J Electron Microsc 42: 41–47 (1993)

PLAINTIFF'S EXHIBIT

SA-470

Characterization of Asbestos Bodies and Uncoated Fibers in Lungs of Hamsters

Kenzo Hiroshima and Yasunosuke Suzuki*

Division of Environmental and Occupational Medicine, Department of Community Medicine, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, New York 10029, U.S.A.

Dimensional changes of asbestos bodies and uncoated fibers and the leaching of magnesium from the surface of chrysotile after a single intratracheal injection (1 mg) were investigated in male hamsters. The animals were sacrificed at 1 day, 6 months, 1 and 2 years later. In addition to histopathological observation, asbestos tissue burdens were investigated in 25 μm thick sections of the lungs. The sections were ashed in a low-temperature asher to be extracted with carbon film. The carbon extracted ashed sections were investigated by high resolution analytical electron microscopy to characterize asbestos fibers and asbestos bodies. Pulmonary interstitial and pleural fibrosis were observed after 6 months and later on. Light microscopically, asbestos bodies were observed after 6 months; their incidence subsequently increased with the lapse of time after the injection. Asbestos bodies measured on light micrographs became longer with the lapse of time. This suggested that the coating of chrysotile fibers had been continuously progressing in the lung. Geometric means of length and width of uncoated chrysotile fibers seen by transmission electron microscopy in animals at 1 year (length: 1.4 μm; width: 0.057 μm) or 2 years (1.2 μm, 0.047 μm) were greater compared with those of animals at 1 day (0.9 μm, 0.041 μm) or 6 months (0.9 μm, 0.037 μm) after instillation. These findings suggested that short and thin chrysotile fibers were cleared from the lung. Energy dispersive X-ray spectrometry revealed that the ratio of the content of magnesium to that of silicon was slightly lower in the fibers detected in the animals with a recovery period of 6 months and longer (6–12%), suggesting that magnesium had been leaching from the surface of chrysotile fibers although its amount was small.

Key words: **chrysotile, intratracheal injection, asbestos body, carbon extraction technique, transmission electron microscopy**

In 1969, we reported experimentally-induced asbestosis in hamsters, with special reference to the development of asbestos bodies.[1] We discovered that alveolar macrophages played an important role in the formation of asbestos bodies, since the cells provided the "iron protein complex" coating of the asbestos bodies. Although chrysotile fibers were subsequently reported to form bodies less frequently compared with amphiboles,[2,3] we showed that chrysotile fibers were also transformed into asbestos bodies. We suggested that longer chrysotile fibers became fragmented and split, and that the asbestos bodies grew in thickness and in length with the continuous addition of iron.

At that time, high resolution analytical electron microscopy was not utilized for the chemical analysis of the asbestos fibers. Further, the importance of size distribution related to the carcinogenicity of asbestos fibers had not yet been reported: this hypothesis was proposed by Stanton and Wrench.[4] Adamson and Bowden reported that long asbestos fibers were essential in the experimental induction of pulmonary fibrosis.[5] In contrast, Churg et al. reported that the longer the fiber, the milder the degree of pulmonary fibrosis in chrysotile miners and millers.[6] While Stanton et al. reported that thin (≤ 0.25 μm) and long (> 8 μm) fibers had the highest probability of induction of mesothelioma in rats.[7] Pott suggested that, in terms of carcinogenesis, many short fibers in the lung may be just as dangerous as a few long fibers.[8]

A question has been raised as to whether size distribution of inhaled asbestos fibers may be different from that of airborne fibers. Timbrell reported that fiber width may be an important factor in penetration to the alveolar regions.[9] Since, in our former experimental study, chrysotile fibers were given in a single administration, the treated animals seemed to be good material to study the fate of intrapulmonary asbestos fibers. This study is a re-analysis of the same material and animals reported in 1969,[1] using currently available high resolution analytical electron microscopy. The histopathology, light microscopic measurement of asbestos bodies, and size distribution of intrapulmonary asbestos fibers were investigated. Our objectives were to clarify the fate of administered fibers, and how the coating of asbestos fibers progressed.

