

DRUG AND CHEMICAL TOXICOLOGY, 10(1&2), 49-86 (1987)

CYTOTOXICITY OF RESPIRABLE DUSTS FROM INDUSTRIAL MINERALS:
COMPARISON OF TWO NATURALLY OCCURRING AND TWO MAN-MADE SILICATES

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INTRODUCTION

Foreword.

Because no single material has ever been found which could replace
chrysotile asbestos in its numerous diversified technological applications,
many different materials have been proposed or are currently used
extensively as substitutes. For technological reasons, the majority of the
substitute materials are intended for those applications where «short»
chrysotile fibers are traditionally used, i.e. in situations where
insulating properties or «inert» inorganic filler materials are desired.
Thus, while few naturally occurring short fibrous materials have been

proposed for asbestos substitution, numerous man-made materials have on the other hand been selected (e.g. «mineral wools»).

Related to this is the fact that comparatively few studies have been carried out that could provide indications as to the relevant merits of these newly introduced materials from a biological and health stand point. In view of the irrefutable evidences on the hazards to human health after exposure to «respirable» particles (for examples, see: «Biological effects of mineral fibers», 1980), many in vitro assays have been developed and used to study the response of target cells to mineral dusts (for examples, see: «The in vitro effects of mineral dusts», 1980). Therefore, because naturally occurring and man-made fibrous materials are often proposed as allegedly safe or safer substitutes for chrysotile asbestos, this fact prompted us to evaluate the relative in vitro cytotoxic response of rat pulmonary alveolar macrophages toward four industrial silicates. In order to establish a comparison between two in vitro assays, the membranolytic profiles of the mineral dusts were also evaluated.

Description of the Silicates Tested.

1. Naturally Occurring Silicates. Chrysotile asbestos is the well known hydrated magnesium silicate which has been in use for many decades. In order to comply with its different commercial uses, several grades of this natural silicate have been fashioned by the mining industry. For example, the so-called «Québec Standard» (QS) grade 3 fibers are very long and are used primarily in the manufacture of different types of yarns and rovings. In turn, these products are used in the manufacture of various textiles, such as protective gloves, clothings and fire blankets, woven-type clutch facings and gaskets, and electrical insulating tapes. Of increasingly shorter length, QS grades 4 and 5 fibers are typically used as reinforcing

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agents in the manufacture of fibre-cement products (pipes, sheets, roofing tiles, etc.). The «shorter» fibers representative of QS grades 6 to 9 are used primarily as fibrous inorganic filler in manufactured products such as thermoplastics compounds, flooring tiles, jointing cements, and asphalt roofing papers.

As found in these commercial grades, the dimensions (length and diameter) of the chrysotile fibers are generally far out of the «respirable» range and therefore, are not readily suitable for biological experimentation. For this reason, «respirable» or biologically pertinent fractions must be prepared from these commercial grades. The most well known are the UICC preparations, which have been obtained by various comminution and extraction methods applied to a mixture of different grades and sources (Timbrell and Rendall, 1971-1972). While these particular preparations represented a valuable effort to provide the investigators with standardized biologically pertinent asbestos samples, they may not be entirely representative of the actual «respirable» fibers which may be emitted from real life situations. Consequently, milder extraction procedures must then be devised to obtain unaltered fiber fractions (Langer et al., 1978).

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Attapulgit, a naturally occurring magnesium aluminium silicate, was named from Attapulgis, Georgia, USA, the source of the first sample studied in the mid-thirties. Attapulgit clay derives its non-swelling, needlelike morphology from its three-dimensional structure, and the shape and size of these needles result in remarkable properties such as a very high surface area (Haden, 1963). The commercial availability of attapulgit in large tonnage and at relatively low costs, has made it an important source of raw material for a variety of industries.

Originally developed for the petroleum industry (drilling muds), attapulgite finds its current largest uses in many unrelated applications such as a carrier, a diluent and a conditioner for agriculture chemicals. It is also used in adhesives, sealants, mastics, paints, pharmaceuticals, and many other applications such as animal bedding, polishes, wax emulsions, laundry washing powders and tape joint cements. Recently, it was even proposed as a component of an asbestos-free friction material. Contrary to many commercial grades of chrysotile, the dimensions of the American attapulgite fibers commercially available are already in the respirable range. For this reason, no further comminution and extraction are required for biological experimentation.

2. Man-Made Silicates. The term man-made mineral fibers (MMMF) embraces a variety of fibrous inorganic materials typically obtained from amorphous molten slags, certain types of rocks, and various glassy composites. Inevitably, the elemental composition vary from one type to another. But, in general, these fibers are either mainly aluminium or calcium silicates; minor components such as iron, boron or titanium oxides are also found associated with these silicates. The MMMF are known under a variety of different names such as «ceramic fibers», «processed mineral fibers», «kaowool», «fibrous ceramic aluminium silicate glass», «rock wool» «slag wool», «mineral wool», «glass fibers», and so on. In the present study, we selected a commercial grade of an aluminium silicate material: Fiberfrax®. This type of silicate is proposed as a substitute for chrysotile asbestos in applications where thixotropic properties are desired; it is also used as an additive to epoxies, plastic mixes, caulking compounds, fine paper production, and injection molding compounds.

In general, mineral and glass wools are formed by blowing a thin stream of the molten mass by means of high pressure air or steam jets.

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Centrifugal spinning is also used, where the melt is allowed to fall onto a high speed rotating disk. As a consequence, the diameters of these MMMF vary considerably. For example, slag wools may contain up to 60% of fibers with a diameter between 0-3 μm , while rock wools and glass wools may respectively contain 36% and 26% of such small diameter fibers. For this reason, extraction methods must also be used to obtain fibrous fractions biologically pertinent.

In contrast to the above-mentioned MMMF, a relatively new material has been introduced on the market for substitution of asbestos in temperature insulation products. In carefully controlled pressure and temperature conditions, the addition of CaO to SiO_2 will cause the production of fine calcium silicate fibers known as xonotlite (Funk, 1965). The dimensions of the fibers, being essentially 100% in the «respirable range», allow the use of this material as such in biological experimentations.

MATERIALS AND METHODS

Source of the Fibrous Silicates.

The separation of very short chrysotile fibers follows the method outlined by Jolicoeur et al. (1981). Briefly, 60 g of Johns-Manville grade 4T30 chrysotile fibers, slightly opened by two passes through a mechanical conditioner (B.O.P.), were mixed with 6 L of demineralized water, and inverted 20 times. After sedimentation, the fine suspension in the supernatant was concentrated by filtration, and the remaining water was removed by a freeze-drying process. The complete procedure will be outlined elsewhere (Kimmerle, Roberge and Nadeau, unpublished results).

The aluminium silicate sample used in this study is of the HSA (High Specific Area) grade of Fiberfrax®, obtained from The Carborundum Company.

Niagara Falls, NY, USA. A «short» fraction was extracted as follows: 20 g of the material was suspended in 7.5 L of demineralized water and inverted 10 times. After 24 h of sedimentation, 5.5 L of the supernatant were aspirated, care being taken not to remove the material floating at the surface. The colloidal suspension was then filtered on 0.8 μ filters (Millipore Ltd., Mississauga, Ontario, Canada), and dried to a cake under an infrared lamp. A typical extraction yields 120-130 mg/L of solid particles.

