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Cytotoxicity of a Short-Fiber Chrysotile Asbestos for Human Alveolar Macrophages: Preliminary Observations

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Studies were performed to compare the cytotoxicity for human alveolar macrophages of a naturally occurring short-fiber chrysotile asbestos (RG 144) to that of a standard reference mixed-fiber (long and short) chrysotile asbestos (UICC chrysotile A, Rhodesian). Parallel studies were also performed with quartz (Min-U-Sil 15), a known macrophage toxin. On a mass basis, and after 24 hr incubation, RG 144 was more cytotoxic than the UICC standard reference fiber and less toxic than quartz (silica). The cytotoxic potential of RG 144 chrysotile was further enhanced after size reduction by milling. These findings may have important biologic implications with respect to the use of short-fiber asbestos in industry.

INTRODUCTION

Controversy exists regarding the biologic potential of asbestos fibers of different lengths (recently summarized by several authors, e.g., Harington *et al.*, 1975; Beck, 1980; Hillerdal, 1980). The central issue of the controversy focuses on the cytotoxicity and pathogenicity of short-fiber asbestos, that is, asbestos in which the longest fibers do not exceed 5 μm in length. In addition to this scientific question, the problem of short-fiber asbestos also has important practical implications. The current protective standard used by the U.S. Department of Labor for workers who may be exposed to asbestos-containing dust in the workplace takes into account only those fibers greater than 5 μm in length (OSHA, 1972; Langer *et al.*, 1978). With increasing usage of short-fiber grades in industry and the preponderance of such fibers in the environment, the biologic potential of short fiber should be further studied.

The present study was undertaken to determine whether there is a difference in the cytotoxicity of a naturally occurring short-fiber chrysotile asbestos, before and after milling, and that of a preparation of chrysotile containing both long and short fibers. In addition, these two preparations have been compared to quartz, a known macrophage toxin. Well-characterized asbestos fibers and quartz particles were incubated with human alveolar macrophages, cells considered to be among the most important targets for inhaled asbestos (Hocking and Golde, 1979; Kagan, 1981). Cytotoxicity was determined by the trypan blue exclusion method.

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MATERIALS AND METHODS

Test materials. UICC chrysotile A fiber was obtained from the Pneumoconiosis Research Unit, Cardiff, Wales, U.K. The mineralogic purity and general physicochemical characteristics of these fibers have been extensively characterized (Rendall, 1970; Timbrell, 1970). Specimen suitability and purity were checked by polarized light microscopy and continuous scan X-ray diffraction. The UICC preparation consists of 98–99% chrysotile fiber, with the remaining mineral assemblage made up of sheet silicates (primarily antigorite, lizardite, and traces of chlorite). Nemalite or fibrous amphibole minerals were not detected. Approximately 25% of the fiber population was greater than 5 μm in length (Table 1).

RG 144, a naturally occurring, predominantly short-fiber chrysotile from the New Idria deposit in California, was obtained in the commercially available form from the Union Carbide, New York. This preparation has been previously described in detail (Langer *et al.*, 1978). Its purity was checked in a fashion similar to that described for UICC chrysotile A; it had identical characteristics in terms of chrysotile content and mineral contaminants. In comparison with the UICC preparation, a very different particle size distribution was found. Only 2% of the fiber population was greater than 5 μm in length (Table 1; Fig. 1).

Fiber size distributions were measured by sizing and counting objects directly from electron micrographs at final magnifications of 10,000×. Counting was assisted by use of a calibrated hand lens (a Porton graticule). Particle numbers for each of the preparations were computed from the size-distribution data (Langer *et al.*, 1978). The mean particle dimension within each size class was used in the computation of mass fractions for each of the classes. For each of the calculations used in determining particle number per mass fraction of the specimen, a chrysotile density of 2.5 g/cm^3 was assumed, as well as an average fibril diameter of 333 Å, and an average fiber diameter of 9 fibrils in the unmilled specimen and of 3 fibrils for both the 60-sec and the 1200-sec milled preparations. Surface area of RG 144 was estimated from values issued by Union Carbide, and by recomputation of particle number for each of the size distributions.

