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An Assessment of the Fibrogenic Potential of Very Short 4T30 Chrysotile by Intratracheal Instillation in Rats[1]

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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 μm) 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.

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

Previous work has suggested that the fibrogenic potential of asbestos is influenced 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 very fibrogenic (Holt and Mills, 1963; Holt *et al.*, 1964), less fibrogenic than long fibers of the same chemical composition (Klosterkötter, 1968; Burger and Engelbrecht, 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 native fibers (Jolicoeur *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 respirable 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 Africa. Very short 4T30 fibers were isolated from Quebec 4T30 chrysotile according to the sedimentation procedure of Jolicoeur *et al.* (1981). Each sample was autoclaved 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 one 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 ζ 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 1
PROPERTIES OF INJECTED MATERIALS

	Short 4T30 chrysotile	UICC chrysotile B
Length	<0.5 μm, 50%	>2 μm, 100%
	<3 μm, 98%	>5 μm, 42%
	<8 μm, 100%	>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 (Å)[a]	7.36 (Å)[b]
	4.56	4.55
	3.65	3.66
	2.44	2.46
	1.53	1.54

[a] As reported by Jolicoeur *et al.* (1981).
[b] As described by Timbrell (1970).

A

B

FIG. 1.
chrysotile

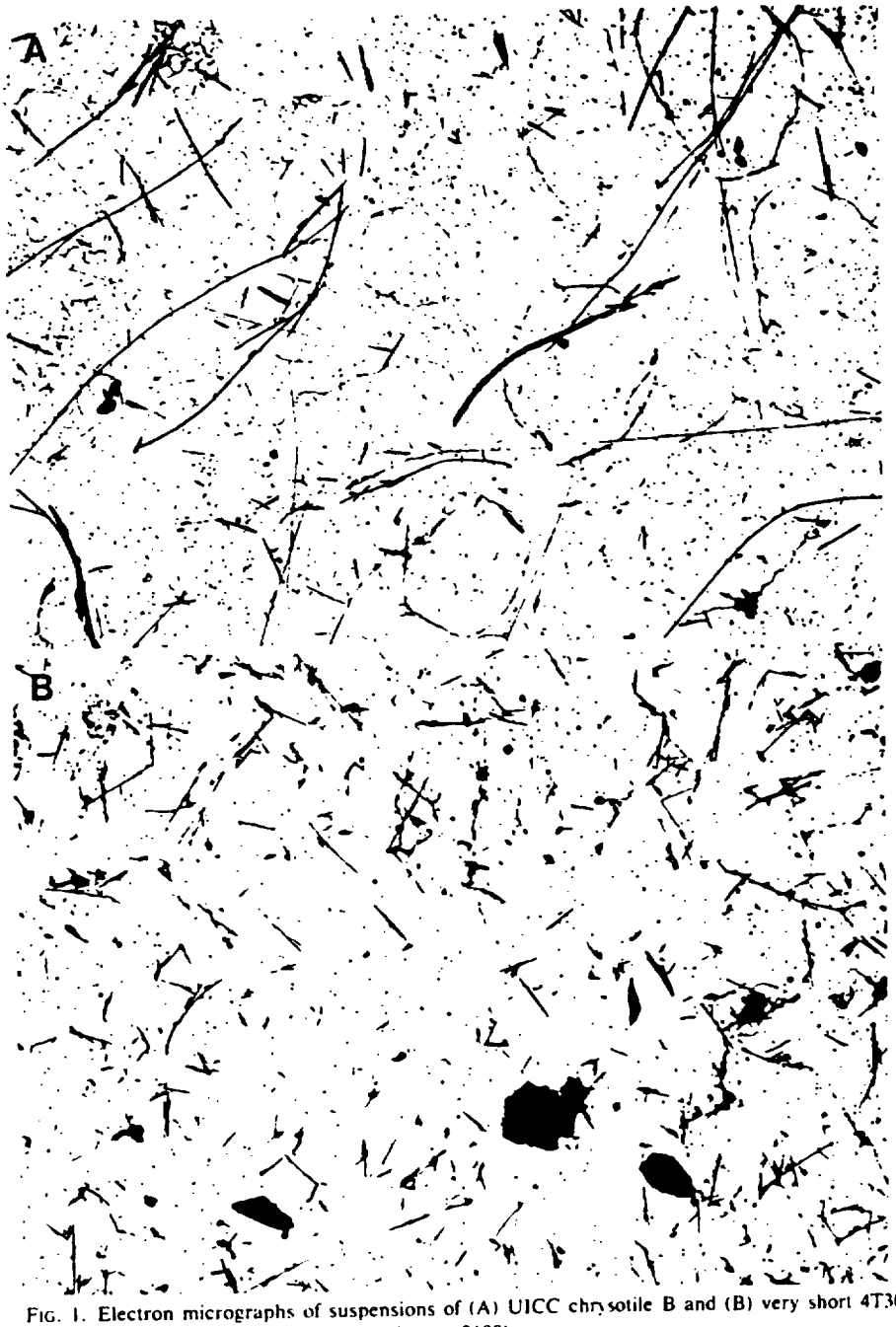

FIG. 1. Electron micrographs of suspensions of (A) UICC chrysotile B and (B) very short 4T30 chrysotile dispersed with Dounce homogenizer (×2100).