The clay mineral attapulgite was obtained from the Quincy deposits (Florida, USA) and the calcium silicate xonotlite was provided by a European supplier. The American attapulgite and the calcium silicate dusts were tested as supplied.

Physico-Chemical Characterization of the Silicates.

1. Surface Area and Zeta Potential. Specific surface area measurements were determined by measuring the quantity of nitrogen adsorbed, using the BET theory (Brunaueur et al., 1938). Before the measurements the samples were conditioned overnight at 110°C, under an helium gas stream (Quantasorb, model QS-10; Quantachrome Corp. Syosset, NY). Zeta potentials were determined in 1 mM KCl (pH7.0) on a Zeta Reader Konline-Sanderson (model ZR11, Noram, Pointe-Claire, Québec, Canada).

2. Size Distribution. For the length and diameter distributions, the following procedure was used: 0.5 mg of each fiber sample was resuspended (25 full strokes of a glass Dounce homogenizer; Dunnigan et al., 1980) in 2 ml of saline (0.19%, P/V) containing 25 μ g/ml of Tween 80 (Sigma Chemical Co., St. Louis, MO). After serial dilutions, the final concentration of Tween 80 was reduced to 1 μ g/ml and, before the filtration step, the fiber

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suspensions were adjusted to yield on the membrane 0.25 $\mu\text{g}/\text{cm}^2$ for chrysotile, 1.0 $\mu\text{g}/\text{cm}^2$ for attapulgite and xonotlite, and 3.0 $\mu\text{g}/\text{cm}^2$ for Fiberfrax. For the transmission electron microscopy (TEM) study, the carbon-coated Nuclepore technique was used (Anderson and Long, 1976; Chatfield et al., 1978). Fifty ml of each suspension were filtered either on a 0.4 μ or on a 0.1 μ Nuclepore membrane (47 mm; Maynard Scientific, Ste-Foy, Québec), with a usable surface of $\sim 10 \text{ cm}^2$. Sections of each membrane ($\sim 10 \text{ mm}^2$) were selected arbitrarily for the TEM study. Basically, after being deposited on 200-mesh copper grids (J.B. EM Services, Pointe-Claire, Québec), the membranes were first coated with carbon, then solubilized with chloroform. Representative pictures of each sample were taken on a Philips 200 TEM, and the length and diameter distributions were determined by computer-assisted image analysis (Bioquant, R & M Biometrics, Inc., Nashville, TN). According to the current convention, any particle presenting an aspect ratio (length/diameter) less than three to one was not counted as a fiber.

In Vitro Biological Assays.

1. Hemolysis. The hemolytic activities of the four silicates were evaluated as follows: red blood cells (RBC) were collected from whole blood drawn from the vena cava of the rats used for the isolation of the macrophages. The heparinized RBC were washed three times in a modified (330 mOsm instead of 290 mOsm) veronal buffer (Harrington et al., 1971) and then resuspended at 4% (V/V) in the same buffer (Dunnigan et al., 1980; Pelé et al., 1983). With a Dounce homogenizer, the fibers were resuspended in the veronal buffer (supplemented with 2 $\mu\text{g}/\text{ml}$ of Tween 80) at a concentration twice the final concentration desired.

For each assay, 1 ml of RBC was mixed with 1 ml of fibers in a siliconized scintillation glass vial capped with a gas permeable membrane

closure (P.M. Cap, type 20; Kimble/Owens-Illinois, Toledo, OH). Blank assays (no fibers) were also included to evaluate the non-specific hemolysis due to the mechanical stresses of the procedure (usually 1-2%). The vials were then incubated at 37°C in a reciprocal water bath set at 96 strokes/min. At any given time, the assays were stopped by the addition of 0.2 ml of 1% (V/V) glutaraldehyde. Following a centrifugation at 500 XG/10 min, the clear supernatants were immediately diluted 1:4(V:V) with the veronal buffer, and the absorbance (O.D.) values were determined at 541 nm. The percentage of release of hemoglobin was evaluated by comparison with a fully lysed sample (100% release), that is by sonicating for 20 sec (Heat Systems Sonicator Cell Disruptor, model W-220F set at level one; Bionetic Ltée/Ltd., St-Laurent, Québec) a blank assay before processing it as above. The percentage of hemolysis was calculated as follows:

$$\% \text{ Hemolysis} = \left[\frac{\text{O.D.}_{541} \text{ sample} - \text{O.D.}_{541} \text{ blank}}{\text{O.D.}_{541} \text{ lysed sample}} \right] \times 100 \quad (4.1)$$

2- Cytotoxicity. The target cells were obtained from 200-250g Long-Evans black hooded rats [male Cr1:(LE)BR]; Charles River Canada Inc., St-Constant, Québec), delivered in filtered cages, and immediately housed under a laminar flow enclosure (BIOCLEAN PC 580, Hazelton Systems, Aberdeen, MD). The animals were fed autoclaved rat chow, had access to water ad libitum, and were maintained under an alternating period of artificial light and darkness (LD 12:12), with light from 07.00 to 19.00 h. The rats were kept as such until the day of the experiment, which was usually within one or two weeks from their shipment. The day before the experiment, the rats were fasted overnight.

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Isolation of pulmonary alveolar macrophages (PAM). The animals were sacrificed with an overdose of sodium pentobarbital (250 mg/kg, i.p.), exsanguinated via the vena cava, and cannulated at the upper trachea level. The bronchoalveolar lavages (BAL), were carried out in situ (4 X 10 ml) with a modified Hanks' balanced salt solution (BSS) developed for that purpose. The composition of the (Ca,Mg)-free BSS is given in Table 1. Then, the BAL were centrifuged at 240 XG/10 min. The individual cell pellets were submitted to a brief osmotic shock to eliminate contaminating RBC. After pooling, the lung free cells (LFC) were washed twice with the BSS solution. The BAL usually yield $\sim 10^7$ LFC/rat, of which $\geq 95\%$ are viable PAM (Trypan blue dye exclusion test). For the establishment of the PAM monolayers, two protocols were used.

Fresh monolayers. In Linbro multi-well plate (24 wells, $\sim 2\text{cm}^2/\text{well}$; Flow Laboratories, Inc., McLean, VA), the LFC were seeded at 4×10^5 cells/well and incubated for 2 h at 37°C in water-saturated atmospheric air. The composition of our incubation medium is shown in Table 2. After this pre-incubation period, the cell monolayers were rinsed with fresh medium and then challenged for 18 h with the mineral fibers.

One day-old cultured monolayers. The cell monolayers were established as described above, with the exception that before being challenged with the fibrous silicates, PAM were allowed to incubate further for 24 h following the 2 h pre-incubation period. As with fresh PAM, cultured cells were rinsed with fresh medium and then exposed to the mineral fibers for 18 h.