* To whom reprint requests should be addressed

MATERIALS AND METHODS

Male hamsters, ranging from 50 to 130 g in weight, were given 1 mg of soft chrysotile asbestos suspended in 0.1 ml of normal saline. This was administrated intratracheally under dibutyl intraperitoneal anesthesia (0.6 ml intraperitoneally). Animals were sacrificed: one at 1 day, one at 6 months, two at 1 year, and one 2 years after instillation. Three additional hamsters were used as controls. The lung tissue was fixed and prepared for both light and electron microscopy. For light microscopy, four μm sections were stained with hematoxylin and eosin (HE) and Prussian blue stain.

One hundred fifty (150) asbestos bodies were measured consecutively using a ruler on light micrographs made from lung slides taken from animals sacrificed at 1 year and 2 years after instillation. The slides were stained with Prussian blue. Since the size distribution of asbestos bodies was skewed toward high values, logarithmic transformation was carried out.

To examine asbestos fibers under an electron microscope, tissue samples were prepared by the carbon extraction technique.[10] Twenty five μm thick paraffin-embedded unstained sections of the lung were ashed in a low-temperature asher at a temperature of 45°C for 40 min. The ashed section was outlined with cellophane tape. This area was then covered with a 10% polyvinyl alcohol (PVA) solution. The PVA was allowed to dry at room temperature overnight, and was peeled off the slide. This thin film was then inverted and placed in a vacuum evaporator, and a layer of carbon was deposited onto the surface. The preparation was then placed face down on a hot water bath, until the PVA was dissolved. The carbon extracted sections were mounted on electron microscope grids and investigated under a high resolution analytical electron microscope. Asbestos fibers were identified using a JEOL 100CX electron microscope equipped with a Tracor Northern energy dispersive X-ray spectrometer. Length, width and aspect ratio (i.e., a length: width ratio) of 400 asbestos fibers were investigated consecutively and measured on electron micrographs using a ruler. The magnifications used in photographing were $\times$16,500 and $\times$20,750. All fibers 0.02 μm in width or wider were counted. Again, because the size distribution of asbestos fibers was skewed toward high values, logarithmic transformation was carried out.

For each of the animals, the ratio of magnesium to silicon of approximately 40 fibers was investigated by an energy dispersive X-ray spectrometer. The sum of the intensities of all the channels within the full width at half the maximum intensity was calculated for each fiber. If the analyzed carbon film is thin and two elements, such as magnesium and silicon, are measured simultaneously, the intensity ratio is directly related to the mass concentration ratio.[11] For UICC chrysotile B, whose contents were analyzed by wet chemical analysis, C_{Mg}/C_{Si} (C_{Mg}: concentration of magnesium; C_{Si}: concentration of silicon) was 1.47, whereas I_{Mg}/I_{Si} (I_{Mg}: emitted intensity from magnesium; I_{Si}: emitted intensity from silicon) was 0.70 using our instrument. Therefore, intensity ratios of magnesium relative to silicon must be multiplied by a standard constant k (1.47/0.70 = 2.10) to obtain mass concentration ratios of magnesium relative to silicon for the fibers investigated. Fibers thinner than 0.1 μm were not investigated since the sum of the intensities obtained from these thin fibers by energy dispersive X-ray spectrometry was too small to be accurately calculated.

To confirm the absence of chrysotile contamination from the environment of our laboratory, blank slides were ashed in a low-temperature asher to be extracted with carbon film. The carbon film was mounted on an electron microscope grid. No chrysotile fibers were found in the samples, indicating that the air of the laboratory, the chamber of the asher and the carbon evaporatory were not contaminated. The ashed UICC chrysotile B fibers were compared with the unashed ones. No obvious structural difference was found between the two, suggesting that ashing did not induce splitting and fragmentation of chrysotile fibers.