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
small
ducts

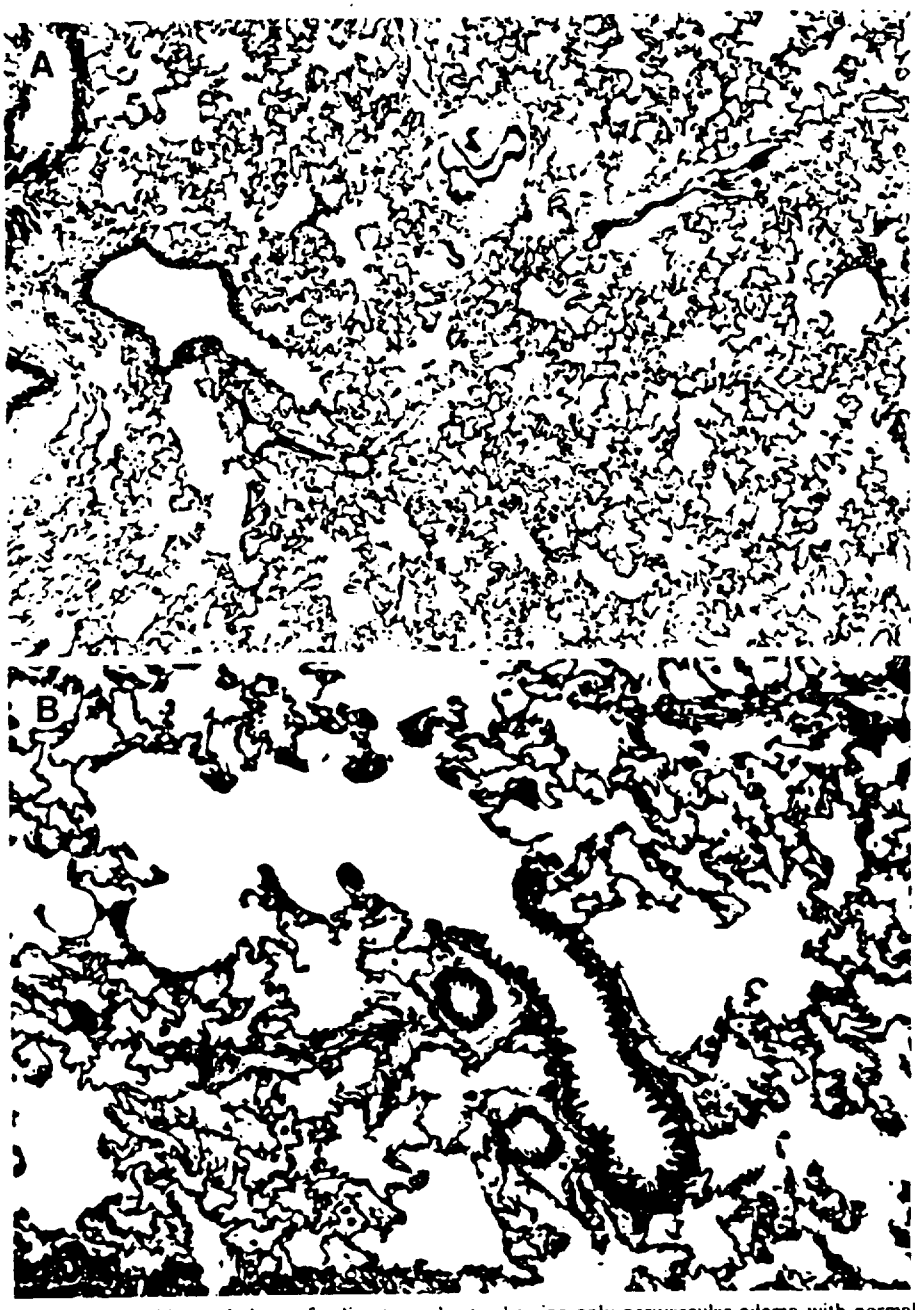

FIG. 2. Lung histopathology of saline-treated rats showing only perivascular edema with normal small airways and alveoli (A, ×46) and normal terminal and respiratory bronchioles with alveolar ducts and sacs (B, ×117).

