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COMPARATIVE CYTOTOXIC EFFECTS OF CROCIDOLITE AND ITS NON-ASBESTIFORM POLYMORPH ON RAT ALVEOLAR MACROPHAGES*

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Abstract—The objective of this study was to compare the cytotoxicity of an asbestiform mineral, crocidolite, to that of its non-asbestiform analogue, riebeckite. Crocidolite had a median fibre length of 11.5 μm and a surface area of 17 $m^2 g^{-1}$ while the count median diameter of riebeckite was 3 μm with a surface area of 2.7 $m^2 g^{-1}$. Crocidolite samples contained 20–40 times more particles per mass than riebeckite samples. Cytotoxicity was determined by treating rat alveolar macrophages in culture with various concentrations of particles for 1–3 days and monitoring membrane integrity, leakage of cytoplasmic and lysosomal enzymes and zymosan-stimulated respiratory burst activity. In general, crocidolite exhibited cytotoxicity at doses as low as 100 $\mu g\ ml^{-1}$. Riebeckite was cytotoxic in most assays at 300 $\mu g\ ml^{-1}$. Therefore, on a mass concentration basis, crocidolite was more toxic than riebeckite. On an equivalent surface area basis, riebeckite was slightly more toxic than crocidolite. On an equivalent particle count basis, riebeckite was far more toxic.

INTRODUCTION

INHALATION of asbestos fibres has been associated with pulmonary fibrosis, lung cancer and mesothelioma (DEMENT *et al.*, 1986). STANTON *et al.* (1981) proposed that fibre dimensions were the major factor in carcinogenicity, i.e. although they tested particles of various chemical, crystallographic and morphological structures, a significant correlation was demonstrated between carcinogenicity and the number of particles having a length >8 μm and a diameter <1.5 μm. In addition, WRIGHT and KUSCHNER (1977) reported that fibrogenicity also depended on fibre length rather than fibre type. Similarly, BROWN *et al.* (1978) reported that *in vitro* activity of particles depended on fibre length.

Other researchers have attributed the cytotoxicity of asbestos to the presence of metals in their chemical structure. Indeed, removal of magnesium from chrysotile by acid leaching or chelation resulted in a decrease in its cytotoxicity (MORGAN *et al.*, 1977; HARINGTON *et al.*, 1971). Others have proposed that iron associated with crocidolite contributes to its cytotoxicity via the production of reactive oxygen species through an iron-catalysed Haber–Weiss reaction (TURVER and BROWN, 1987; SHATOS *et al.*, 1987; GARCIA *et al.*, 1988).

As indicated by the discussion above, the relative importance of chemistry vs morphology in determining the pathogenicity of particles is still unresolved. Therefore, the objective of the present investigation was to compare the cytotoxicity of crocidolite (an asbestiform mineral) to that of riebeckite (the non-asbestiform analogue of the

*This paper was included in Poster Session 4 and the discussion included in the summary presented in Section 12.

same mineral). Cytotoxicity was determined by treating rat alveolar macrophages in culture with these minerals and monitoring the effects on cellular viability and function.

METHODS

Crocidolite and its non-asbestiform polymorph, riebeckite, were obtained from a NIOSH reference mineral set in which samples were milled to provide particles of a size range which was more representative of that encountered in occupational environments. Transmission electron microscopy clearly showed that crocidolite samples were fibrous while those of riebeckite were rectangular (Fig. 1). Particle dimensions were determined by light microscopy. The median fibre length of the crocidolite particles was 11.5 μm while the count median diameter of riebeckite particles was 3 μm. It should be noted that small fibres would be undercounted using light microscopy. Surface areas, measured by the gas adsorption method, were 17.1 $m^2\ g^{-1}$ for crocidolite and 2.7 $m^2\ g^{-1}$ for riebeckite. Estimates of particle counts per mass of material were determined by electron and light microscopy and indicate that crocidolite samples contained 1.3×10^9–1.6×10^9 particles per mg while riebeckite samples contained 3.6×10^7–6.3×10^7 particles per mg. Based on these estimates, crocidolite samples contained 20–40 times as many particles per unit mass as the riebeckite samples.