In our comparisons to assess the dimensional changes in asbestos bodies, in uncoated fibers, and in the ratios of magnesium to silicon in uncoated fibers, we used analysis of variance, followed by multiple comparison testing (Student-Newman-Keuls test)[12] when the F value was significant ($p<0.05$). We used UICC chrysotile B as a standard sample for the calculation of the ratio of magnesium to silicon, since our original (1969) sample was not available.

RESULTS

Light microscopically, 1 day after instillation severe acute inflammation, diapedesis of the erythrocytes, and edema in the alveolar spaces were observed.[1] Asbestos fibers were rarely seen in HE stained slides. At 6 months, obvious fibrosis was seen in the lung, particularly in the peribronchiolar interstitium. One and 2 years after instillation, the severity of fibrosis had progressed and hyperplasia of both bronchiolar and alveolar epithelial cells were observed. Pleural fibrosis was also seen. Macrophages, including multinucleated giant cells were present in both alveolar space and fibrotic interstitium, frequently containing hemosiderin granules. Asbestos bodies, golden-brown with HE and blue with iron staining, were often observed 1 and 2 years after instillation; they were not observed 1 day after instillation and rarely observed at 6 months. Neither interstitial fibrosis nor asbestos bodies were observed in the lungs of control animals.

Electron microscopically, a large number of chrysotile fibers were observed 1 day after instillation (Figs. 1, 2), although it was almost impossible to see them by light microscopy. Six months or more after instillation, typical asbestos bodies, as well as immature asbestos bodies, were observed under an electron microscope (Figs. 3, 4). Most

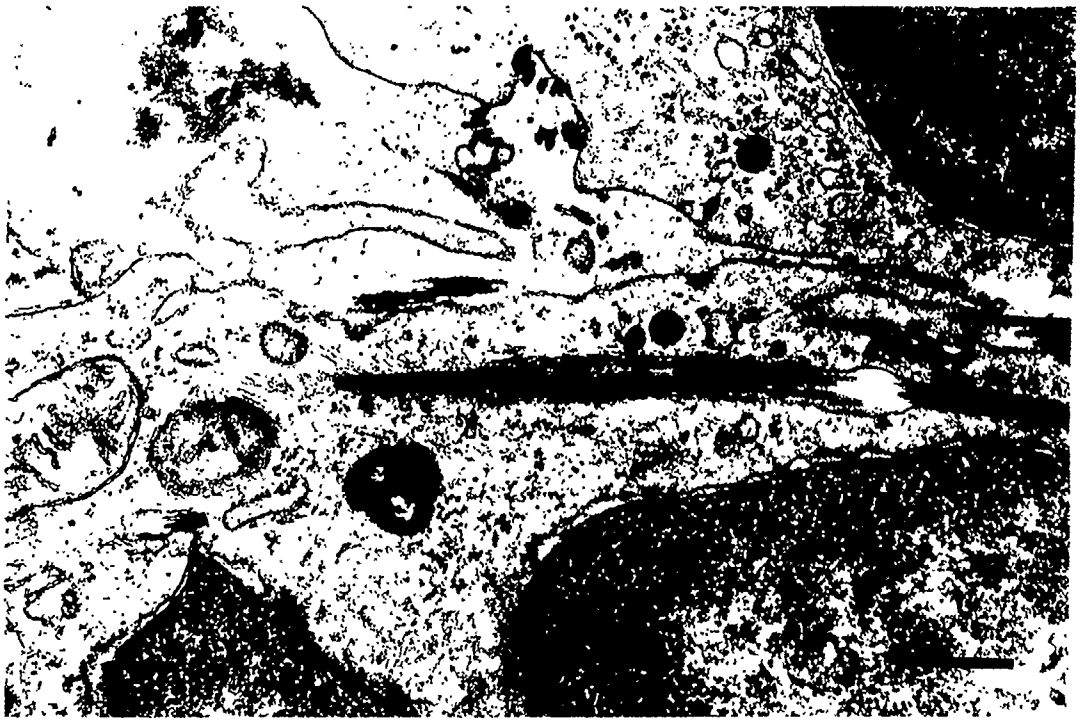

Fig. 1. Chrysotile fibers in a phagosome of an alveolar macrophage and chrysotile fibers which exist in the extracellular space. Chrysotile fibers are also observed in a recess of the plasma membrane of a neutrophil or in contact with it. Transmission electron microscopy of an ultrathin section. One day after administration. Scale = 1 μm.