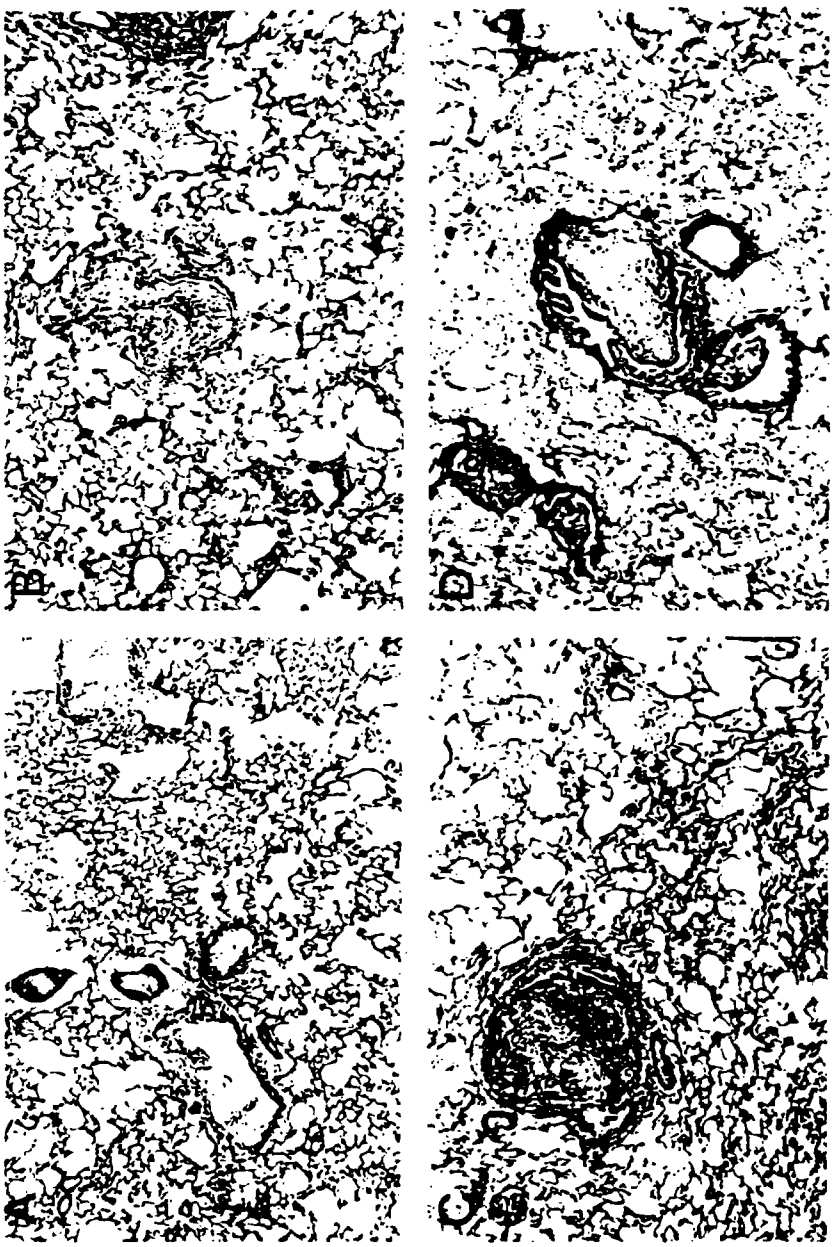

FIG. 3. Time course of lung histopathological changes following intratracheal instillation of UICC chrysotile B. No significant change was seen 1 day after exposure (A). Severe small airway distortion and stenosis by granulation tissue and fibroblastic proliferation were observed at 7 (B), 21(C), and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

