

Comparative Toxicities of Different Forms of Asbestos on Rat Pleural Mesothelial Cells

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The effects of UICC crocidolite and chrysotile A, either oxalic acid-leached or unleached, on the viability, morphology and growth characteristics of rat pleural mesothelial cells (PMC) were examined; DQ12 quartz particles were also used.

When asbestos fibers were added for 48 hr at the beginning of exponential growth, 20 or 50 $\mu\text{g}/\text{mL}$ of chrysotile fibers were cytotoxic and no growth occurred; with 5 or 10 $\mu\text{g}/\text{mL}$ a latent period was observed, and the mean population doubling time was increased. Chrysotile ingestion was associated with morphological changes (spreading, intense vacuolation); moreover, a large proportion of the cells was binucleated (more than 30% with 10 $\mu\text{g}/\text{mL}$). The oxalic acid-leached chrysotile inhibited growth at a concentration of 50 $\mu\text{g}/\text{mL}$; with 5 or 10 $\mu\text{g}/\text{mL}$, no spreading occurred, but a shrinkage of some cells was observed. A few large vacuoles were seen in the cytoplasm of the cells; there were fewer binucleated cells. Addition of 5 or 10 $\mu\text{g}/\text{mL}$ of crocidolite, either leached or unleached, did not significantly change the growth rate, in spite of the presence of a large number of fibers inside the cells which persisted when the cells reached confluency. With 20 or 50 $\mu\text{g}/\text{mL}$, the mean population doubling time was increased in a dose-dependent manner. A slight vacuolation of the cells occurred. The sample of quartz did not modify the parameters studied in this report.

The results confirm the different *in vitro* reactivities of the two kinds of unleached asbestos fibers. Leaching of chrysotile fibers decreased their reactivity; alternatively, leaching of crocidolite increased the effects on PMC.

Introduction

When crocidolite or chrysotile fibers are injected into the pleural cavity of rats, they both induce mesothelioma (1). However, when tested with *in vitro* systems, these fibers give different responses (2). In these *in vitro* experiments, the cells used did not come from the pleura. The aim of the work reported here was to study the *in vitro* effects of asbestos fibers on the morphology and growth characteristics of rat pleural mesothelial cells in short-term experiments.

Since acid-leached chrysotile fibers have been found to be nontumorigenic (3, 4), leached chrysotile and leached crocidolite were also tested. In order to

compare the results with the activity of noncarcinogenic particles, a sample of quartz was also used.

Material and Methods

Asbestos Fibers and Control Particles

The asbestos fibers were obtained from the UICC. Chrysotile A and crocidolite were used. Chrysotile and crocidolite were leached with 0.1 *N* oxalic acid (5). DQ12 quartz from Dörentrup was chosen as a control. The particles were dispersed by sonication in the culture medium (50 kHz, 20 W, 5 min.)

The size parameters of the samples were determined by electron microscopy as described elsewhere (4). The leached chrysotile had a slightly higher mean diameter than the unleached samples (0.091 and 0.082 μm) and a lower mean length (1.8 and 3.1 μm).

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Table 1. Percentage of binucleated PMC 72 hr after contact with the particles.

Concentration	6-8-12 passages, chrysotile (Ch) ^a	11-14-15 passages, leached Ch ^a	8-9-10-13 passages, crocidolite (Cr) ^a	6-8-16 passages, leached Cr ^a	17-10 passages, quartz
Control	1.7 ± 0.8	1.1 ± 1.6	1.7 ± 0.8	1.8 ± 1.4	0.2 ± 0.3
5 µm/mL ^b	29.3 ± 0.2*	8.2 ± 0.8	2.2 ± 0.9	3.4 ± 3.1	1.1 ± 1.5
10 µg/mL	37.3 ± 2.5*	12.7 ± 4.5*	4.3 ± 1.2	8.2 ± 1.2*	0.6 ± 0.8
20 µg/mL	nd ^c	19.8 ± 5.3*	7.5 ± 0.4	13.8 ± 1.3	0.6 ± 0.8
50 µg/mL	nd ^c	19.3 ± 4.0*	16.5 ± 10.0†	25.9 ± 2.6*	2.2 ± 3.1

^a For each treatment different cell strains were used^b When expressed as µg/cm² the concentrations were from 1 to 10 µg/cm².^c nd = not determined.* *p* < 0.1.† *p* < 0.5.