The silica used was quartz, Min-U-Sil 15, obtained from the Pennsylvania Glass and Sand Company, Pittsburgh, Pennsylvania. Its physical properties have been previously described (Nolan *et al.*, 1981). Estimates of surface area were based on data provided by the vendor and recomputations as outlined for the asbestos preparations.

Harvesting of human alveolar macrophages. Seven healthy volunteers between 21 and 26 years of age were bronchoscoped with a fiberoptic bronchoscope for procurement of human alveolar macrophages. One volunteer had two lavages and another had three; lavages were separated by an interval of at least 4 weeks. Three of the volunteers were nonsmokers; one smoked tobacco and another marijuana. Because there were no significant differences in the response of the cells from smokers and nonsmokers to challenges by particles the results have been expressed together. Volunteers gave appropriate informed consent, and the protocol for the study was approved by the Human Research Committee of the Georgetown University Medical Center, where the lavages were performed.

Bronchial lavage was performed as previously described (Yeager *et al.*, 1974). Anesthesia of the nose and upper airway was accomplished with lidocaine spray. No other medication was administered. An Olympus fiberoptic bronchoscope was passed transnasally and wedged into one of the segments of the right lower lobe. The lung was irrigated with 30- to 50-ml quantities of warm sterile physiological saline, and the lavage fluid was withdrawn by gentle suction with a 10-ml syringe. Approximately 150 ml of saline was instilled, of which 30–50% was recovered. The aspirated solution was centrifuged at 200*g* for 15 min in an International refrigerated centrifuge, and the supernatant fluid discarded.

The bronchoalveolar cells obtained were resuspended and washed three times in Gey's saline (Microbiological Associates, Bethesda, Md.). They were finally resuspended in RPMI 1640 medium with 25 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and L-glutamine (GIBCO, Grand Island, N.Y.), supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, Rockville, Md.), penicillin at 100 μg/ml, and streptomycin at 100 μg/ml, hereafter referred to as RPMI supplemented medium. The cells obtained were 88% viable by trypan blue staining (Paul, 1965).

Culture of alveolar macrophages and cytotoxicity tests. One hundred thousand (1×10^5) bronchoalveolar cells were plated into eight-chamber Lab-Tek tissue culture chambers (Miles Laboratories, Naperville, Ill.) in 0.4 ml of RPMI supplemented medium, and incubated for 1 hr at 37°C in a 5% CO_2 incubator. More than 95% of the adherent cells were macrophages as judged by light microscopic appearance, phagocytosis of latex beads, and nonspecific esterase staining.

After a 5-sec resuspension with the Vortex Genie Mixer, the appropriate asbestos or silica suspensions were added in fresh medium to the macrophages. The mixtures were allowed to incubate 1 hr at 37°C in a humidified 5% CO_2 incubator, after which nonengulfed particulate material and medium were removed, and fresh medium was added. The cultures were then incubated for 24 hr at 37°C in the 5% CO_2 atmosphere. At the end of this period, the adherent cells were removed by incubating 1 min in 0.25% trypsin/0.2% EDTA (GIBCO) in Hanks' solution at 25°C (Paul, 1965). The detached cells were then counted in a hemocytometer, and the number of cells excluding trypan blue dye was determined (Paul, 1965).

Statistics. Data were analyzed using analysis of variance and Duncan's multiple range test (Snedecor and Cochran, 1980).

RESULTS

The physical characteristics of the minerals studied are given in Table 1. The UICC chrysotile A sample contained a larger percentage of long fibers compared to the unmilled RG 144 specimen. However, when the absolute number of fibers per microgram of mass (No./μg) was computed, the number >5 μm in length was virtually identical in each preparation. This point is stressed, because comparison of the percentage of specific-size fractions in specimens with orders of magnitude difference in particle number per unit mass of dust is misleading. For example, specimen "A" with 100,000 total fibers per microgram of dust, with a 30% content of fibers >5 μm in length contains 30,000 such fibers per microgram,