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

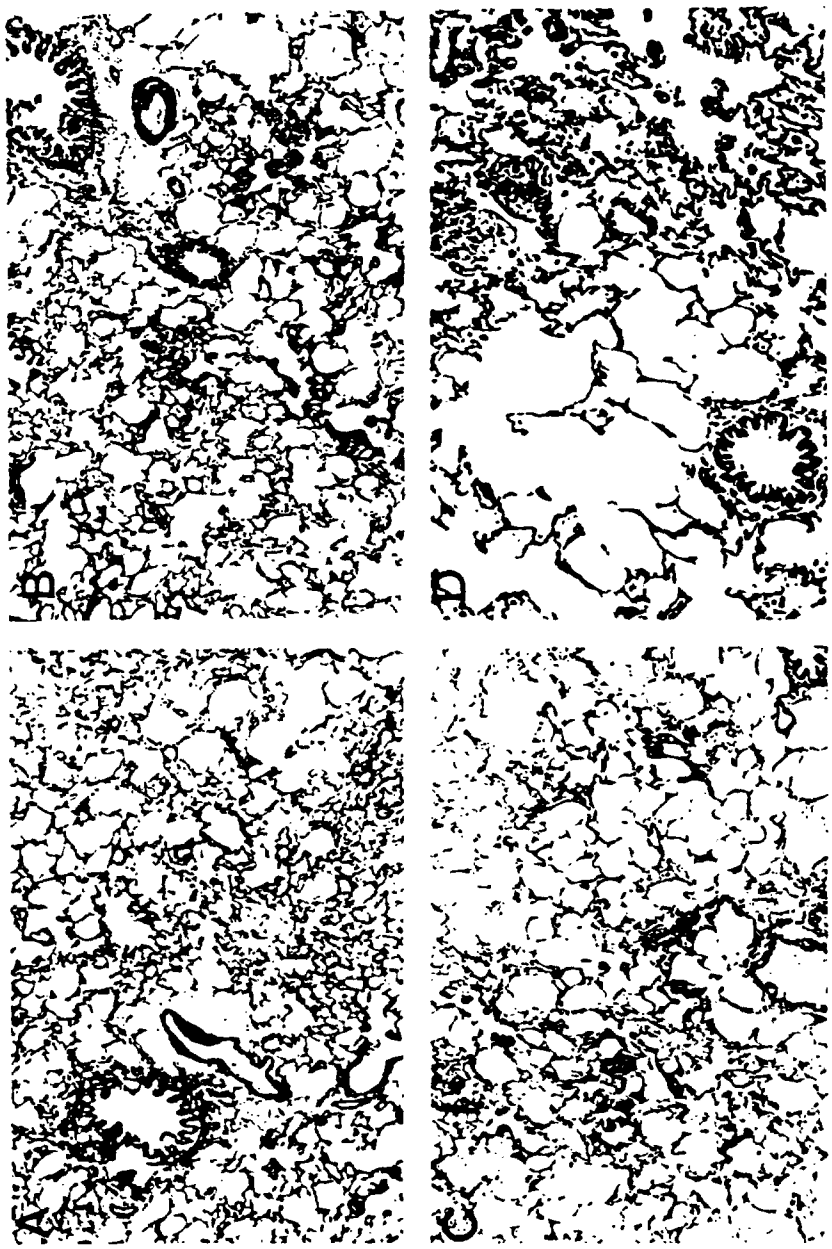

FIG. 5. Lung tissue reaction to intratracheal instillation of very short 4T30 chrysotile fibers. Only minimal alteration of alveolar septa with normal airways were seen 1 day after exposure (A). Multifocal septal thickening and alveolar distortion caused by interstitial mononuclear cell infiltration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

FIG. 6
(A) Macr
focal sept
furcation

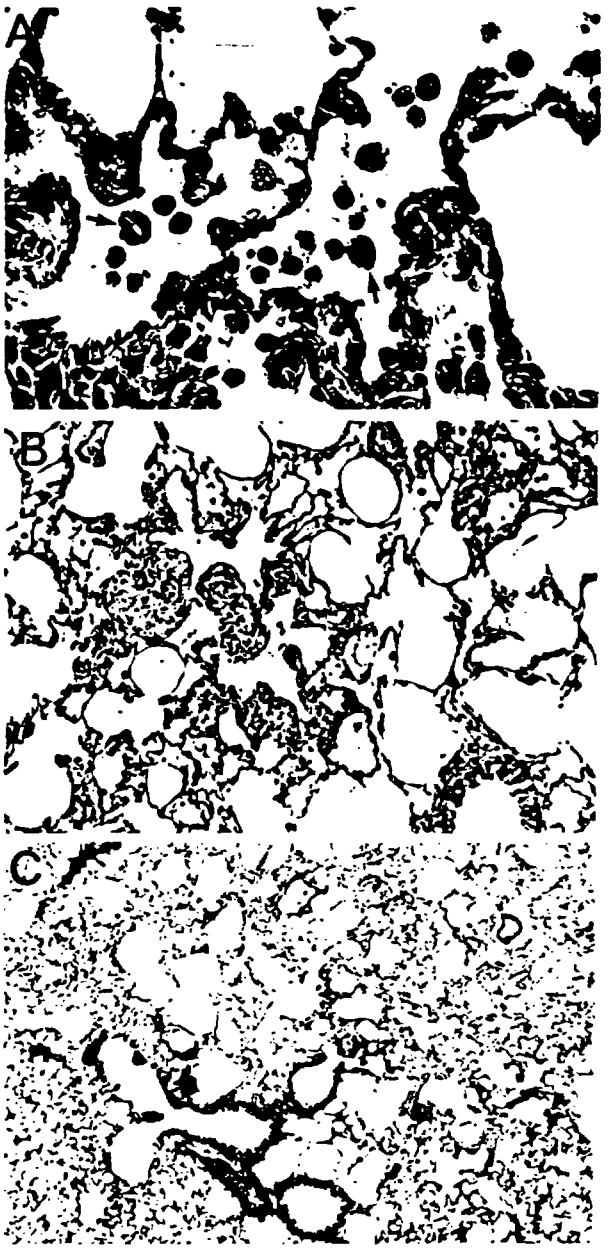

FIG. 6. Representative histopathology of lungs of rats exposed to very short 4T30 chrysotile fibers. (A) Macrophage accumulation in alveolar spaces (×367). Note binucleate cells (arrows). (B) Multifocal septal thickening by mononuclear cell infiltrates (×112). (C) Terminal airway with normal bifurcation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

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 installation. The small airways are normal. (A, B, C, ×44; D, ×91).

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 fundamental lesion of asbestosis, the rat was found to be the best available model (Davis, 1979) for studies aiming at the assessment of 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 baboons (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 abnormal 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 rapidly 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 pathogenicity of asbestos fibers.

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