Rat Pleural Mesothelial Cells (PMC)

PMC were cultured as described elsewhere (6). The cells were used between the 6th and 17th passage. The PMC came from six different rats.

Morphological Studies

PMC morphology was observed by use of phase-contrast microscopy and electron microscopy as described by Jaurand et al. (7).

Growth Analysis

The growth curve analysis was performed as described elsewhere (7). Briefly, PMC were plated at 5×10^5 cells per flask, and 24 hr after plating the particles were added to the culture medium for 48 hr. The difference between the number of treated and untreated cells was assessed by the *F* test. The experiments were carried out in triplicate or quadruplicate for each particle type.

The percentage of mitosis or of binucleated cells, following either 48 hr or 72 hr of incubation with the particles, was determined. The difference between the percentage observed in treated and untreated cells was assessed by the *t* test.

Results and Discussion

Morphological Studies

The results describing the effects of chrysotile and crocidolite fibers have been reported elsewhere (7). Chrysotile-treated PMC had numerous vacuoles; there was an increase in the cell volume, and a large proportion of binucleated cells appeared (Table 1). Crocidolite fibers did not induce such morphological changes except at the highest concentration (50 µg/mL), but these effects were less than with 5 µg/mL of chrysotile fibers.

PMC treated with acid-leached chrysotile also showed a vacuolation of the cytoplasm but the vac-

uoles were less numerous and generally larger than with unleached chrysotile (Fig. 1). Shrinkage of the cells was sometimes observed. After 72-hr contact with acid-leached fibers, some binucleated cells were observed, but their percentage was less than with the untreated chrysotile (Table 1). The difference between the number of binucleated cells in treated and untreated cultures was significant with 10 µg/mL of leached chrysotile fibers. With the unleached fibers, it was significant with 5 µg/mL; moreover, it was twice the percentage observed in the leached chrysotile-treated culture. Thus, the effect of leached fibers was less than the effect of the unleached.

Very few vacuoles were seen in the cultures treated with leached crocidolite (Fig. 2). Electron microscopy revealed the presence of intracellular fibers (Fig. 3). The number of binucleated cells was less than in the cultures treated with either leached or unleached chrysotile (Table 1). However, the leached sample of crocidolite had more effect on PMC than the unleached sample, since 10 µg/mL induced a significant increase in the proportion of binucleated cells as compared to the control cultures. Treatment with 50 µg/mL of crocidolite fibers was necessary to obtain a significant increase of this parameter.

The particles of quartz did not modify the morphology of the culture (Fig. 4), in spite of the presence of intraphagosomal particles (Fig. 5).

When the proportion of binucleated PMC was examined, a differential effect of leaching was observed as the proportion of binucleated cells decreased with leaching of chrysotile and increased with leaching of crocidolite.