The cytotoxicity of crocidolite and riebeckite was determined by monitoring their effects on alveolar macrophages in culture. Particles were sterilized at 160°C for 90 min and then suspended in Medium 199. Crocidolite was added to cell cultures at final concentrations of 50, 100, 200 and 300 $\mu g\ ml^{-1}$ and riebeckite was tested at 200, 300 and 1000 $\mu g\ ml^{-1}$. Cell viability and function were monitored after 1, 2 or 3 day treatments with particles by measuring trypan blue exclusion, zymosan-stimulated oxygen consumption and hydrogen peroxide secretion and supernatant levels of lactate dehydrogenase and α-galactosidase.

Alveolar macrophages were obtained by bronchoalveolar lavage of specific pathogen-free male Sprague-Dawley rats (200–250 g). Briefly, animals were anaesthetized with sodium pentobarbital (200 mg kg^{-1} body weight). The trachea was cannulated and the lungs lavaged 10 times with 8 ml aliquots of Ca^{2+}–Mg^{2+}-free phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, 5.5 mM dextrose; pH = 7.4). Cells were centrifuged at 500 ***g*** for 5 min and resuspended for determination of cell number and purity using an electronic cell counter equipped with a cell sizing attachment. Cells were then centrifuged and resuspended in Medium 199 supplemented with 1 mM glutamine, penicillin (100 units ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$), kanamycin (100 $\mu g\ ml^{-1}$) and 10% fetal bovine serum. Alveolar macrophages (1×10^7 cells per 4 ml) were transferred to 25 cm^2 Corning cell culture flasks and incubated for 1.5–2 h at 37.5°C and 80% relative humidity. After this pre-incubation period, 1 ml of Medium 199 or particles was added to the control or test culture flasks, respectively. After 1, 2 or 3 days macrophages were harvested by cooling the flasks to 2°C and shaking vigorously. The harvested cell suspensions were then centrifuged at 500 ***g*** for 5 min. The supernate was saved for analysis of enzymes and the cells were suspended in HEPES-buffered medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, 5.5 mM dextrose; pH = 7.4).

Cell counts and viability were determined microscopically (PHILLIPS, 1973). An aliquot of cells was mixed with 0.04% (w/v) trypan blue for 4 min before the addition of