Each fibrous material was autoclaved for 10 min at 121°C prior to its dispersion. The silicates were then resuspended in a saline-Tween 80 solution (0.19% and 25 $\mu\text{g}/\text{ml}$, respectively) with 25 full strokes of a Dounce homogenizer in order to have dispersions of the testing materials

TABLE 1. Composition of the (Ca,Mg)-Free BSS Developed for the BAL

Components	Concentrations	Conditions (37°C)
KCl ^a	0.40 mg/ml	
NaCl ^a	8.12 mg/ml	
NaHCO ₃ ^a	0.35 mg/ml	
KH ₂ PO ₄ ^a	0.06 mg/ml	
Na ₂ HPO ₄ ·7H ₂ O ^a	0.09 mg/ml	pH: adjusted to 6.80 ± 0.05
Na ₂ SO ₄ ^a	0.07 mg/ml	
Hepes (Ultral) ^b	2.38 mg/ml	
Glucose ^a	1.00 mg/ml	Osmolality: 298 ± 2 mOsm
Penicillin (base) ^c	100 U/ml	
Streptomycin (base) ^c	100 µg/ml	
Fungizone ^c	250 ng/ml	
Tetracycline (HCL) ^d	5 µg/ml	
Tylocine ^c	60 µg/ml	

(a) Fisher Scientific Co., Fair Lawn, NJ.

(b) Calbiochem-Behring, San Diego, CA.

(c) GIBCO Canada Ltd., Burlington, Ontario.

(d) Sigma Chemical Co.

similar to those used for the size distribution study. As with the hemolysis assay, the final concentration of Tween 80 in the cytotoxicity assay was 1 µg/ml. Two doses, 50 µg (33.3 µg/ml; 25 µg/cm²) and 250 µg (166.7 µg/ml; 125 µg/cm²) were selected for the evaluation of the cytotoxic effects of the silicates on PAM.

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TABLE 2. Composition of the Incubation Medium Developed
for the PAM Cytotoxicity Assay

Components	Concentrations	Conditions (37°C)
KCl ^a	0.40 mg/ml	
NaCl ^a	6.66 mg/ml	
CaCl ₂ ·2H ₂ O ^a	0.74 mg/ml	
MgCl ₂ ·6H ₂ O ^a	0.51 mg/ml	
MgSO ₄ ·7H ₂ O ^a	0.62 mg/ml	
NaHCO ₃ ^a	0.35 mg/ml	pH: adjusted to 6.80 ± 0.05
KH ₂ PO ₄ ^a	0.06 mg/ml	
Na ₂ HPO ₄ ·7H ₂ O ^a	0.09 mg/ml	Osmolality: 300 ± 5 mOsm
Hepes (Ultrol) ^b	2.38 mg/ml	
Fetal bovine serum ^c (heat-inactivated)	4% (V/V)	Incubation: water-saturated air
Glucose ^a	1.00 mg/ml	
Amino acids ^c	MEM (1X)	
Non-essential amino acids ^c	MEM (1X)	
Vitamins ^c	MEM (1X)	
L-Glutamin ^c	292 µg/ml	
Penicillin (base) ^c	50 U/ml	
Streptomycin (base) ^c	50 µg/ml	
Fungizone ^c	125 ng/ml	
Tetracycline (HCL) ^d	5 µg/ml	

- (a) Fisher Scientific Co.
 (b) Calbiochem-Behring.
 (c) GIBCO Canada Ltd.
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Biochemical Analyses.

For each type of fiber and every dose studied, duplicate cell incubations were made; incubation blanks (no fiber) were also included. After being centrifuged at 240 XG/10 min to obtain cell-free supernatants, the medium of one of the duplicate incubation was used for the determination of the extracellular enzyme leakages and the production of lactic acid. For the quantification of ATP, the very few cells recovered from the previous centrifugation were combined to their corresponding cell monolayer. The counterpart of the duplicate cell incubations was treated with Triton X-100 (final concentration: 0.1%), then sonicated briefly for 15 sec (Heat Systems Sonicator Cell disruptor, model W-10 set at level one; Bionetics Ltée/Ltd.) to determine the total enzymatic activities (cells + medium) and DNA contents.

1. Extracellular Enzyme Leakages. The two enzymatic markers of cytotoxicity were measured as follows:

LDH. L-lactate dehydrogenase (EC 1.1.1.27) was determined by a modification of the procedure of Wacker et al. (1960): NAD (10 mM) and lactic acid (70 mM) in TAPS buffer (85 mM), pH 8.5 (Beckman Canada Inc., Montréal, Québec). The assay is based on the rate of formation of NADH at 340 nm, and the activity is measured as milli- International Units (mIU)/ml.

β -GAL. The β -galactosidase (EC 3.2.1.23) assay is based on the procedures of Levy and McAllan (1963) and Christomanou et al. (1977): p-nitrophenyl-D-galactoside (4 mM) in citrate buffer (50 mM), pH 4.4. The reaction is stopped by the addition of 0.5 M glycine buffer (pH 10.5), and the activity is measured as nmoles of p-nitrophenyl released/min/ml.

2. Production of Lactic Acid. Lactate synthesis by PAM was evaluated by measuring the amount of the metabolite released into the incubation medium (Paradis and Nadeau, 1982).

3. Cellular ATP. PAM (cell pellet + monolayer) were successively treated with 1 ml of dimethyl sulfoxide (DMSO; Sigma Chemical Co.) and 3 ml of demineralized water to release the nucleotides. Following the extraction, the diluted DMSO extracts were immediately frozen in liquid nitrogen and kept at -60°C . The ATP was measured later by a bioluminescence assay, using the LKB Luciferin-Luciferase Monitoring Reagent (Fisher Scientific Co.). Before being assayed, freshly thawed samples were properly diluted with an Hepes buffer (25 mM, pH 7.75) supplemented with MgSO_4 (10 mM) and NaN_3 (0.02%, P/V). For the assay, 150- μl aliquots were mixed with 40 μl of ATP reagent, and the bioluminescence was recorded for 10 sec at room temperature with the integral mode of the luminometer (Berthold Biolumat LB 9500, Labserco Ltd., Oakville, Ontario). An internal standard of ATP (10 μl) was added to evaluate the presence of quenching factors (Turner Designs, 1981).

The ATP concentration was evaluated as follows:

$$\text{ATP (ng/ml)} = \left[\frac{\text{Volume STD}}{\text{Volume S}} \right] \times \left[\frac{\text{BLU} \times \text{Concentration STD}}{\text{BLS} - \text{BLU}} \right] \times \text{DIL} \quad (4.2)$$

where : STD = internal standard of ATP
 S = sample
 BLU = recorded bioluminescence of the sample
 BLS = recorded bioluminescence of the sample after the addition of the internal standard
 DIL = dilution of the sample

On average, 10^6 PAM contain ~1500 ng of ATP.

4. Cellular DNA. DNA contents were measured with a fluorescence enhancement technique, by modifying the method of Adams and Storrle (1981); their procedure is based on the lysis of cells with Triton X-100 and the use of the Hoechst dye #33258 (Calbiochem, La Jolla, CA). From a stock solution of the bisbenzimidazole fluorochrome (200 $\mu\text{g/ml}$) kept refrigerated in the dark, a working solution is made freshly the day of the experiment. The assay buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 7.0; Brunk et al., 1979) was set at a final dye concentration of 100 ng/ml, and kept protected from the light at all time.