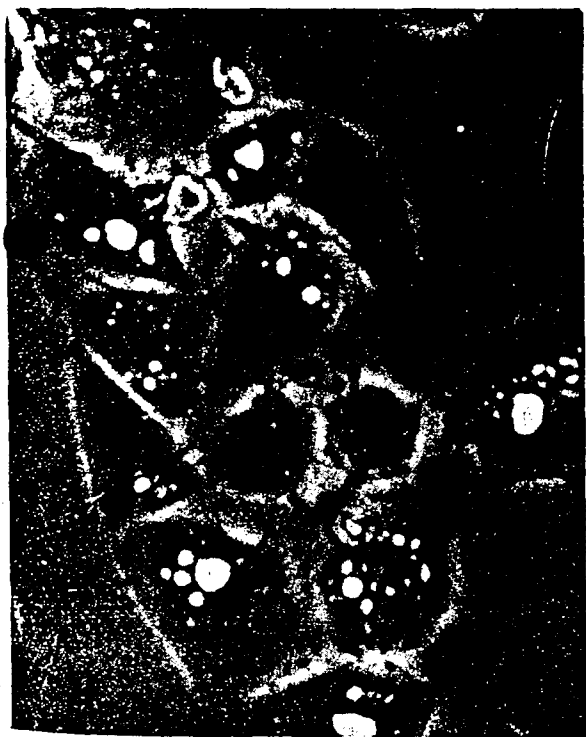
Table 2 shows that there was no effect, due to the treatment with the particles, on the percentage of mitosis determined 48 hr after their addition. However, during this time, the cells were in contact with the particles. This result means that in the five groups of treated PMC there was no inhibition of passage to the M phase and that the toxicity may



(a)

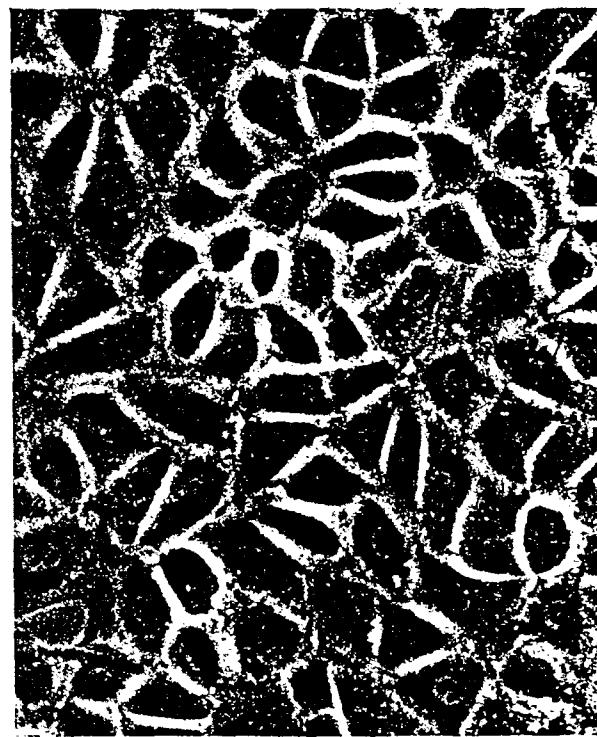


(a)



(b)

FIGURE 1. Phase contrast microscopy of PMC in culture ($\times 500$); 24 hr following trypsinization, PMC were treated with (a) chrysotile, 10 $\mu\text{g}/\text{mL}$, or (b) leached chrysotile, 50 $\mu\text{g}/\text{mL}$ for 48 hr.



(b)

FIGURE 2. Phase contrast microscopy of PMC in culture ($\times 310$): (a) PMC at the 8th passage treated with 20 $\mu\text{g}/\text{mL}$ of leached crocidolite for 48 hr; (b) control culture.



FIGURE 3. Electron microscopy of PMC at the 17th passage treated with 10 µg/mL of leached crocidolite fibers for 48 hr ($\times 9000$).