TABLE 1

PHYSICAL PROPERTIES OF MINERAL DUSTS USED TO CHALLENGE MACROPHAGES

Materials	Percentage of particles						Absolute Number		Surface area, air permeability and others (m^2/g)
	0.1 μm or less	0.11–0.5 μm	0.51–1.0 μm	1.1–2.0 μm	2.1–5.0 μm	5.1 μm or larger	Particle No./μg	Particles 5 μm/μg	
UICC A (Rhodesian) chrysotile[a]	0.0	4.9	13.4	19.8	36.5	25.4	0.1×10^9	2.5×10^6	6.8
RG 144 (Calidria) chrysotile unmilled[b]	20.3	49.2	12.4	8.5	7.5	2.1	1.1×10^9	2.1×10^6	60
RG 144, 60-sec milled[b]	12.7	58.6	16.7	8.6	3.2	0.2	10×10^9	2.0×10^6	>60
RG 144, 1200-sec milled[b]	58.2	39.4	2.3	0.1	0.0	0.0	23×10^9	$<2.0 \times 10^6$	>>60
Quartz (Min-U-Sil 15)[c]	0.0	0.0	0.0	67.0	22.0	11.0	3.0×10^4	0.3×10^4	(probably <2.0)

[a] Data from Timbrell (1970). Particle size distribution by transmission electron microscopy.

[b] Data from Langer *et al.* (1978). Particle size distribution by transmission electron microscopy. (~1000 particles sized in each specimen).

[c] Data from Nolan *et al.* (1981). Particle size distribution by polarized light microscopy.

as does specimen "B" with 1,000,000 fibers per microgram of dust but with a 3% content of this same size class of fibers.

As milling time for RG 144 increased, the particle number per unit mass increased as well. Although the percentage of fibers >5 μm in length decreased after 60 sec of milling, the absolute number per unit mass remained constant. At milling times of 1200 sec, fibers >5 μm in length were not detected in a population defined by almost 1000 measurements (see Table 1 and Fig. 1).

The results of treating human alveolar macrophage cultures with various asbestos fiber preparations and silica are shown in Table 2. The unmilled RG 144 chrysotile fiber preparation was more cytotoxic than the UICC preparation. Moreover, after it was milled, the cytotoxic action of the RG 144 chrysotile was further enhanced. This effect was correlated with an increased number of short fibers and the total fiber number, rather than with the absolute number per microgram of fibers >5 μm in length. Values were found for the cytotoxicity of silica that were parallel to those found in previous studies (Kagan and Miller, 1981).

Macrophage viability decreased at higher particulate concentrations, but a clear cut dose–response relationship was not observed. The results obtained were reproducible, since repeat studies in the same person yielded similar findings on three occasions in one individual and on two occasions in another volunteer.

DISCUSSION

The question of the health effects of short-fiber asbestos has been largely neglected, and attention has been mainly focused on asbestos fibers of 5 μm or more in length. The latter have been shown to be toxic to macrophages *in vitro*, and fibrogenic and tumorigenic in animals *in vivo* (Harington *et al.*, 1975; Beck, 1980).

Certain observations, however, suggest that short-fiber asbestos may also be harmful. A case of diffuse interstitial lung fibrosis was reported in which only submicroscopic particles of chrysotile asbestos were present (Miller *et al.*, 1975). Crapo *et al.* (1980) found that rats exposed to short-fiber preparations (NIEHS "short range" asbestos size) developed significant lung toxicity, as manifested by alterations in epithelium, interstitial space, and alveolar macrophages after 3 months of exposure. These toxic effects, however, were not as severe as those noted in rats which had inhaled "intermediate range" chrysotile asbestos. In another study, Sebastien *et al.* (1979) found a preponderance of short-fiber chrysotile (<5 μm) in the pleural tissue of humans with "asbestos-related disease."