Fig. 2. Intrapulmonary chrysotile fibers in an ashed 25 μm thick section. Transmission electron microscopy. One day after administration Scale = 1 μm.

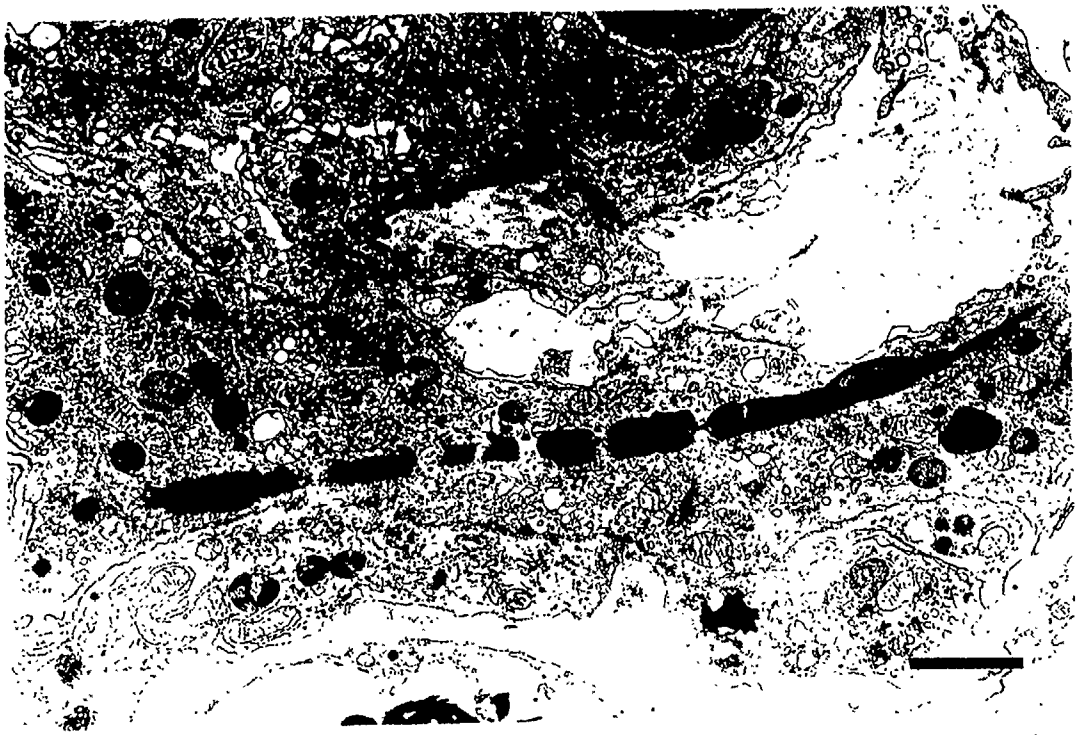

Fig. 3. An asbestos body seen in an interstitial histiocyte. Transmission electron microscopy of an ultrathin section. Two years after administration. Scale = 2 μm.

Fig. 4. Intrapulmonary chrysotile fibers in an ashed 25 μm thick section. An asbestos body is observed. Transmission electron microscopy. Two years after administration. Scale = 1 μm.

asbestos bodies were longer than 10 μm, but there were bodies as short as 3 μm in length. The shape of the asbestos bodies varied. Straight, segmented, corrugated, parallel, curved and comma forms were observed.