The DNA standard stock solution, made with highly polymerised calf thymus DNA (Sigma Chemical Co.), was adjusted spectrophotometrically (O.D. at 260 nm for a 50 $\mu\text{g/ml}$ DNA solution = 1.0) and stored at 4°C. Daily, a standard curve (0-500 ng) was made by mixing one volume of the DNA standard stock solution with two volumes of a concentrate (1.6X) of the culture medium and 0.2 volume of Triton X-100 1.6% (P/V). As with the cell monolayers, each point of the standard curve was sonicated briefly for 15 sec (Brunk et al., 1979).

For the DNA determinations, 100- μl aliquots (triplicate) of the sonicated DNA standards or samples were mixed with 3 ml of the assay buffer. The fluorescence intensity was measured on a Perkin-Elmer Luminescence Spectrometer (model LS-5; Perkin-Elmer Canada Ltd., Montréal, Québec), with the excitation and emission wavelengths respectively set at 350 and 455 nm. As recommended by Downs and Wilfinger (1983), 10 nm slit widths were used. Although the DNA determinations were usually done on freshly sonicated cell samples, it was found that the measurements could be postponed up to 48 h later if EDTA (100 mM, final concentration) was added to the sonicated samples to inhibit DNases (Junowicz and Spencer, 1973). Moreover, since we noted an interference of the attapulgitic fibers on the

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intensity of the fluorescence reaction, probably by adsorption of the fluorochrome, it might be necessary to spin down some cellular extracts before taking the 100- μ l aliquots for the DNA determinations. On average, 10^6 PAM contain ~10 μ g of DNA.

Statistical analyses.

The experimental data were evaluated first with a one-way analysis of variance (Tallarida and Murray, 1981). Then, when the F-values were significant, the fiber-exposed cell monolayers were compared to their respective control incubations by performing a Dunnett's multiple comparison test (Tallarida and Murray, 1981). This test was selected for its ability to compare several treated groups to a single control group (Dunnett, 1955). The level of significance was set at $p \leq 0.05$ and, for each parameter evaluated, no statistical difference was ever observed between the two control groups (one for each fiber concentration) of either incubation condition (fresh or one day-old cultured cell monolayers).

RESULTS

Physico-Chemical Properties of the Industrial Silicates.

As seen in Fig. 1 to 4, the TEM micrographs show that three of the four silicates are in a close dimensional range, that is chrysotile, attapulgite and xonotlite. Quantitative size analysis (Table 3) revealed that the average fiber length of these three minerals was ~1.0 μ m. The diameters were also very similar, with the exception of the xonotlite sample which had an aspect ratio about half the value of the chrysotile and attapulgite fibers. All the short chrysotile fibers had a length ≤ 5.0 μ m and a diameter ≤ 0.2 μ m. The attapulgite fibers were $100\% \leq 1.0$ μ m in length

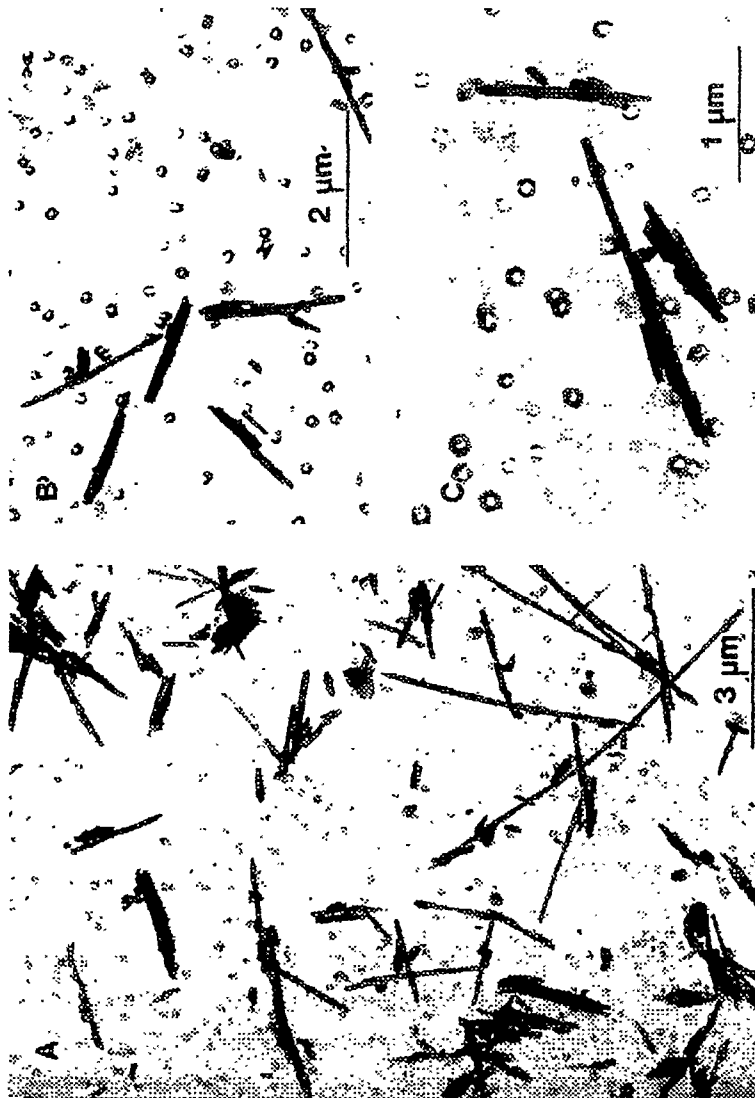


Fig. 1 Transmission Electron Micrograph of the «Very Short» Fiber Fraction of Chrysotile Isolated by Water Sedimentation. (A) X 8,000; (B) X 11,600; (C) X 20,700.

Fig. 1 Transmission Electron Micrograph of the «Very Short» Fiber Fraction of Chrysotile Isolated by Water Sedimentation. (A) X 8,000; (B) X 11,600; (C) X 20,700.



Fig. 2 Transmission Electron Micrograph of the American Attapulgitic Fibers. (A) X 10,000; (B) X 11,600; (C) X 20,700.



