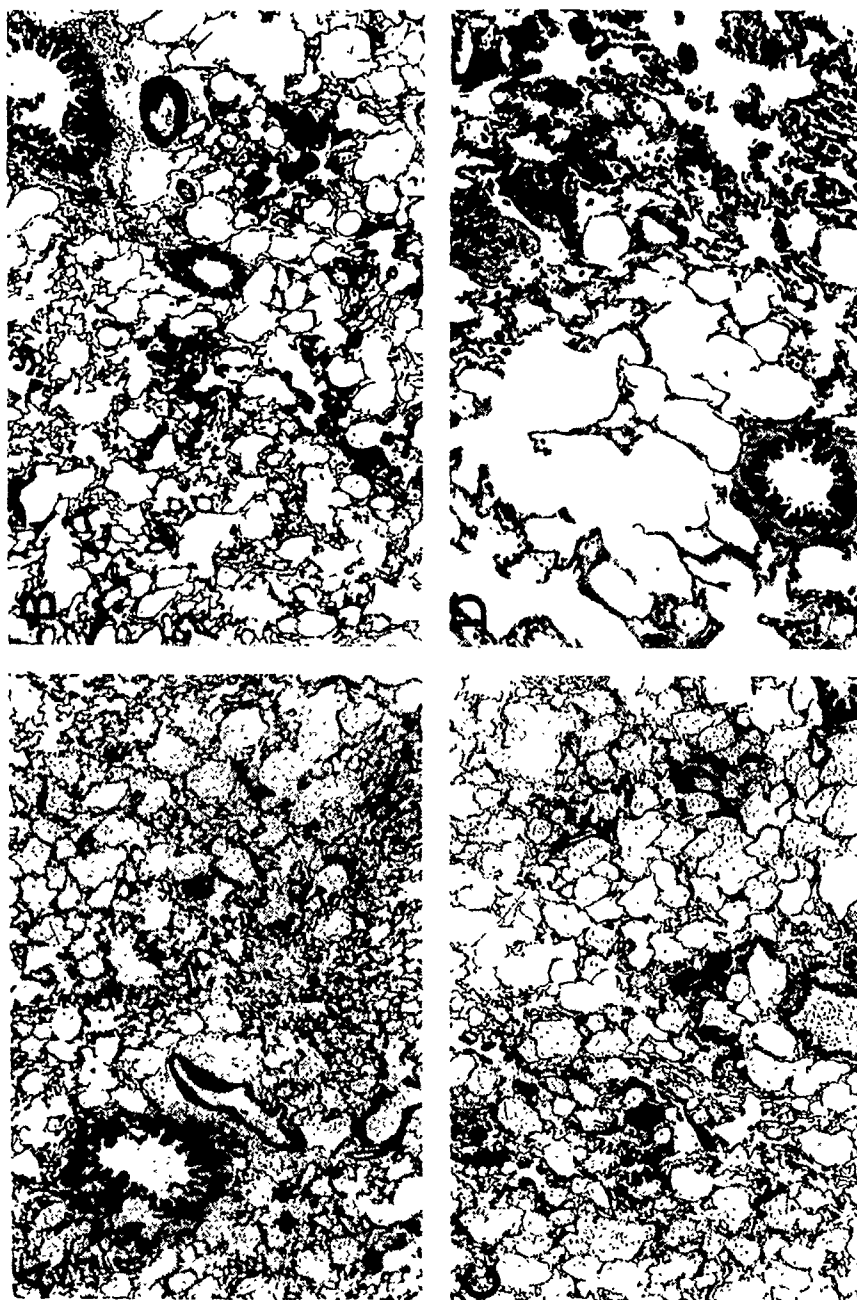


Fig. 5. Lung tissue reaction to intratracheal instillation of very short 4T30 chrysotile fibers. Only minimal alteration of alveolar septa with normal airways were seen 1 day after exposure (A). Multifocal septal thickening and alveolar distortion caused by interstitial mononuclear cell infiltration were observed at Days 7(B), 21(C), and 60(D) after instillation. The small airways are normal. (A, B, C, $\times 44$; D, $\times 91$).