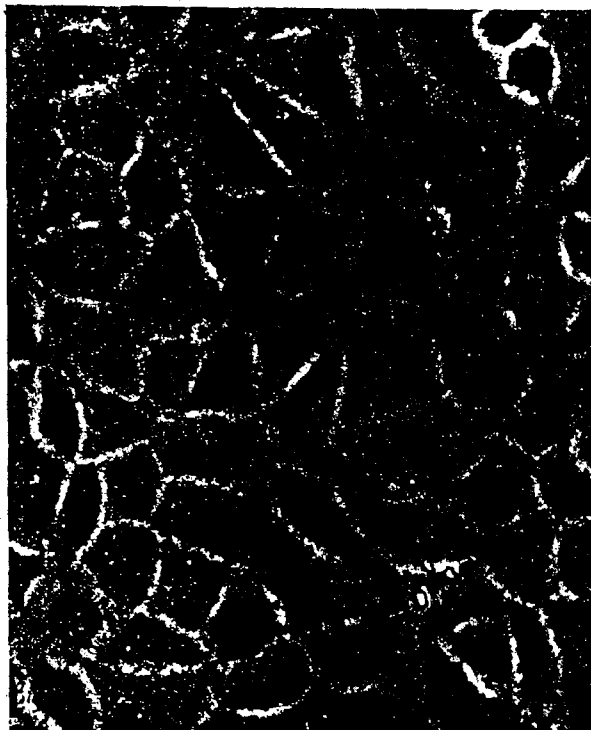


FIGURE 4. Phase contrast microscopy of PMC at the 20th passage treated with 10 µg/mL of DQ12 for 48 hr ($\times 310$).



FIGURE 5. Electron microscopy of PMC at the 4th passage treated at confluency with 5 µg/mL of DQ12 for 48 hr ($\times 55000$).

not be dependent on the phase of the cell cycle. Our previous results (7) have shown that the phases of cell mitosis can be observed in chrysotile-treated PMC. The presence of binucleated PMC may result from an inhibition of cell division or from a fusion of the PMC; however, no indication of fusion between PMC was observed. When mitosis occurred in a chrysotile-treated culture, the cells adhered and spread on the plastic at the end of telophase, rather than before the beginning of mitosis. An incomplete synthesis of the plasma membrane may, therefore, result in a binucleated cell.

Growth Analysis

The results concerning the mean population doubling time θ are expressed in Table 3. DQ12 quartz had no significant effect on the value of θ . Leached crocidolite and chrysotile, either leached or unleached, were cytotoxic, but the concentration needed to prevent growth depended on the nature of the fibers. Unleached crocidolite had some effect if added at the highest concentration (50 µg/mL).

Table 2. Percentage of cells in mitosis after 48 hr of contact with the particles: talc, 1.4 folds T

Concentration	6-8-12 passages, chrysotile (Ch) ^a	11-14-15 passages, leached Ch ^a	8-9-10-13 passages, crocidolite (Cr) ^a	6-8-16 passages, leached Cr ^a	10-11-17 passages, leached quartz ^a
Control	1.4 ± 0.3	2.0 ± 0.7	1.4 ± 0.3	0.6 ± 0.6	1.8 ± 0.7
5 µg/mL	1.6 ± 0.6	1.3 ± 0.5	0.7 ± 0.2	1.7 ± 0.3	0.8 ± 0.3
10 µg/mL	1.6 ± 0.8	1.2 ± 0.8	1.0 ± 0.1	1.7 ± 0.3	2.3 ± 2.4
20 µg/mL	0.9 ± 0.2	0.9 ± 0.2	2.1 ± 0.2	1.5 ± 0.7	0.2 ± 0.3
50 µg/mL	0.7 ± 0.8	0.7 ± 0.8	1.0 ± 0.3	1.4 ± 1.2	1.0 ± 1.4

^a For each treatment, different cell strains were used: for chrysotile and leached chrysotile, the same strain was used; for crocidolite and leached crocidolite, different strains were used.

Table 3. Mean population doubling time in hours of PMC incubated for 48 hr with the particles.