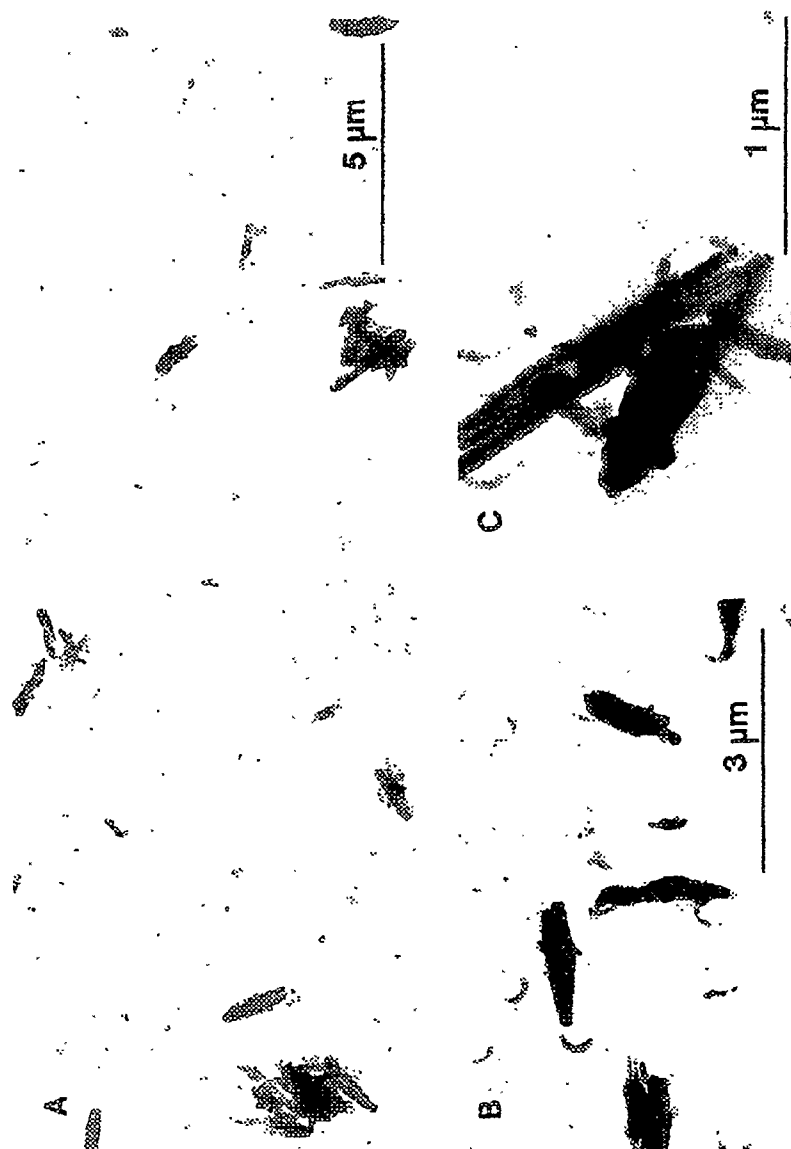


Fig. 3 Transmission Electron Micrograph of the Calcium Silicate Xonotlite Fibers. (A) X 6,400; (B) X 11,600; (C) X 34,100.



Fig. 3 Transmission Electron Micrograph of the Calcium Silicate Xonotlite Fibers. (A) X 6,400; (B) X 11,600; (C) X 34,100.



Fig. 41 Transmission Electron Micrograph of the Aluminum Silicate Fiberfrax «Short» Fiber Fraction Isolated by Water Sedimentation. (A) X 2,100; (B) X 6,400.

TABLE 3. Physico-Chemical Properties of the Fibrous Silicates

Types of Silicate ^a	Average Measurements			Intrinsic Properties	
	Length (μm)	Diameter (μm)	Aspect Ratio	Specific Surface Area (m^2/g)	Zeta Potential (mV)
Chrysotile	0.9	0.1	12.3	36 (17) ^c	+ 45 (+34) ^c
Attapulgite	0.8	0.1	15.3	133	- 29
Xonotlite	1.2	0.2	5.8	42	- 24
Fiberfrax ^b	8.3	0.2	38.5	15 (4) ^c	- 43 (-38) ^c

(a) As specified in Material and Methods, the attapulgite and xonotlite samples were used as obtained, while the chrysotile and the aluminum silicate samples were fractionated in demineralized water to recover a «short» fiber fraction.

(b) This «short» fiber fraction contains ~60% of non-fibrous particles with diameters ranging between 1.4 and 3.3 μm .

(c) The values in parentheses represent the properties of the starting material from which each «short» fiber fraction was isolated.

and $\leq 0.1 \mu\text{m}$ in diameter. For the xonotlite sample, 100% of the fibers had a length $\leq 3.0 \mu\text{m}$ and a diameter $\leq 0.70 \mu\text{m}$. The Fiberfrax sample was more heterogenous in size and, contrary to the other three silicates which did not contain any fiber longer than $5 \mu\text{m}$, only 50% of the aluminium silicate fibers were in this size range (data not shown). The diameters of the Fiberfrax fibers ranged from 0.2 to $1.0 \mu\text{m}$, and the mean aspect ratio was ~ 39 (Table 3).

As expected, the chrysotile sample has a positive surface charge due to its brucite layer (Table 3). The other silicates have a typical negative charge, the highest value being for the aluminium silicate sample. In decreasing order of magnitude, the specific surface areas were: attapulgite > xonotlite > chrysotile > Fiberfrax (Table 3). One can also observe that the isolation of «short» fractions of chrysotile and Fiberfrax caused a two-to fourfold increase in the specific surface area of these two samples.

Biological Effects of the Fibrous Silicates.

As seen in Table 4, the chrysotile, attapulgite and xonotlite samples were markedly hemolytic to the rat erythrocytes. Comparatively, the Fiberfrax particles did not show any significant hemolytic activity, even at the highest dose tested ($1000 \mu\text{g/ml}$). Chrysotile hemolysis was the strongest, followed very closely by xonotlite; attapulgite fibers were consistently slightly less hemolytic than the other two silicate fibers. In view of the different surface charges between these mineral dusts (Table 3), it is interesting to note that both negatively and positively charged minerals are able to induce hemolysis. Similarly, there appears to be no straightforward relationship between the fiber surface areas (Table 3) and the hemolytic activities. Nonetheless, it is well known

between 1.4 and 3.3 μm .

(c) The values in parentheses represent the properties of the starting material from which each «short» fiber fraction was isolated.

TABLE 4. Membranolytic Profiles of the Fibrous Silicates

Types of Silicate ^a	Dose of Fibers ($\mu\text{g/ml}$)				Percentage of Hemolysis ^b			
	100	250	500	1000				
Chrysotile	26.5 $\pm$ 1.9	52.0 $\pm$ 3.4	74.3 $\pm$ 2.5	82.0 $\pm$ 2.8	87.8 $\pm$ 2.2			
Attapulgite	11.8 $\pm$ 2.4 ^c	29.3 $\pm$ 4.4 ^c	49.8 $\pm$ 8.3 ^c	62.0 $\pm$ 7.1 ^c	75.0 $\pm$ 7.7 ^c			
Xonotlite	19.8 $\pm$ 2.1 ^c	40.8 $\pm$ 1.7 ^c	64.0 $\pm$ 2.7 ^c	74.5 $\pm$ 1.7	82.0 $\pm$ 2.6			
Fiberfrax	0	0	0	0	2.0 $\pm$ 0.3			

(a) See legend Table 4.3

(b) The data represent the mean values $\pm$ S.D. ($n = 4$), after 60 min of incubation.(c) $p \leq 0.05$ when compared to chrysotile (Dunnett's multiple comparison test, two-sided, using chrysotile as the control group).