Fig. 6. Representative macrophage accumulation and septal thickening observed at Day 7 (A) after instillation ($\times 44$).

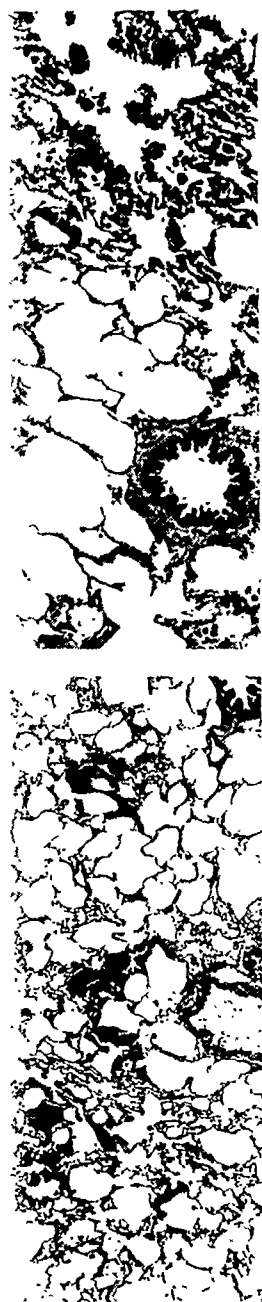


FIG. 5. Lung tissue reaction to intratracheal instillation of very short 4T30 chrysotile fibers. Only minimal alteration of alveolar septa with normal airways were seen 1 day after exposure (A). Multifocal septal thickening and alveolar distortion caused by interstitial mononuclear cell infiltration were observed at Days 7(B), 21(C), and 60(D) after instillation. The small airways are normal. (A, B, C, $\times 44$; D, $\times 91$).