The present study has shown that a naturally occurring short-fiber asbestos (RG 144) is cytotoxic to human alveolar macrophages *in vitro*. Per unit mass, the RG 144 preparation was more lethal for macrophages than the UICC chrysotile A reference sample, even though both samples had comparable numbers of asbestos fibers >5 μm in length. Furthermore, the cytotoxic effect was increased after milling for 60 sec, thus creating more short fibers, and was increased even more after 1200 sec of milling, to a point where it was virtually comparable to that of quartz. Since the milling process produced both an increase in the total number of fibers and an increase in the total surface area of the sample (Fig. 1), each or both

FIG. 1. Montage of transmission electron micrographs of UICC Rhodesian fiber (A), RG 144 Calidria fiber, as received (B), and RG 144 milled for 1200 sec (C). Fiber in A is about 5 μm in length. Magnification for each micrograph is the same. The Rhodesian fiber specimen consists of many fibers greater than 5 μm in length; RG 144, as received, contains fewer long fibers, as does the 1200-sec milled preparation. The selected area electron diffraction pattern (inset in C) shows the chrysotile structure was retained even after the 1200-sec milling treatment.

TABLE 2

PERCENTAGE VIABILITY OF HUMAN ALVEOLAR MACROPHAGES (HAM) AFTER 1-HR PULSE WITH MINERAL DUST AND 24-HR INCUBATION IN FRESH MEDIUM[a]

Culture	No. runs	Percentage viability	
		50 μg/culture	100 μg/culture
(1) HAM + no dust[b]	10	88.3	88.3
(2) HAM + UICC chrysotile A[b]	10	67.2	62.7
(3) HAM + unmilled RG 144 chrysotile[b]	10	47.4	42.0
(4) HAM + 60 sec. milled RG 144[c]	6	39.7	27.4
(5) HAM + 1200 sec. milled RG 144[c]	6	23.7	14.4
(6) HAM + quartz (Min-U-Sil 15)[b]	10	29.3	9.3

[a] Incubation conditions as described under Materials and Methods.

[b] SEM for all groups from analysis of variance was 3.05. All values in groups 1, 2, 3, and 6 were significantly different from each other at $P < 0.05$ by Duncan's multiple range test.

[c] SEM for all groups from analysis of variance was 4.02. There was a significant difference between the results obtained with the 60-sec milled and 1200-sec milled preparations ($F(1,15) = 12.99$, $P < 0.005$).

of these parameters may in part explain the enhanced cytotoxic action of milled asbestos on human alveolar macrophages.

No clearly defined dose–response relationship was observed in the dose range used. There are a number of possible explanations. It is conceivable that clumping of particles, or their removal after 1 hr of exposure, or adsorption of protein onto the particle surface may have modified the cytotoxic properties of the dusts at higher mass concentrations. It is of interest that a similar rank order of toxicity of these particulates was seen in preliminary experiments with rabbit alveolar macrophages (H. Yeager, unpublished data).

Differential phagocytosis of different fiber sizes is a possible bias in these experiments. Nevertheless, on light microscopy, fibers >5 μm in length were seen both in cultures treated with RG 144 and in those treated with the UICC preparation.

The findings in the present study are at variance with those reported elsewhere by other investigators. Thus Chamberlain *et al.* (1980) reported that ball-milled UICC chrysotile B (Canadian) was less cytotoxic to Chinese hamster lung cells than unmilled UICC chrysotile B. Such lack of biologic potential may have been produced as the result of a vigorous comminution process, since such has been shown to alter fiber structurally and blunt at least some of its activity (Langer *et al.*, 1978). Tilkes and Beck (1980), using short-fiber chrysotile prepared by physical separation (Spurny *et al.*, 1979), observed that "extra-fine" fibers were less toxic for phagocytic tumor cells than the "parent" fiber preparation.

The explanation for the differences among the various studies is not apparent. It is possible that the naturally occurring short fiber used in the present study is somehow unique and not representative of all short-fiber chrysotile asbestos. Other factors, such as differences in culture systems, doses used, etc., may have accounted for the discrepancies between our results and those of the aforemen-

tioned authors. Nevertheless, since the type of short fiber used by us is currently employed in the United States and has been shown to be cytotoxic to human alveolar macrophages *in vitro*, it would seem of importance that further studies of the possible harmful effects of this material should be pursued.