All asbestos bodies observed on light micrographs were measured for length and width, and plotted on a scattergraph for 1 and 2 years after instillation (Figs. 5a, b). The geometric mean (GM) length, width and aspect ratio of asbestos bodies measured by light microscopy are listed in Table 1. The difference in length and aspect ratio between the animals 1 year and 2 years after instillation was statistically significant ($p<0.01$), but the width was not.

Chrysotile fibers found by transmission electron microscopy were measured for length and width, and the cumulative percentages of the undersize, by length and width, were plotted against the length and width in the log scale (Fig. 6). The GM length, width and aspect ratio of uncoated fibers are listed in Table 2. Fibers 1 or 2

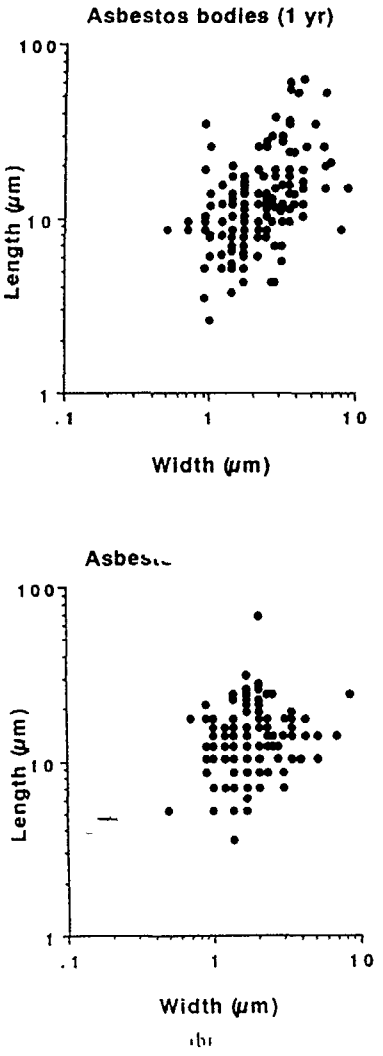

Fig. 5. Distribution by length and width of asbestos bodies measured on light microscopic photographs. (a—upper) One year and (b—lower) two years after administration.

Table 1. Characteristics of asbestos bodies in hamster lung following intratracheal injection of chrysotile (1 mg).

Dimension	Geometric mean of dimension[a]	
	Recovery period	
	1 year	2 years
Length (μm)[b]	12.1 [1.8]	16.1 [1.9]
Width (μm)[b]	2.1 [1.7]	2.2 [1.7]
Aspect ratio	5.7 [1.8]	7.3 [1.8]
Number measured	150	150

[a] Values in brackets indicate geometric standard deviation. [b] Measured on light micrographs.

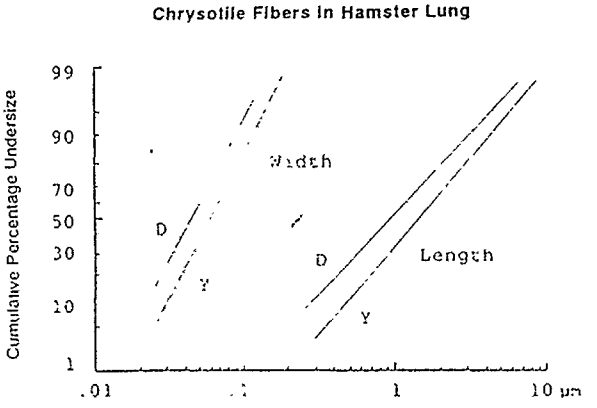

Fig. 6. Cumulative percentage of undersize expressed by length and width in log scale. D: one day after administration. Y: one year after administration. Transmission electron microscopy.

Table 2. Characteristics of chrysotile fibers in hamster lung following intratracheal injection (1 mg).

