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Some Observations on the *in Vitro* Cytotoxicity of Chrysotile Prepared by the Wet Dispersion Process

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Samples of the chrysotile taken during and after treatment by the wet dispersion process have been tested for their cytotoxic effect *in vitro* and the results compared with both a UICC chrysotile A sample and a dust prepared from a standard chrysotile textile yarn. Results were obtained from three different *in vitro* assay systems utilizing P388D₁, V79-4 and A549 cells. A sample which still contained the wetting agent used in the wet dispersion process failed to show activity in any of these assays. The other samples, however, were all active with these dusts obtained by milling the final product and by sampling the air of the factory consistently proving significantly more cytotoxic than the standard chrysotile controls. Preliminary results from a parallel *in vivo* study suggest that these samples are also more active in producing mesotheliomas in rats.

Introduction

In industrialized countries, man is continuously introducing alternative processes for the production and exploitation of new mineral substances. In many cases, the hazards to health resulting from the use of minerals are well established, but, when new minerals or processes are involved, their potentially pathogenic effects are unknown. As epidemiological studies often take many years to complete, during which time a considerable number of people are exposed to a possibly hazardous situation, much effort has been expended in investigating short-term laboratory techniques that are capable of detecting pathogenic minerals. These techniques have involved both *in vivo* and *in vitro* approaches.

The effects of mineral dusts in animals, mainly rats, have been extensively studied, with the animals being exposed to a given dust by inhalation or intratracheal instillation and then investigated for disease over their lifespan. As macrophages are considered to be the cells most likely to first come into contact with mineral particles in the lung, most of the *in vitro* assays have been based on the cytotoxicity of a given mineral for these cells. Both animal

macrophages (1-3) and more recently permanent lines of macrophagelike cells (4-6) have been used to investigate the cytotoxicity of mineral dusts and this effect seems to give an indication of the fibrotic potential of a given mineral dust. In addition, it has been suggested that assays using phagocytic cells can also be used to give an indication of the malignant potential of such dusts (7, 8). New forms of cytotoxicity assay have also been developed by Chamberlain and Brown (9) using other types of permanent cell lines, and it is suggested that these may indicate the carcinogenic potential of such dusts rather than their fibrogenicity.

The wet dispersion process is now commonly used by industry to produce an asbestos fabric from chrysotile, and an experimental sample of this material was found to be particularly cytotoxic in an *in vitro* assay with P333D₁ cells, a mouse macrophagelike cell line (10). This material was subsequently found to induce mesotheliomas in rats at an earlier time than UICC chrysotile A (11). It seemed possible that these phenomena might be related to wet dispersion processes in general and so the present study was undertaken using several different samples, some of which had been treated in order to observe the effects of extraneous chemicals used during the chrysotile treatment.

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Materials and Methods

Dust Samples

The wet dispersion (WD) process is one used by a number of manufacturers in order to prepare a particularly durable chrysotile yarn that can be spun or woven into cloth. Although the technique varies slightly from manufacturer to manufacturer, the basic process consists of the disaggregation, using a wetting agent, of bundles of chrysotile fibers into a slurry consisting of predominantly single fibrils. This slurry is then passed through fine nozzles, and the addition of electrolytes at this point causes the fibrils to reaggregate with a very firm bonding between them. The resulting strands of yarn can then be woven or spun to produce asbestos textile products (12). The samples used in the present study consisted of: (1) the original experimental sample tested previously (10) [original WD chrysotile (WDC)]; (2) a finished textile yarn from a long-running factory WD process which was milled in order to generate dust (milled WDC); (3) a sample from the milled process in (2) above from which the wetting agent had not been removed (unextracted WDC); (4) a sample of material from the process in (2) above from which all traces of the wetting agent had been removed by heat cleaning (heat cleaned WDC); (5) a dust sample collected from the factory air at the plant producing sample (2). This sample was collected from a section of the factory where only WDC products were handled (factory WDC).

The WDC samples were compared to both a UICC chrysotile A sample and to dust prepared from a standard chrysotile textile yarn made from exactly the same type of chrysotile used in the WD process. All of the samples tested were collected from an airborne dust cloud before use in either *in vitro* or *in vivo* studies. A number of these samples were extremely difficult to suspend in tissue culture medium due to their tendency to form tangled masses of fibers during the suspension process. The samples used *in vitro* were therefore ultrasonicated and, in some cases, forced through a hypodermic syringe needle (21 gauge) in an attempt to disperse them.

Cytotoxicity Assays

P388D₁ Assay. The techniques used have been fully described previously (5, 10). In brief, 5×10^5 viable cells of the macrophagelike cell line P388D₁ were exposed to 10 and 50 $\mu\text{g/mL}$ of the dust in 5 mL of culture medium. After 24 and 48 hr the cultures were assayed for cell viability by using the trypan blue exclusion technique, lactate dehydrogenase release (13), *N*-acetyl- β -D-glucosaminidase (14),

lactic acid (15) and cellular protein content (16). The controls consisted of undusted cells and cells exposed to 80 μg titanium dioxide/mL (nontoxic control) or 20 μg quartz DQ₁₂/mL (17) (toxic control).