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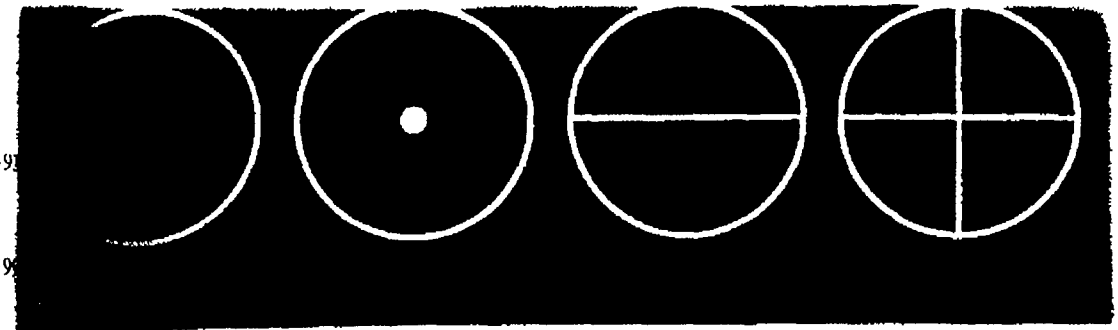

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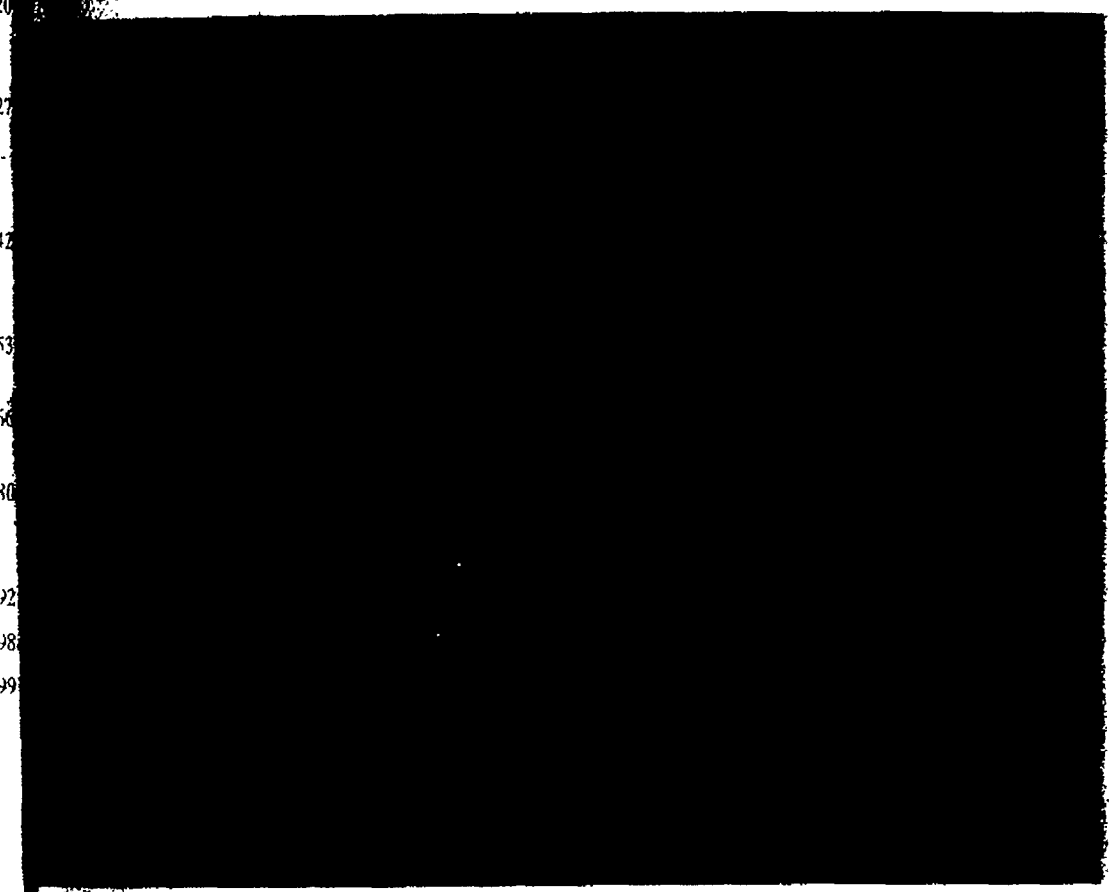

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