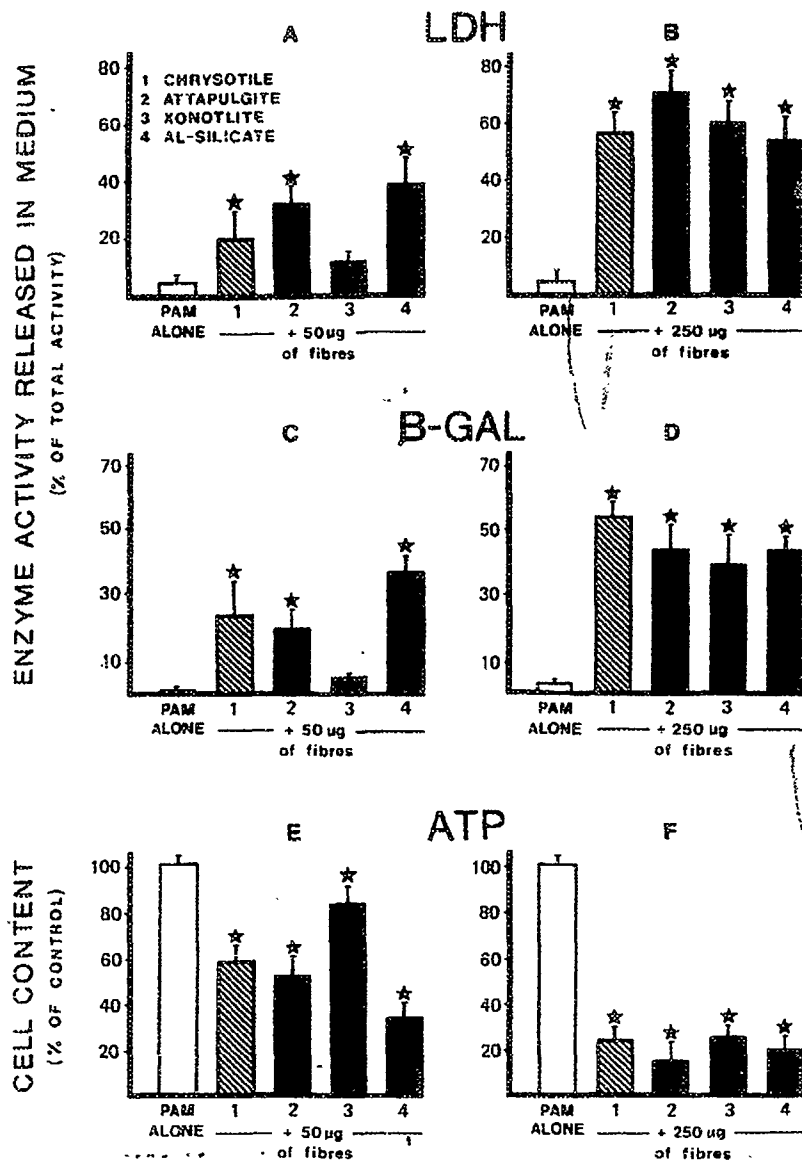
that both factors can participate in the membranolytic effect of mineral dusts (Harrington et al., 1971; Light and Wei, 1977; Schnitzer and Pundsack, 1970; Morgan et al., 1977; Jaurand et al., 1979²).

The *in vitro* cytotoxic effects of the silicates are illustrated in Fig. 5 and 6. Using fresh PAM monolayers (Figure 5), one can observe that at the highest dose (250 μ g), all four mineral dusts were highly cytotoxic. The extracellular releases of the enzymatic markers LDH and β -GAL were respectively ~60-70% and ~40-50% (Fig. 5B and 5D). The resulting low viability of the cells was confirmed by the 80% decrease in ATP cell contents (Fig. 5F). When exposed to a lower dose of mineral dust (50 μ g), only chrysotile, attapulgite and the aluminium silicate fibers presented a significant cytotoxic effect, as judged by the enzyme releases and the ATP cell contents (Fig. 5A, 5C and 5D). Nonetheless, the xonotlite fibers can also be considered slightly toxic at this dose, since the ATP contents of the exposed-cell monolayers were significantly reduced by ~20%. It is interesting to note that at either dose of the attapulgite, the calcium and the aluminium silicate fibers, the LDH releases were always higher than for the lysosomal enzyme. For the chrysotile fibers, the magnitude of the enzyme releases were roughly similar.

Using a more common experimental protocol, the cytotoxicity of the silicate dusts was then evaluated on one day-old cultured PAM monolayers (Fig. 6). Again, one can observe that all four fibers were cytotoxic at 250 μ g (Figures 6B, 6D and 6F). However, it is striking to see that, with older cells, the cytotoxicity of the chrysotile fibers was greatly reduced (Fig. 5 vs. Fig. 6). Moreover, at 50 μ g (Fig. 6A, 6C and 6D), the chrysotile dust induced only a «selective» release of the lysosomal marker of β -GAL (Davies et al., 1974), while both the attapulgite

(b) The data represent the mean values $\pm$ S.D. (n=4), after 60 min of incubation.
(c) $p \leq 0.05$ when compared to chrysotile (Dunnett's multiple comparison test; two-sided, using chrysotile as the control group).

RAT PULMONARY MACROPHAGE RESPONSE (FRESH MONOLAYER)



SPONSE



and aluminium silicate fibers were still highly cytotoxic. At this low dose, and in these conditions, the calcium silicate fibers were not very toxic to the cells.