Concentration	6-8-12-14 passages, chrysotile (Ch) ^a	11-14-15 passages, leached Ch ^a	9-10-8-13 passages, crocidolite (Cr) ^a	6-9-16 passages, leached Cr ^a	10-11-17 passages, leached quartz ^a
Control	29.7 ± 4.1	32.3 ± 5.5	29.6 ± 3.8	46.3 ± 9.3	43.0 ± 5.0
5 µg/mL	33.2 ± 11.3	36.0 ± 4.4	31.8 ± 4.3	49.3 ± 8.1	42.7 ± 8.9
10 µg/mL	42.7 ± 20.9	42.6 ± 6.0	32.8 ± 3.4	50.7 ± 11.9	46.3 ± 7.2
20 µg/mL	> 100	87.0 ± 15.6	35.5 ± 5.8	74 ± 33	49.0 ± 10.5
50 µg/mL	< 0.0	273 ± 249	90.7 ± 5.1	> 100	50.5 ± 14.7

^a For each treatment, different cell strains were used.

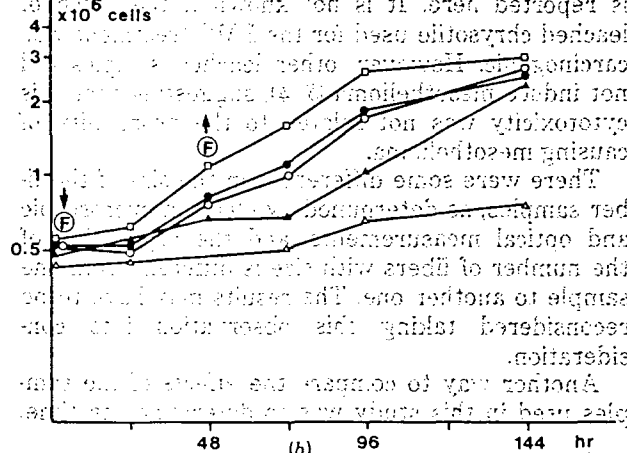
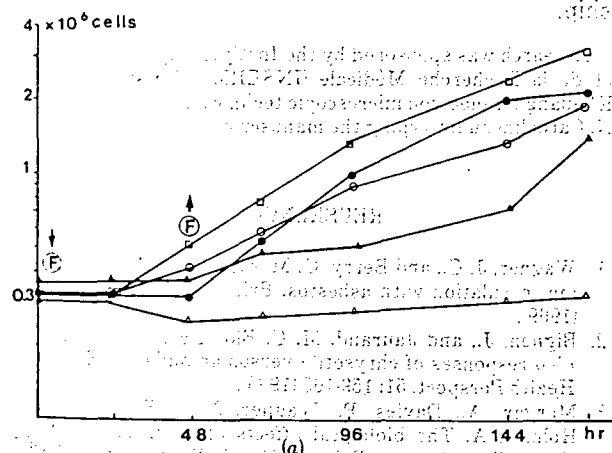


FIGURE 6. Growth curves of one strain of PMC treated with (a) leached-chrysotile or (b) leached-crocidolite: (□) control culture; (●) 5 µg/mL; (○) 10 µg/mL; (▲) 20 µg/mL; (△) 50 µg/mL.

The growth curves showed that a lag time occurred with the chrysotile-treated cultures but this was not observed with leached-chrysotile or with crocidolite, either leached or unleached (Fig. 6).

It is known that the number of cells N during the exponential growth is

$$N = N_0 2^{t/\theta} \quad (1)$$

where N_0 is the number of PMC at the beginning of growth, t the time and θ the population doubling time. Then the derivative of Eq. (1) may be written

$$dN/dt = 1/\theta (N \log 2) \quad (2)$$

When the particles are introduced into the medium, an inhibition can occur; it may be proportional to a power (α) of the particle concentration c , and it causes a decrease in the number of PMC:

$$dN/dt = kNc^\alpha \quad (3)$$

where k is a constant. Then, the variation in the number of PMC will be

$$dN/dt = N(1/\theta \log 2 - kc^\alpha) \quad (4)$$

If θ' is the mean population doubling time in the presence of the particles, the variation of θ' with c can be written

$$\theta' = a\theta/(a - c^\alpha) \quad (5)$$

In this relationship, when $c \rightarrow 0$, then $\theta' \rightarrow \theta$; and when $c^\alpha \rightarrow a$, $\theta' \rightarrow \infty$. From the value a it is possible to deduce $a^{1/\alpha}$, which is the value of the concentration at which no growth occurs (c_{lim}).