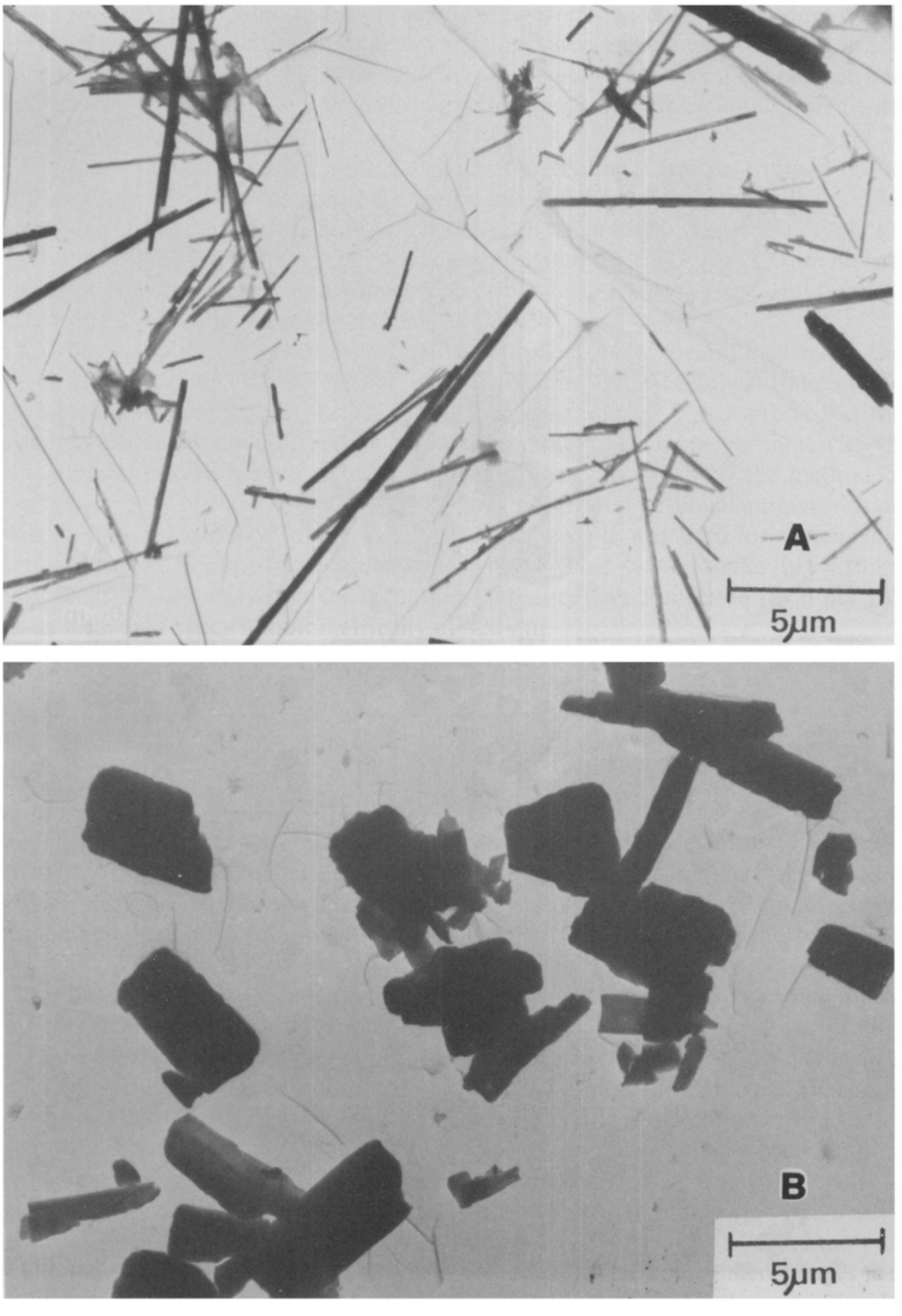

FIG. 1. Transmission electron micrographs of crocidolite (A) or riebeckite (B) samples.