V79-4 Cell Assay. The reduction in colony formation by V79-4 cells was measured using the techniques described by Chamberlain and Brown (9).

Giant Cell Formation in A549 Cultures. The formation of giant cells in A549 cells was also measured by using the techniques described by Chamberlain and Brown (9). The measurements of cell size were made by using a Graphic Instruments GDS1 image analyzer. However, the WD chrysotile samples were very cytotoxic when added to these cells, and so a higher cell inoculum (2.0×10^6 cells/25 cm² tissue culture flask) was used with lower dust concentrations (25 and 50 $\mu\text{g/mL}$).

In both the V79-4 and A549 assays, a sample of UICC crocidolite was added as an additional control so that the results could be compared with those reported by Chamberlain and Brown (9).

Animal Experiments

The techniques used in this part of the study have been fully described by Bolton et al. (11). The five WDC samples and the two chrysotile reference samples were each injected into groups of thirty-two 10-week-old male AF/HAN random-bred SPF Wistar laboratory rats. The elutriated dust samples were dry heat-sterilized at 60°C for 30 min, mixed with sterile Dulbecco's phosphate buffered saline, and 2 mL of PBS containing 25 mg of the dust were then injected intraperitoneally. The animals were observed daily and killed when distressed or moribund.

Results and Discussion

The mean results from a series of three independent experiments using the P388D₁ assay are shown in Table 1. The results for the biochemical estimations were in good agreement with those for the cellular viability as reported previously (10, 18), and so only the viability results are reported. These are presented as the percentage of those found for the titanium dioxide controls. It can be seen from Table 1 that, although there were slight differences (probably due to experimental variation) in the ranking of the dusts according to their cytotoxicity, four out of the five samples of WDC were more cytotoxic than the UICC chrysotile control. The only exception to this was the sample of unextracted WDC. It was noteworthy that three samples of the finished product—heat cleaned WDC, milled WDC and the environmentally collected factory sample—were al-

Table 1. Order of cytotoxicity of WDC samples based on viability estimations.

Time, hr	Sample (10 µg/mL)	Viability ^a	Sample (50 µg/mL)	Viability ^a
24	Unextracted WDC	96 ± 14	Unextracted WDC	87 ± 9
	UICC chrysotile A	84 ± 6	UICC chrysotile A	47 ± 8
	Milled chrysotile	79 ± 10	Original WDC	34 ± 3
	Original WDC	68 ± 11	Milled chrysotile	34 ± 14
	Heat-cleaned WDC	62 ± 10	Milled WDC	25 ± 4
	Factory WDC	50 ± 8	Heat-cleaned WDC	22 ± 7
	Milled WDC	44 ± 6	Factory WDC	20 ± 8
48	Unextracted WDC	75 ± 4	Unextracted WDC	59 ± 7
	UICC chrysotile A	52 ± 6	UICC chrysotile A	21 ± 5
	Milled chrysotile	43 ± 12	Milled chrysotile	18 ± 7
	Original WDC	30 ± 11	Original WDC	17 ± 5
	Factory WDC	25 ± 3	Factory WDC	10 ± 3
	Heat-cleaned WDC	25 ± 5	Milled WDC	8 ± 2
	Milled WDC	22 ± 4	Heat-cleaned WDC	6 ± 1

^aViability = (no. live cells for test dust/no. live cells for TiO₂ control) × 100% ± SD. The dusts are ranked in order of increasing cytotoxicity.

Table 2. Cytotoxicity of WDC samples by the V79-4 assay.

Sample	LD ₅₀ , µg of dust ^a
Unextracted WDC	87.0 ± 19.1
Titanium dioxide	84.8 ± 14.8
Original WDC	46.2 ± 39.2
Quartz DQ ₁₂	28.8 ± 12.4
Heat-cleaned WDC	20.4 ± 15.8
UICC chrysotile A	13.5 ± 5.6
UICC crocidolite	11.6 ± 2.3
Milled chrysotile	9.7 ± 4.6
Factory WDC	8.4 ± 5.9
Milled WDC	5.0 ± 3.4

^aThe concentration of dust causing a 50% reduction in the cloning efficiency of V79-4 cells.

ways the most cytotoxic, regardless of time or dose, and these three samples reduced the viability to approximately half of that seen with the UICC chrysotile sample.

The concentration of dust causing a 50% reduction in the cloning efficiency of the V79-4 cells (9) was calculated for each dust sample from five independent experiments, and the results are shown in Table 2. The results are broadly in agreement with those obtained using the P388D₁ assay in that the unextracted WDC sample was the least cytotoxic, whereas the factory and milled WDC were the most cytotoxic. The result for UICC crocidolite was in good agreement with that originally described by Chamberlain and Brown (9), who reported an LD₅₀ of 9 µg/mL for this substance. However, quartz DQ₁₂ was always found to be active in this system with a mean LD₅₀ of 28.8 µg/mL, in contrast to the results reported for Min-U-Sil and South African silica reported by Chamberlain and Brown (9).