Fiber dimension	Geometric mean of dimension[a]			
	Recovery period			
	1 day	6 months	1 year	2 years
Length (μm)[b]	0.9 [2.4]	0.9 [2.9]	1.4 [2.1]	1.2 [2.2]
Width (μm)[b]	0.041 [1.8]	0.037 [2.0]	0.057 [1.8]	0.047 [1.8]
Aspect ratio	22 [2.1]	24 [2.3]	24 [2.0]	26 [1.9]
Number measured	400	398	394	384

[a] Values in brackets indicate geometric standard deviation. [b] Transmission electron microscopy.

Table 3. Magnesium to silicon ratios of chrysotile fibers* in hamster lung following intratracheal injection (1 mg).

	Mg:Si ratio	No. of fibers
Standard (UICC chrysotile B)	1.47±0.16[a]	54
1 day	1.44±0.14	40
6 months	1.30±0.12[bc]	55
1 year	1.34±0.14[bc]	44
2 years	1.38±0.14[bd]	40

* Energy dispersive X-ray spectrometry. [a] Mean magnesium to silicon ratio ± standard deviation. [b] Ratio was significantly different from the standard sample using the Student-Newman-Keuls test ($p<0.01$). [c] Ratio was significantly different from the animal 1 day after instillation using the Student-Newman-Keuls test ($p<0.01$). [d] Ratio was significantly different from the animal 1 day after instillation using the Student-Newman-Keuls test ($p<0.05$).

years after instillation had greater GM length and width compared with those 1 day or 6 months after instillation ($p<0.01$). GM aspect ratios of those fibers at 6 months, 1 year or 2 years after instillation were greater than those 1 day after instillation ($p<0.05$).

The ratio of the number of asbestos bodies to total chrysotile fibers were 0, 0.005, 0.015, 0.04 in animals 1 day, 6 months, 1 year and 2 years after instillation, respectively.

The means of the ratio of content of magnesium to silicon in chrysotile fibers are shown in Table 3. The ratios of chrysotile fibers in animals after 6 months and longer were significantly smaller than in standard sample ($p<0.01$), but the magnitude of decrement was small (6–12%). The ratios in animals after 6 months and longer were also slightly smaller than in the animal after 1 day, and the differences were statistically significant ($p<0.05$). There was no progressive decrement of the ratios after 6 months.

DISCUSSION

The range of size distribution of the asbestos fibers is said to be an important factor related to fibrogenesis and carcinogenesis. Adamson and Bowden reported that a high dose of exclusively short crocidolite fibers produced minimal fibrosis in mice, whereas fibrosis was seen in peribronchiolar locations after exposure to long crocidolite fibers.[5,13] In contrast, Churg et al. reported that the longer the fiber, the milder the degree of fibrosis in chrysotile miners and millers.[6] Stanton et al. reported that fibers > 8 μm in length and ≤0.25 μm in diameter had the highest probability of inducing mesothelioma in rats,[7] although the importance of chemical properties influencing carcinogenicity has only recently been emphasized.[14]

It has been reported that transformation of asbestos fibers into asbestos bodies in human lungs is very common in amphiboles and much less in the chrysotile.[2,3] Similar findings have been obtained in our recent study on experimental asbestosis in baboon lungs induced by inhalation of amphiboles and chrysotile.[15] It was obvious, that, as in the hamster, chrysotile fibers in baboon lungs could become core fibers of asbestos fibers.

In the present hamster study, it was found that the ratios of asbestos bodies to total fibers were from 0.5 to 4%, depending on the length of the time after instillation; the longer the observation period, the higher the ratio. It has been reported that the ratio of asbestos bodies to total fibers was 0.01% in the human general population,[16] and 1–5% in patients with moderate and heavy exposure.[17]

While it has been reported that asbestos bodies were rarely formed on fibers < 10 μm in length,[2,3] in this study, we observed both the incipient stage and fully formed asbestos bodies as short as 3 μm in the lung using electron microscopy. Since we observed asbestos bodies on 4 μm thick sections with light microscopy, some of the long asbestos bodies might have been cut off when the block was sliced. However, this could occur in every case with the same probability. Our data showed that the mean length of asbestos bodies increase with time after a single intratracheal administration of chrysotile. We have already reported that the formation of asbestos bodies (coating of asbestos fibers with iron protein complex) needs a slow but continuous process.[1] It is suggested that it takes more time for longer, thicker fibers to be invested with the coating substances compared with shorter, thinner ones. Our previous report suggested that the raw materials of the coating substance were produced in macrophages and were continuously discharged to the surface of phagocytized asbestos fibers to form asbestos bodies.[1]