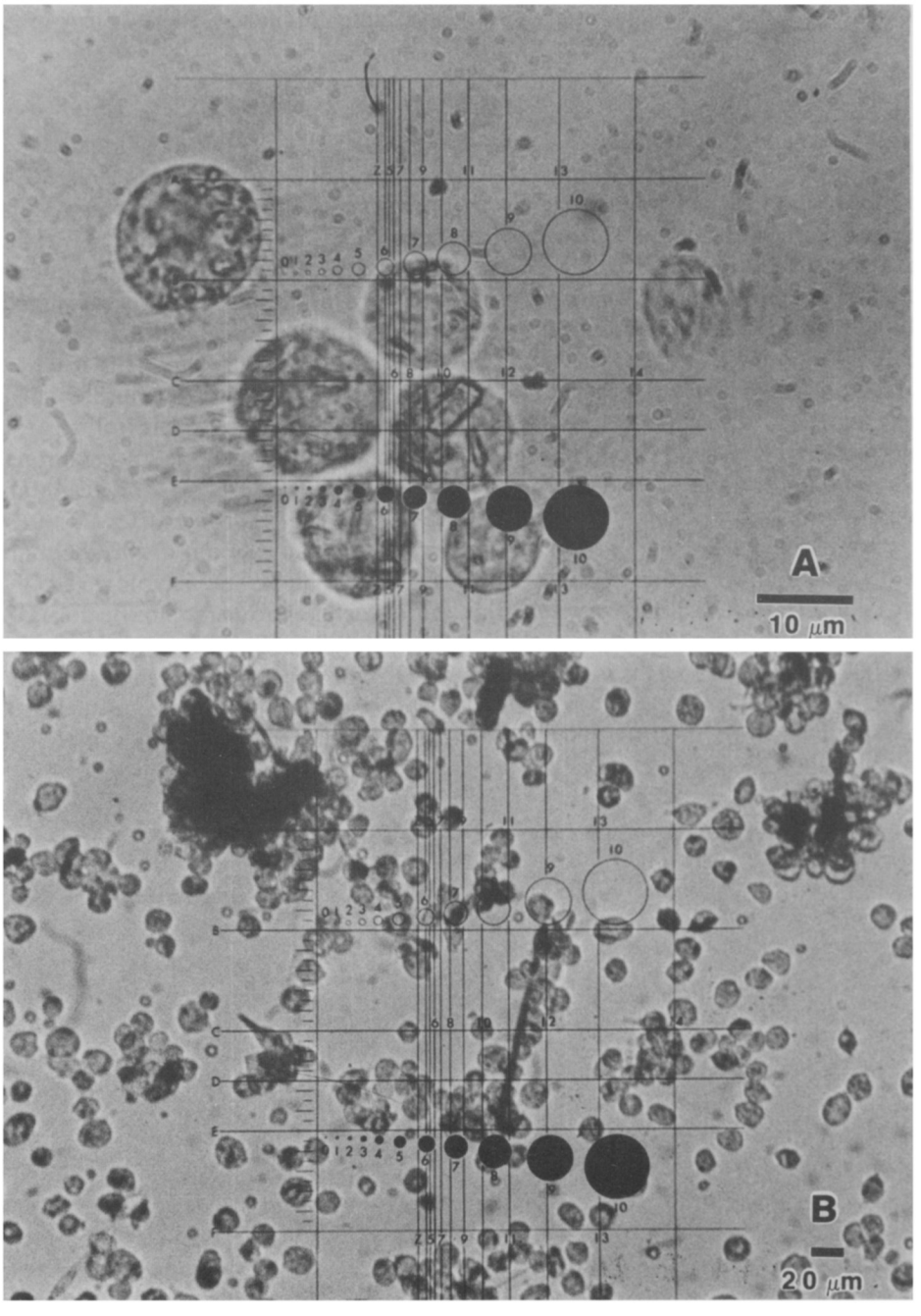

FIG. 2. Light micrograph of culture alveolar macrophages exposed to riebeckite (A) or crocidolite (B).

buffered-formalin (1%). Cells were counted under a light microscope using a haemocytometer and viability determined as the percentage of cells excluding dye.

Oxygen consumption by alveolar macrophages was measured at rest and in the presence of zymosan (2 mg ml^{-1}) using an oxygraph equipped with a Clark electrode (VAN SCOTT *et al.*, 1987). The oxygraph was calibrated using media equilibrated with gases of known oxygen content.

Hydrogen peroxide secretion from alveolar macrophages was determined in the presence of zymosan (2 mg ml^{-1}) by measuring the oxidation of scopoletin. The fluorescence of scopoletin (1.2 μM) was measured in the presence of type IX horseradish peroxidase (6.6 units per ml) at an excitation wavelength of 350 nm and an emission wavelength of 460 nm. Fluorescence changes were converted to nmoles of H_2O_2 using a standard curve (VAN SCOTT *et al.*, 1987).

Lactate dehydrogenase activity of culture supernates was determined using the method of CABAUD and WROBLEWSKI (1958) as given in Sigma Technical Bulletin No. 500 (Sigma Chemical Co., St. Louis, U.S.A.). Specific activity was expressed as the amount of enzyme that would reduce 4.8×10^{-4} μmoles of pyruvate per min at 25°C.

β-galactosidase activity of culture supernates was determined by the method of CONCHIE *et al.* (1959). The substrate was 2.5 mM *p*-nitrophenyl-D-galactopyranoside in citrate-phosphate buffer (pH = 3.6). A 0.25 ml sample was used for the enzyme reaction which was stopped by the addition of 0.4 M glycine–NaOH buffer (pH = 10.8). The specific activity was expressed as nmoles of *p*-nitrophenyl released per h per 10^6 cells as measured spectrophotometrically at 420 nm.

Statistical significance was determined at $P < 0.05$ using the Student's *t*-test.

RESULTS

Figure 2 demonstrates the interaction of riebeckite (A) and crocidolite (B) particles with alveolar macrophages in culture. In both cases, macrophages phagocytized many particles. However, as shown in Fig. 2(B), some crocidolite particles were too long to be engulfed and were often found with macrophages attached along the length of these particles.

The time- and dose-dependent effects of crocidolite on alveolar macrophages are summarized in Table 1. For the most part, 50 μg ml^{-1} crocidolite did not exhibit substantial cytotoxicity even after macrophages were exposed for 3 days. In contrast, significant cytotoxicity was found at 200 and 300 μg ml^{-1} in all of the bioassays monitored. This indicates that both membrane integrity and cellular function had been compromised. At 100 μg ml^{-1} crocidolite, zymosan-stimulated oxygen consumption was significantly inhibited while membrane integrity was only slightly decreased, suggesting that functional assays were more sensitive indicators of toxicity than assays of membrane integrity.