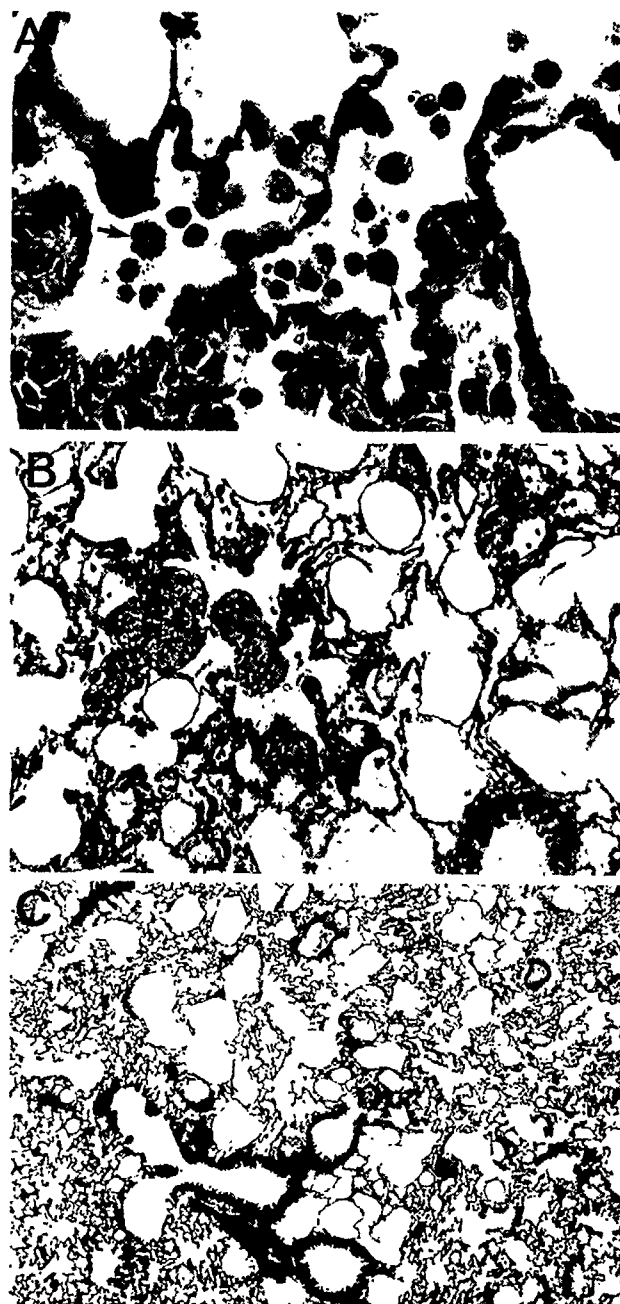


FIG. 6. Representative histopathology of lungs of rats exposed to very short 4T30 chrysotile fibers. (A) Macrophage accumulation in alveolar spaces ($\times 367$). Note binucleate cells (arrows). (B) Multifocal septal thickening by mononuclear cell infiltrates ($\times 112$). (C) Terminal airway with normal binucleate cells ($\times 44$).

distortion (Fig. 6B). No evidence of collagen deposition was found. In all animals the small airways were normal (Fig. 6C) indicating that the lesions were confined to the alveolar structures. The alveolitis was still present on Days 21 and 60 (Fig. 5A-D) and the extent of lung injury did not resolve during that period.

DISCUSSION

In the present study a preparation of very short 4T30 chrysotile fibers was tested in comparison with UICC chrysotile B asbestos for its ability to induce lung fibrosis. Using the rat as an animal model and intratracheal instillation as the method of exposure, we have demonstrated that the lung reactions to very short 4T30 chrysotile and UICC chrysotile B differed considerably both in nature and localization. While the predominant pulmonary lesion produced by very short 4T30 chrysotile was minimal in character consisting of mononuclear cells, few giant cells, and no fibrosis, UICC chrysotile B caused severe fibrotic lesions of terminal bronchioles. Moreover the lesions found in chrysotile B-treated rats were located in and around terminal bronchioles with some present at small airway bifurcations. By contrast very short 4T30 chrysotile fibers affected predominantly the alveolar structures. These findings are consistent with previous suggestions (Allison, 1974) that long chrysotile fibers are deposited by interception mainly at bifurcations of smaller bronchioles due to their stretched and curved form while shorter fibers penetrate more deeply into the lungs and reach the alveolar compartment.

Our results also demonstrate that 2 months after exposure, there was still no evidence of a progressive fibrotic reaction in rats exposed to very short 4T30 chrysotile. The 2-month observation period chosen may appear to be a relatively short time scale to assess the fibrogenicity of asbestos dusts. Nevertheless it differentiates rapidly the lung reactions to these two chrysotile samples. Whether very short 4T30 chrysotile fibers will produce lung fibrosis at a later period or at a higher dose is unknown but is currently under investigation. Very short 4T30 asbestos fibers have been reported to have hemolytic (Pelé and Calvert, 1983) and cytotoxic activities (Kimmerle *et al.*, in preparation) and to alter lung fibroblast DNA synthesis (Lemaire *et al.*, 1982) although to a lesser extent than UICC chrysotile B asbestos. Extrapolation of these *in vitro* observations to predict a possible fibrogenic effect of very short 4T30 fibers after a longer period of observation is difficult, especially since there is still controversy concerning the correlation of hemolytic and cytotoxic potential with fibrogenicity (Robock and Klosterkötter, 1973; Harington *et al.*, 1975).

The use in our study of a preparation of chrysotile with well defined fiber length and unchanged chemical composition brings additional support to the concept that fiber length is an important factor for fibrogenicity (Harington *et al.*, 1975; Davis *et al.*, 1978). Our observations that small-sized 4T30 chrysotile fibers, in spite of their large surface area, are less fibrogenic than chrysotile B further suggest that fibrogenicity is related to fiber length but not to the specific surface area of the asbestos dusts.