From the values reported in Table 3, the calculation gives the values of c_{lim} expressed in Table 4. When the particles are compared by weight, the toxicity will increase in the order $Cr < LCr < LCh < Ch$, where Cr , Ch are crocidolite and chrysotile,

Table 4. Calculated values of the concentration where no growth occurred.

Treatment	c_{lim} , $\mu\text{g/mL}$
Chrysotile	22.6
Leached Ch	28.8
Crocidolite	237
Leached Cr	51

respectively, and LCr and LCh are the respective leached fibers.

Our results are in good agreement with previous data reported by Neugut et al. (8) and Reiss et al. (9) using chrysotile and amphiboles on various cell lines. These authors found a higher toxicity of chrysotile than of crocidolite. It seems that proliferative epithelioid cell lines react to asbestos treatment by somewhat similar responses, possibly related to the amount of particles ingested. Reiss et al. (9) found that the toxicity of the leached samples of amphiboles increased, whereas that of chrysotile decreased with leaching. A similar result is reported here. It is not known if the batch of leached chrysotile used for the PMC treatment was carcinogenic. However, other leached samples did not induce mesothelioma (3, 4), suggesting that this cytotoxicity was not related to the probability of causing mesothelioma.

There were some differences in the size of the fiber samples, as determined by electron microscopic and optical measurements, and the repartition of the number of fibers with size is different from one sample to another one. The results may have to be reconsidered taking this observation into consideration.

Another way to compare the effects of the samples used in this study was to determine the time,

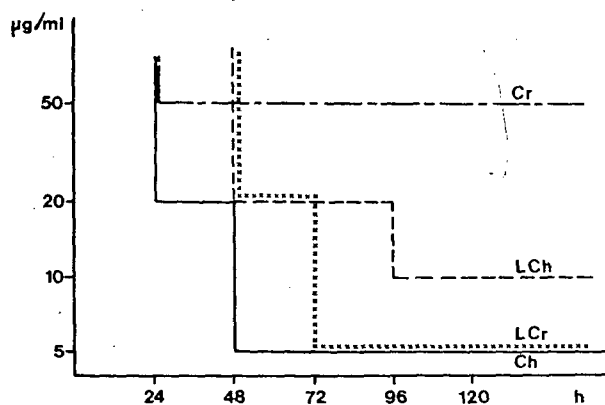


FIGURE 7. Level at which the number of treated cells was lower than that of the untreated cells in all of the cell strains used to determine the growth rate (see Table 3). For example, with chrysotile fibers, the difference was significant following 24 hr of incubation with a concentration equal to or higher than 20 $\mu\text{g/mL}$, or following 48 hr with a concentration equal to or higher than 5 $\mu\text{g/mL}$.

for a given concentration, where the value of F , which measures the difference between the number of PMC, in treated cultures and the number of PMC in the control culture is such that $p < 0.5$. For each treatment, the F test was applied every day, and the results are expressed in Figure 7. It can be seen that chrysotile and leached crocidolite were both effective at a concentration of 5 $\mu\text{g/mL}$, but the time necessary to observe a significant effect was longer with leached crocidolite than with chrysotile. This may be due to similar "toxic power" of the samples and to a different sensitivity of the mesothelial cells (expressed by the lag time and possibly related to the intensity of phagocytosis). With the leached samples, no significant effect was observed after 24 hr of contact with the fibers, and with 10 $\mu\text{g/mL}$, the relationship was $\text{Cr} < \text{LCh} < \text{LCr} < \text{Ch}$.

When considering the effects on growing cells, the comparison between different varieties of fibers must be made carefully, since an effect may be reversed due to the form of the dose-effect relationship.

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