The time- and dose-dependent effects of riebeckite on alveolar macrophages are summarized in Table 2. At all treatment times zymosan-stimulated hydrogen peroxide secretion, i.e. a functional assay, was significantly inhibited at 200 μg ml^{-1}. All other assays, showed cytotoxicity consistently at 300 and 1000 μg ml^{-1}. Doses of 100 μg ml^{-1} or less riebeckite exhibited little toxicity (data not shown).

TABLE 1. EFFECT OF CROCIDOLITE ON ALVEOLAR MACROPHAGES IN CULTURE

Treatment (duration/dose)	Trypan blue exclusion (% viable)	Zymo-stim O_2 consumption (nmoles min^{-1} per 10^6 cells)	Zymo-stim H_2O_2 (nmoles min^{-1} per 10^6 cells)	LDH (units per 10^6 cells)	β-Gal (nmoles h^{-1} per 10^6 cells)
1 day					
Control	77.8±1.6	5.1±0.4	4.5±0.6	20.3±6.4	7.2±1.2
50 $\mu g\ ml^{-1}$	72.2±1.4*	4.9±0.5	3.9±0.5	28.8±6.2	21.2±5.8
100 $\mu g\ ml^{-1}$	74.2±4.0	2.8±0.3*	—	—	—
200 $\mu g\ ml^{-1}$	62.7±1.2*	3.3±0.5*	2.9±0.4*	32.9±4.4	21.1±1.8*
300 $\mu g\ ml^{-1}$	56.8±1.1*	2.9±0.7*	2.5±0.3*	39.6±6.4*	30.8±1.9*
2 days					
Control	76.3±1.8	8.1±0.8	9.0±1.4	50.8±6.4	17.0±3.2
50 $\mu g\ ml^{-1}$	68.8±1.2*	8.0±0.5	7.9±0.8	60.0±5.8	20.5±3.4
100 $\mu g\ ml^{-1}$	77.2±3.4	3.4±0.9*	—	—	—
200 $\mu g\ ml^{-1}$	61.3±1.9*	4.5±0.8*	6.1±0.6*	71.1±10.2	26.6±1.6*
300 $\mu g\ ml^{-1}$	56.7±1.6*	4.8±1.1*	5.5±0.5*	81.8±11.9*	34.8±1.6*
3 days					
Control	71.4±2.4	8.4±0.4	8.9±0.5	100.9±15.8	48.6±11.2
50 $\mu g\ ml^{-1}$	62.9±1.8*	7.8±1.2	8.3±0.8	119.3±13.2	58.6±8.3
100 $\mu g\ ml^{-1}$	66.2±4.7	4.6±1.4*	—	—	—
200 $\mu g\ ml^{-1}$	56.5±2.5*	4.5±1.0*	5.8±0.7*	123.2±9.9	49.8±5.2
300 $\mu g\ ml^{-1}$	55.1±2.3*	3.7±1.0*	3.9±0.4*	145.9±12.1*	61.5±4.3

Value are means±standard errors of between six and 10 experiments.
*Indicates a significant difference from control ($P<0.05$).