Chrysotile fibers have been reported to be cleared from the lung at a greater rate than amphiboles.[18–20] In animal studies, the mean length of asbestos fibers increases progressively suggesting that short fibers are cleared from the lung,[21–23] and the mean width of chrysotile fibers decreases with time suggesting that they are breaking up longitudinally into fibrils.[21] Churg and DePaoli reported that there was no difference in size distribution of intrapulmonary chrysotile and tremolite fibers in chrysotile miners and millers with a long extension period since their last exposure (22–25 years).[24] Our data show that the mean length and width of chrysotile fibers became greater with the extension of time, suggesting that clearance of short and thin chrysotile fibers had been occurring. It is not clear why our findings on the mean width are different from that of Roggli and Brody.[23] However the mean width of fibers in the animal 1 day after instillation in our study was 0.041 μm; perhaps such thin fibers are not separated further.

Chemical changes in chrysotile fibers in the lung remains a controversial issue. Morgan et al. reported that as much as 35% of the magnesium dissolved in the first month after intrapleural administration of chrysotile in rats.[25] In contrast, Churg et al. observed guinea pigs

until 1 month after intratracheal instillation of a mixture of amosite and chrysotile, and found that there was no major magnesium loss in chrysotile fibers.[26] In humans, it was suggested that the leaching of magnesium was not homogeneous and differed from one fiber to another, and, even in a single fiber, it differed along the axis of the fiber.[27] Churg and DePaoli reported that in the lungs of chrysotile miners and millers who died after more than 12 years since their last exposure 20% of the magnesium was lost from the fibers.[24] The current study showed that leaching of magnesium from chrysotile occurred in animals at least 6 months after instillation, but the magnitude of the decrement was small (6–12%). Soft chrysotile, which was used in this study, is the fiber type which has the ability to be woven into valuable products. This term was used in commercial literature. Its components are the same as UICC chrysotile, or chrysotile used in industry. We used UICC chrysotile B as the standard sample, since there was no difference in the content of chemical components among the three distinct types of chrysotile standard samples (UICC chrysotile A, UICC chrysotile B, and Calidria chrysotile) which we measured preliminary. The biological significance of the small altered chrysotile fibers in the lung is not known; however, there is still debate about the carcinogenicity of altered chrysotile fibers. It was reported that the incidence of mesothelioma induced by 50%-magnesium-depleted chrysotile was similar to that with intact chrysotile, but with 90% depletion, the incidence was considerably less, and that *in vitro* the cytotoxicity of chrysotile fibers decreased in magnesium depleted fibers.[28] It must be remembered that the composition of chrysotile fibers may be different after residence in the lung, resulting in some difficulties for the identification of intrapulmonary chrysotile fibers due to a decrease in magnesium.

Acknowledgments. The authors express their appreciation to Dr. I. J. Selikoff for his continuous encouragement to our research on asbestos, and Dr. N. Kohyama, National Institute of Industrial Health, Japan, for supplying UICC chrysotile B with the information of wet chemical analysis and for his help for the analysis of the ratio of a chemical component by analytical electron microscopy. The authors also thank Mr. I. Weisman, Mr. R. Ashley, Mr. S. R. Yuen, Ms. A. Calderaro for their technical assistance, and Ms. M. Burger for her editorial assistance. This work has been partially supported by ES00928 Center Grant from the National Institute of Environmental Health Sciences.