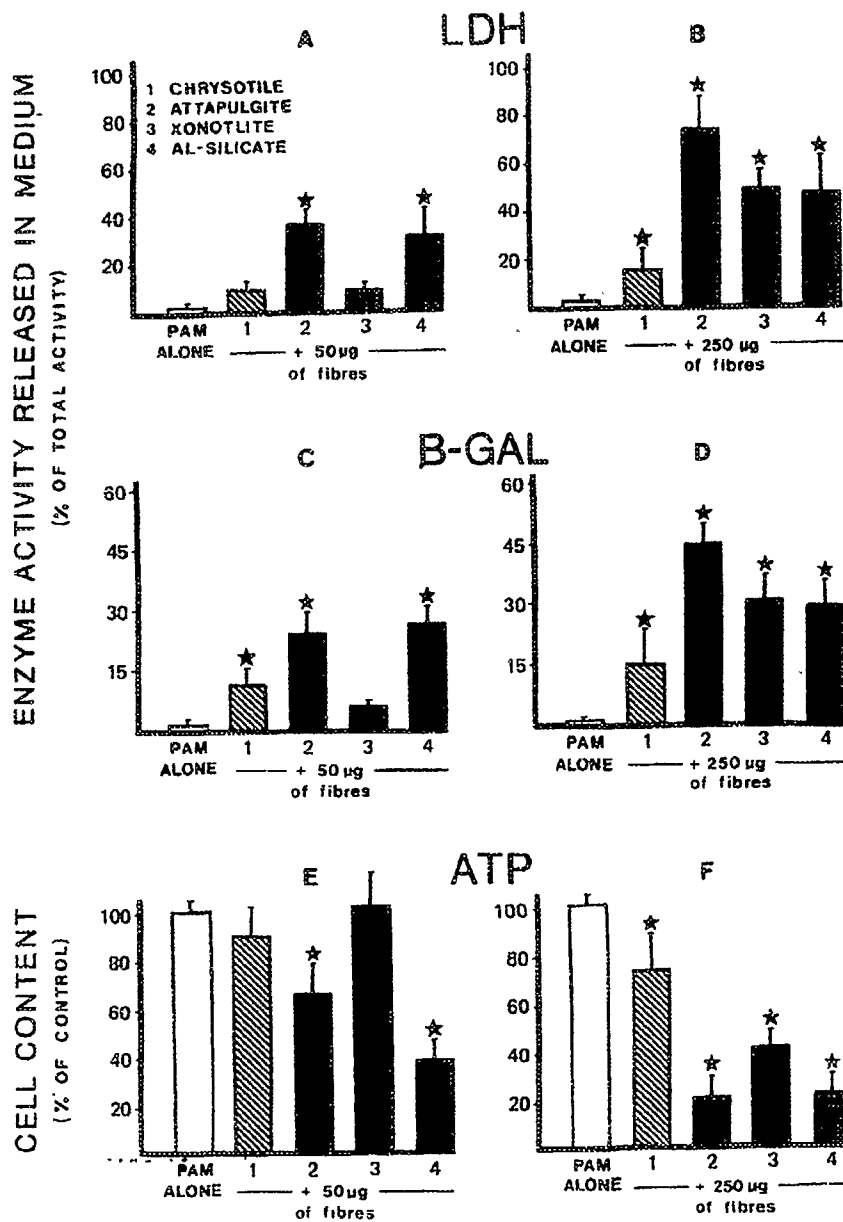
These results are in agreement with our earlier observations to the effect that tissue culture conditions can modulate the *in vitro* response of PAM toward mineral or man-made fibers (Nadeau et al., 1983^a, Dunnigan et al., 1984). For the evaluation of the viability of the cell after the challenge with the mineral dusts, two parameters were used: the ATP cell content (Fig. 5 E,F and 6 E,F), and the production of lactic acid (Table 5). Although useful for an estimation of the metabolic activity of PAM, one can see that the production of lactate is not as sensitive as ATP, especially with older cells. With a low dose of fibers, slight stimulations of the cell metabolism can even be observed (Table 5). Finally, no significant effect of the silicate fibers on total ADN cell contents was noted (data not shown).

DISCUSSION

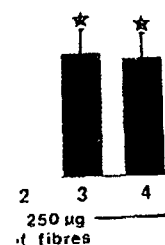
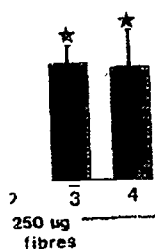
The significance of *in vitro* cytotoxicity data on mineral dusts cannot be easily extrapolated to their *in vivo* pathogenic potential. However, several authors have examined the correlation that exists between the *in vitro* and *in vivo* biological reactivity of many mineral dusts (Morgan et al., 1977; Brown et al., 1978; Chamberlain et al., 1979; Richards et al., 1980; Davis et al., 1985). For the assessment of the biological effects of industrial dusts, it is generally recognized that one should always use

Fig. 5 Cytotoxic Effects of Chrysotile, Attapulgit, Xonotlite and Fiberfrax (Al-Silicate) Fibers on Rat PAM. Releases of Lactate Dehydrogenase (LDH; A and B) and β -Galactosidase (β -GAL; C and D), and ATP Contents (E and F) of Freshly Cultured Cells Exposed to 50 μ g (A, C and E) and 250 μ g (B, D and F) of Mineral Fibers.

RAT PULMONARY MACROPHAGE RESPONSE (MONOLAYER CULTURED FOR 24 HRS)



SPONSE



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multiple assay systems and parameters. However, the utilisation of short-term *in vitro* assays like hemolysis and PAM cultures are of scientific relevance, because cytotoxicity tests can provide practical informations on the pathogenic events linked to the exposure of an organism to «respirable» particles (Tilkes, 1985).

Three of the four silicates tested certainly fall into that dimensional range: the chrysotile, xonotlite and attapulgite samples are 100% respirable. For the Fiberfrax sample, the classification is less well defined; nevertheless, our «short» fraction can be considered highly respirable. If we look first at the aluminium silicate fibers, we found that this sample was non-hemolytic, but highly cytotoxic. This duality of the responses between the two *in vitro* assays is not uncommon: for example, the amphibole asbestos fibers are very well known to be poorly hemolytic while being highly cytotoxic to macrophages (Harrington, 1976; Davies, 1980). While hemolytic dusts cannot be automatically classified as fibrogenic (Manyai et al., 1969), inversely, non-hemolytic or poorly hemolytic minerals dusts are not necessarily non-fibrogenic (Richards et al., 1980). On the other hand, dusts cytotoxic to macrophages *in vitro* usually cause fibrosis *in vivo* (Marks and Nagelschmidt, 1959; Styles and Wilson, 1973; Davies et al., 1980). It is not known at this time if the presence of non-fibrous particles in the Fiberfrax sample can account for the observed cytotoxic effect. However, the presence of long and thin fibers in the aluminium silicate sample is certainly an important factor to consider (Chamberlain et al., 1979). Notwithstanding the morphology of the Fiberfrax sample, it is interesting to

Fig. 6 Cytotoxic Effects of Chrysotile, Attapulgite, Xonotlite and Fiberfrax (Al-Silicate) Fibers on Rat PAM. Releases of Lactate Dehydrogenase (LDH; A and B) and β -Galactosidase (β -GAL; C and D), and ATP Contents (E and F) of One Day-Old Cultured Cells Exposed to 50 μ g (A, C and E) and 250 μ g (B, D and F) of Mineral Fibers..:

TABLE 5. Production of Lactic Acid by PAM Exposed to the Fibrous Silicates

		Culture Conditions	
		Fresh Monolayer	1 Day-Old Monolayer
		Percentage of Production ^a	
Controls		100 ± 14.7 ^b	100 ± 7.3 ^c
Chrysotile	50 µg	78.7 ± 20.7	106.6 ± 12.3
	250 µg	39.9 ± 31.4 ^d	102.0 ± 12.6
Attapulgate	50 µg	62.7 ± 20.1 ^d	81.2 ± 5.5
	250 µg	28.9 ± 12.5 ^d	57.6 ± 11.7 ^d
Xonotlite	50 µg	95.5 ± 13.9	113.7 ± 7.6
	250 µg	36.2 ± 17.6 ^d	87.5 ± 9.1
Fiberfrax	50 µg	71.7 ± 16.8	94.0 ± 17.4
	250 µg	55.4 ± 28.0 ^d	82.0 ± 20.0

(a) The data represent the mean value ± S.D. (n = 5).

(b) For this set of conditions, the mean production of lactic acid was 121.7 µg.

(c) For this set of conditions, the mean production of lactic acid was 184.2 µg.