TABLE 2. EFFECT OF RIEBECKITE ON ALVEOLAR MACROPHAGES IN CULTURE

Treatment (duration/dose)	Trypan blue exclusion (% viable)	Zymo-stim O_2 consumption (nmoles min^{-1} per 10^6 cells)	Zymo-stim H_2O_2 (nmoles min^{-1} per 10^6 cells)	LDH (units per 10^6 cells)	β-Gal (nmoles h^{-1} per 10^6 cells)
1 day					
Control	76.7±1.4	5.2±0.4	4.7±0.8	23.6±1.7	12.9±1.4
200 $\mu g\ ml^{-1}$	71.4±1.6*	4.9±0.4	2.9±0.5*	38.3±4.2*	26.0±4.2*
300 $\mu g\ ml^{-1}$	70.3±1.8*	4.4±0.5	2.5±0.6*	48.6±3.2*	28.7±3.8*
1000 $\mu g\ ml^{-1}$	49.2±2.1*	1.9±0.4*	0.0±0.0*	248.4±14.0*	252.8±42.0*
2 days					
Control	69.1±2.7	7.9±0.7	11.4±1.5	67.6±9.7	24.4±6.2
200 $\mu g\ ml^{-1}$	65.8±2.1	7.3±0.5	5.7±0.8*	77.8±10.3*	26.6±6.3
300 $\mu g\ ml^{-1}$	61.9±1.5*	6.6±0.8	5.7±0.8*	115.4±13.5*	52.9±13.0
1000 $\mu g\ ml^{-1}$	43.9±1.4*	2.8±0.5*	0.1±0.1*	318.7±25.9*	301.5±48.5*
3 days					
Control	63.8±2.3	9.3±0.6	10.0±1.2	126.6±7.7	38.2±6.3
200 $\mu g\ ml^{-1}$	61.1±1.4	7.7±0.5	6.3±1.7*	149.6±16.1	71.5±12.0
300 $\mu g\ ml^{-1}$	56.5±1.9*	6.6±0.8	4.2±1.2*	180.3±22.2*	69.8±18.1
1000 $\mu g\ ml^{-1}$	46.5±1.9*	2.8±0.4*	0.0±0.0*	342.1±19.1*	269.3±20.4*

Values are means±standard errors of between six and 10 experiments.
*Indicates a significant difference from control ($P<0.05$).

DISCUSSION

The present study investigated the effects of exposure of alveolar macrophages in culture to crocidolite and its non-asbestiform polymorph, riebeckite. A previous study by our laboratory has shown that this *in vitro* system was capable of distinguishing between pathogenic chrysotile and non-toxic latex beads (PAILES *et al.*, 1984). Wollastonite, a naturally occurring fibre which has been proposed as a non-toxic substitute for asbestos (SHASBY *et al.*, 1979), was also found to be relatively non-reactive in this assay system (PAILES *et al.*, 1984). Therefore, this *in vitro* system may be useful in screening various proposed asbestos substitutes for potential pathogenicity.

Data in Table 2 indicate that riebeckite exhibits cytotoxicity to alveolar macrophages in culture at doses equal to or less than those for crocidolite, i.e. when dose is determined on an equivalent surface area or particle count basis.

In contrast, MOSSMAN and SESKO (1990) reported that riebeckite was non-toxic to alveolar macrophages at doses where crocidolite exhibited substantial activity. The discrepancy between these studies results from the unit employed to measure dose. MOSSMAN and SESKO (1990) determined dose on a mass basis. Using mass, riebeckite was relatively non-toxic in the present study as well. However, the crocidolite sample used in our study had 6.3 times the surface area and over 20 times the number of particles per mass as riebeckite. It is clear that the conclusion as to the toxicity of riebeckite *in vitro* is dictated by the means in which dose is determined. This impacts greatly on whether environmental sampling should be on a mass per m^3 or particle count per m^3 basis.

In the present study, 50 $\mu g\ ml^{-1}$ crocidolite was not cytotoxic to alveolar macrophages. Using a similar assay system, PAILES *et al.*(1984) reported that 50 $\mu g\ ml^{-1}$ chrysotile was cytotoxic. Similarly, other laboratories have reported that chrysotile is more cytotoxic *in vitro* than crocidolite (MOSSMAN and SESKO, 1990; BROWN *et al.*, 1986). In contrast, crocidolite has been associated with a greater incidence of mesothelioma in workers than chrysotile (CRAIGHEAD and MOSSMAN, 1982). This discrepancy suggests that the macrophage bioassay may not be a predictor of carcinogenicity, rather its usefulness may be limited to estimating the fibrogenic potential of particles.

In conclusion, the results of the present study indicate that on a mass basis fibrous crocidolite was more toxic *in vitro* than non-asbestiform riebeckite. However, on an equivalent surface area basis, the cytotoxicity of riebeckite was slightly greater than crocidolite. Finally on an equivalent particle count basis, riebeckite was far more cytotoxic than crocidolite. These results point to the means in which dose is determined as a vital factor in evaluating the potential toxicity of occupational dust exposure. Since non-asbestiform riebeckite exhibited toxicity equal to or greater than its asbestos polymorph, crocidolite, the data suggest that fibre shape may not be the exclusive variable affecting cytotoxicity and that surface chemistry may also play a role.

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