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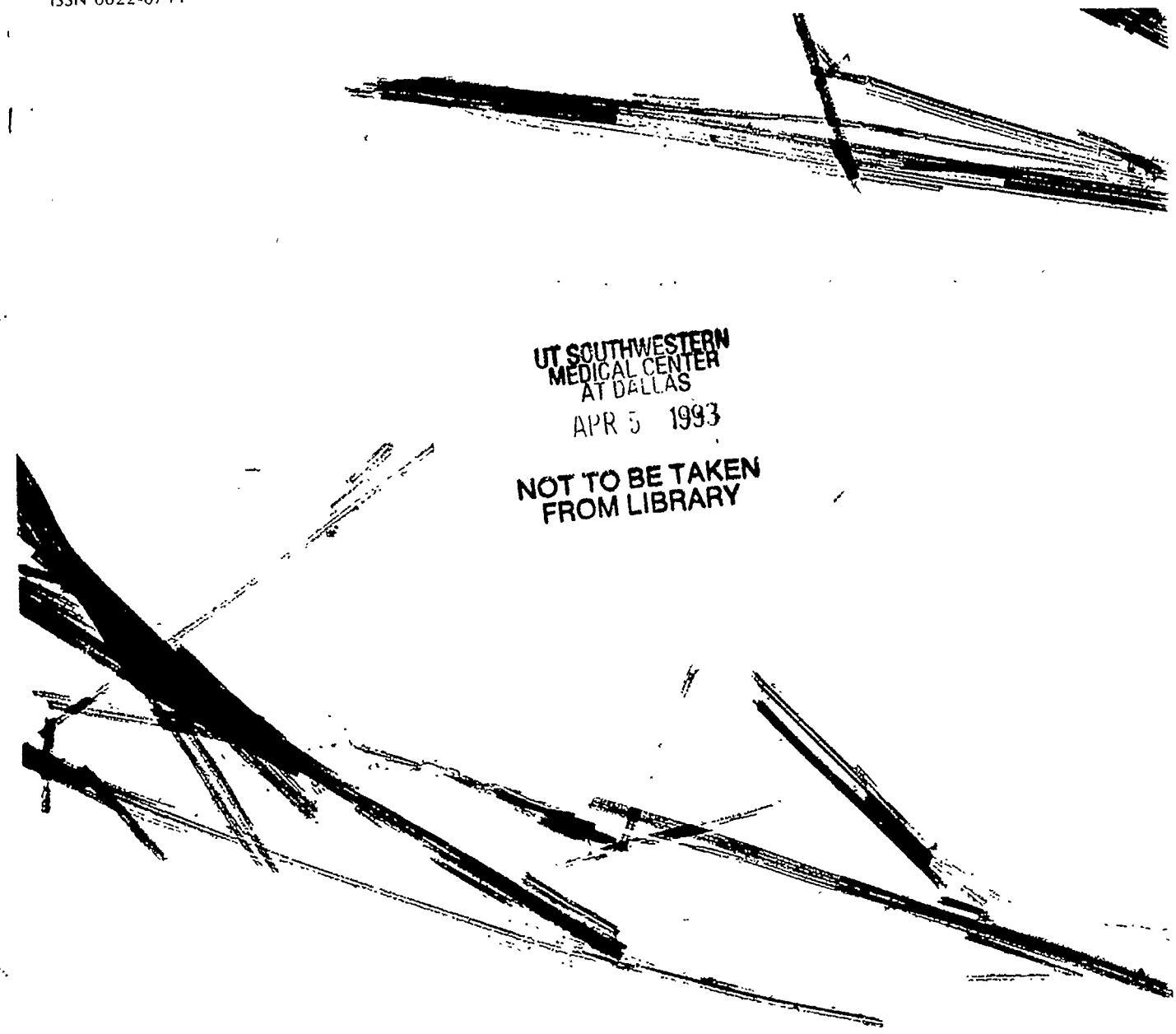
(Received September 4, 1992; accepted November 24, 1992)

Journal of Electron Microscopy

ISSN 0022-0744

Volume 42 • 1993 Number 1



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