(d) p<0.05 when compared to the untreated cells (controls).

note that aluminium silicate fibers have recently been reported to be both fibrogenic and carcinogenic to rats (Davis et al., 1983).

is Silicates

id Monolayer

$\pm 7.3^c$

± 12.3

± 12.6

± 5.5

$\pm 11.7^d$

± 7.6

± 9.1

± 17.4

± 20.0

The direct comparison of the other three fibrous silicates, namely attapulgite, xonotlite and chrysotile, is facilitated considering their average dimensions. As shown before, «very short» chrysotile fibers were found to be both hemolytic and cytotoxic (Dunnigan et al., 1980; Nadeau, 1981; Pelé et al., 1983). Similarly, our results confirm that attapulgite fibers from various sources are highly hemolytic and cytotoxic (Jaurand et al., 1979^b; Nadeau, 1981; Nadeau et al., 1983^b; Harvey et al., 1984), and can even stimulate squamous metaplasia *in vitro* (Woodworth et al., 1983). Calcium silicates particles are also known to be biological active *in vitro* (Hunt et al., 1981; Skaug et al., 1984). However, contrary to chrysotile and attapulgite, which have a unequivocally fibrous nature, the morphology of this type of mineral dust vary greatly with its origin (Skaug et al., 1984). Nonetheless, these authors showed that a highly fibrous synthetic tobermorite containing xonotlite was the most cytotoxic sample among the five types of calcium silicates tested.

Although the biological relevancy of «short» fibers is very often questioned, recent reports state clearly that shorter fibers should not be considered innocuous, but rather that the biological reactivity of fibers is diminishing with fiber length (Gormley et al., 1985; Davis et al., 1985). In fact, the «very short» chrysotile fibers used in this study have been shown to produce some degree of alveolitis both in the rat (Lemaire et al., 1985) and in the sheep (Bégin et al., in press). In the rat model, although no fibrotic lesions like those induced by the UICC 8 chrysotile fibers were apparent, the alveolitis persisted during the 60 days of the experiment; 9 months after the single 5-mg exposure, the alveolitis reaction was still present in some animals (I. Lemaire, personal communication). In the sheep

model, similar results were seen 60 days after the exposure to a single 100-mg dose of the same short chrysotile fibers; yet, some areas of early fibrosis were even observed, although to a lesser degree when compared to the longer UICC B fibers. In their study, Bégin et al. also examined the lung reactivity to the American attapulgite. They concluded that attapulgite fibers can induce lesions comparable to those of short chrysotile fibers, except that early peribronchiolar lesions were seen more often with the attapulgite sample. Although of lower intensity than those of the highly fibrogenic UICC B chrysotile sample, they nonetheless predicted that the lung lesions induced by the American attapulgite fibers should have a fibrotic evolution (Lee et al., 1981). So, at least as far as pulmonary fibrosis is concerned, there is a growing interest to the effect that various forms of attapulgites could present in humans a risk similar to asbestos (Sors et al., 1979; Bignon et al., 1980; Bégin et al., in press).

These in vivo findings are particularly important in view of our in vitro data. The density of the mineral dusts are: chrysotile, 2.5 g/cm^3 (Jolicoeur et al., 1981); attapulgite, $\approx 2.5 \text{ g/cm}^3$ (Haden, 1963); Fiberfrax, 2.7 g/cm^3 (The Carborundum Company, 1984); xonotlite, 2.7 g/cm^3 (Kudoh and Takéuchi, 1979). So, in combination with the data in Table 4.3, on a basis of fiber number rather than weight, one should expect to have a cytotoxicity scale like: attapulgite $\approx$ chrysotile $>$ xonotlite $\gg$ Fiberfrax. However, with the exception of the aluminium silicate sample, which contains long and thin fibers potentially more cytotoxic (Brown et al., 1978; Beck and Tilkes, 1980; Gormley et al., 1985), the decreasing order of cytotoxicity observed for the other three short silicates was: attapulgite $\gg$ xonotlite $>$ chrysotile. This ranking, based on the relative potency of each dust with the two PAM assays, correlate very well with the available in vivo data on the short chrysotile and American attapulgite fibers (Lemaire et al., 1985; Bégin et al., in press).

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In conclusion, it is believed that the combined use of fresh and cultured PAM for the evaluation of the in vitro biological activity of mineral dusts may be useful to extrapolate their in vivo pathogenicity. The direct comparison of different «respirable» dusts can then be achieved, more so if the particles tested have similar morphologies.

SUMMARY

The membranolytic and cytotoxic properties of two naturally occurring (chrysotile asbestos; attapulgitic clay) and two man-made (Fiberfrax[®], an aluminium-silicate, and xonotlite, a calcium silicate) industrial minerals were compared. «Short» fiber fractions of chrysotile and Fiberfrax were obtained by sedimentation in demineralized water, while the attapulgitic and xonotlite samples were used as obtained. The aluminium silicate fibers were found to be non-hemolytic, while for the other three silicates, chrysotile had the strongest hemolysis potential, followed very closely by xonotlite; attapulgitic was less hemolytic than the former two silicates, but was nevertheless highly hemolytic to the rat erythrocytes.

Using rat pulmonary alveolar macrophages, the in vitro cytotoxicity assays showed that with fresh cell monolayers, all four silicates were equivalent in causing cell damages at a dose of 250 µg; at a lower dose (50 µg), the intensity of the cytotoxic effect was in the decreasing order: Fiberfrax > attapulgitic > chrysotile > xonotlite. With one day-old cultured cell monolayers, a dose of 250 µg of the silicates fibers was less cytotoxic, with the exception of the attapulgitic fibers which remained essentially as cytotoxic as with the fresh cell monolayers. The reduced cytotoxic response was especially noticeable with the chrysotile fibers. At 50 µg, the cytotoxicity scale of the mineral dusts with one day-old cell monolayers was essentially the same as the one obtained with the fresh cell monolayers, that is: Fiberfrax ≈ attapulgitic > chrysotile > xonotlite.

Overall, these in vitro tests imply: 1) that all four industrial silicates tested can be considered to be «biologically active»; 2) that on the basis of their different reactivities with the two types of cell culture conditions used, their biological reactivity in vivo might be quite distinct. This might be especially true for at least the chrysotile, attapulgite and xonotlite short fibers, considering that these three types of silicate dusts have very similar dimensions. Moreover, for the chrysotile and attapulgite samples, fiber numbers is probably not an important factor, since the density of the two silicates is roughly the same. The unaltered cytotoxic responses of the American attapulgite fibers in the two macrophage assays correlate well with the fact that short attapulgite fibers seems to have a stronger in vivo reactivity than short chrysotile fibers. In fact, it might be the strongest of the four industrial silicates tested. On that basis, it is concluded that the combined use of fresh and cultured PAM for an in vitro assay may be useful to extrapolate the pathogenicity of «respirable» dusts, more so if the comparison is done with particles with similar morphologies.

ACKNOWLEDGMENTS

We wish to thank Mr. Bruno Gagné¹, Mr. Alain Lemieux², Ms. Irène Lévesque¹, Mr. Daniel Ouellet³ and Ms. Martine Senneville¹ for their able technical assistance, and Ms. Ginette Prince³ for typing this manuscript. This work was supported by L'Institut de l'amiante, Division de la recherche (Grant #A19).

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