Although various animal models (Holt *et al.*, 1964, 1965; Vorwald *et al.*, 1951; Wagner *et al.*, 1974; Davis *et al.*, 1978; Davis, 1963; Goldstein *et al.*, 1978) have

been used to reproduce the best available model of the bioeffects of a large amount of a single intratracheal instillation similar to that of asbestos (Davis *et al.*, 1974; Davis and Goldstein, 1978; Goldstein and Davis, 1978). A striking process with the application of asbestos (Davis *et al.*, 1964, 1965) as soon as 14 days after exposure. This phenomenon is probably a model but rather to the amount of chrysotile instillation at bifurcations of the bronchioles. The normal connective tissue density of asbestos is due to low asbestos concentration. The results of our study over a long time span in rats confirm the fundamental role of asbestos in the development of fibrosis. This is a relative fibrogenicity of the fibers, and many other various respirable fibers have a relative importance in the fibrogenicity of asbestos.

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n was found. In all animals the lesions were confined to the lung on Days 21 and 60 (Fig. 1) during that period.

4T30 chrysotile fibers was chosen for its ability to induce fibrosis after intratracheal instillation as compared to the lung reactions to very short fibers. The reaction produced by very short fibers consisted of mononuclear cells, few macrophages and severe fibrotic lesions of the lung. In chrysotile B-treated rats were present at small airway bifurcations, fibers affected predominantly the alveolar septa with previous suggestions that fibers are intercepted mainly at the bifurcation and curved form while short fibers can reach the alveolar com-

mon. After exposure, there was still no evidence of fibrosis in rats exposed to very short 4T30 fibers. It may appear to be a relatively insensitive test system for asbestos dusts. Nevertheless, in the present study, chrysotile samples. Whether the test system for fibrosis at a later period or at an earlier period. Very short 4T30 fibers (Pelé and Calvert, 1983) did not induce fibrosis (Pelé and Calvert, 1983) and to alter lung fibroblasts to a lesser extent than UICC chrysotile. In vitro observations to predict a correlation between the effect after a longer period of observation. The controversy concerning the relative fibrogenicity (Robock and

Robock, 1975) with well defined fiber length and diameter. The support to the concept of relative fibrogenicity (Harrington *et al.*, 1975; Goldstein *et al.*, 1978) showed 4T30 chrysotile fibers, in contrast to chrysotile B further support to the concept but not to the specific surface

area (Goldstein *et al.*, 1978) have been used to reproduce the fundamental lesion of asbestosis, the rat was found to be the best available model (Davis, 1979) for studies aiming at the assessment of the bioeffects of a large number of particulate materials. In our rat model exposed to a single intratracheal instillation of UICC chrysotile B, the lung reaction is quite similar to that seen in chronically exposed rats (Holt *et al.*, 1964; Wagner *et al.*, 1974; Davis *et al.*, 1978), guinea pigs (Davis, 1963; Holt *et al.*, 1965), or hamsters (Goldstein *et al.*, 1978) and is best characterized by peribronchiolar fibrosis. A striking result of our investigation is the rapid onset of the fibrotic process with the appearance of well defined fibrotic lesions within 7 days. Holt *et al.* (1964, 1965) also reported the appearance of fibrosis in rats and guinea pigs as soon as 14 days following inhalation of chrysotile asbestos. Therefore, this phenomenon is probably not related to the method of exposure or the animal model but rather to the type and the concentration of fibers used. It may be that the amount of chrysotile B fibers together with their length and preferential localization at bifurcation sites result in significant accumulation of long fibers at discrete areas of the lung which in turn leads to an acute irritation process with normal connective tissue response and fibrosis. Because of the mode and intensity of asbestos exposure, our data do not relate to the very early lung reactions to low asbestos inhalation exposure in humans and animals. Furthermore, the results of our study do not suggest that short fibers cannot be fibrogenic over a long time span in man. Rather the rat model described here reproduces very closely the fundamental lesion of asbestosis, the peribronchiolar fibrosis, and may allow us to investigate some of the changes more directly associated with the onset of fibrosis. This test system clearly differentiates in a short period of time the relative fibrogenicity of two types of asbestos, namely long and short chrysotile fibers, and may therefore be valuable in assessing the fibrogenic potential of various respirable particles. Furthermore it could permit investigation of the relative importance of surface chemistry versus physical characteristics in the fibrogenicity of asbestos fibers.

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