The results obtained when the samples of WDC were tested for their ability to cause giant cell for-

mation in A549 cells are shown in Tables 3 and 4. Although these large cells have been described as "giant cells" (9), they are not considered to be of similar origin to the classical foreign body giant cell commonly found associated with dust *in vivo*. These results, which are taken from a single representative experiment in which 100 cells were measured for each sample, are broadly in agreement with those reported above with the unextracted sample having the least effect while the other four samples caused more giant cell formation than UICC chrysotile A or milled chrysotile. The quartz sample was not active in this assay system and so was in agreement with the results reported by Chamberlain and Brown (9). In contrast to their results, however, it should be noted that UICC crocidolite had only a minimal effect in our experiments. This result was to be expected in view of the increased cell number and decreased dust dose which was used in this study to overcome the marked toxicity of the WDC samples.

When the results of all three assay systems were considered, the unextracted WDC sample was always found to be the least active of the WDC samples. The remaining four samples were more active than either UICC chrysotile A or milled chrysotile in the P388D₁ and A549 assay, and two samples—factory and milled WDC—were more cytotoxic than the standard chrysotile samples in all three assay systems.

Only preliminary data from the animal experimentation are currently available for comparison with the cytotoxicity results. The mean induction period for the first 15 tumors after intraperitoneal injections of 25 mg of the samples is shown in Table 5. These data suggest that three of the WDC samples induced tumors at a faster rate than either

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Table 3. Giant cell formation in A549 cells.

Sample (25 µg/mL)	Size, µm ± SD	Cells in size range, %			Cells > 25 µm, %
		0-25 µm	25-40 µm	> 40 µm	
Titanium dioxide	17.78 ± 2.40	100	0	0	0
Unextracted WDC	18.13 ± 3.11	98	2	0	2
Quartz DQ ₁₂	18.18 ± 3.58	98	1	1	2
Undusted control	18.32 ± 2.19	99	1	0	1
UICC crocidolite	19.22 ± 3.66	95	5	0	5
UICC chrysotile A	20.68 ± 4.69	83	17	0	17
Milled chrysotile	21.38 ± 5.19	78	22	0	22
Heat-cleaned WDC	22.71 ± 5.09	74	26	0	26
Factory WDC	24.18 ± 5.71	56	44	0	44
Milled WDC	24.51 ± 5.82	60	39	1	40
Original WDC	25.08 ± 6.85	56	41	3	44

Table 4. Giant cell formation in A549 cells.

Sample (50 µg/mL)	Size, µm ± SD	Cells in size range, %			Cells > 25 µm, %
		0-25 µm	25-40 µm	> 40 µm	
Quartz DQ ₁₂	17.54 ± 5.68	99	1	0	1
Titanium dioxide	17.80 ± 1.94	99	1	0	1
Undusted control	18.32 ± 2.19	99	1	0	1
UICC crocidolite	19.19 ± 3.23	93	7	0	7
Unextracted WDC	19.75 ± 4.41	92	7	1	8
Milled chrysotile	22.46 ± 4.19	72	28	0	28
UICC chrysotile A	23.21 ± 5.43	69	31	0	31
Factory WDC	23.49 ± 6.36	63	36	1	37
Heat-cleaned WDC	24.39 ± 5.99	57	41	2	43
Original WDC	24.72 ± 6.22	62	35	3	38
Milled WDC	26.96 ± 7.12	44	50	6	56

Table 5. Preliminary data on mesothelioma production by WDC samples in rats.

Sample	Mean tumor induction time for 15 tumors in each group, days
Heat-cleaned WDC	367
Milled chrysotile	357
Unextracted WDC	355
UICC chrysotile A	354
Milled WDC	300
Factory WDC	282
Original WDC	276

milled chrysotile or UICC chrysotile A, and two of these, milled and factory WDC, caused a marked response in all three cytotoxicity assays. The third sample, original WDC, was more active than the chrysotiles in two out of the three assay systems.

The *in vivo* results for the remaining two WDC samples were not in agreement with the *in vitro* data, as heat-cleaned WDC which was active *in vitro* had the longest tumor induction time, and the unextracted sample, which was the least active of the WDC samples *in vitro*, produced mesotheliomas in rats after the same approximate period as the standard chrysotile samples. Obviously a full comparison of the *in vivo* and *in vitro* data cannot be

made until the *in vivo* experiments have been completed. There are, however, possible reasons for the discrepancies. The unextracted WDC was difficult to suspend for the *in vitro* work, and separation of the fibers may have been incomplete, whereas adequate separation for tumor induction may well have occurred during many months of residence in the animal tissues. Alternatively, the wetting agent materials present in this sample could have a protective effect in the short-term *in vitro* tests but be removed during the longer-term *in vivo* experiments. The discrepancy between the *in vitro* and *in vivo* data for heat-cleaned WDC may be related to the heat treatment, as there is evidence to suggest that this was carried out at a temperature high enough to break down some of the chrysotile to forsterite. Although chrysotile samples heated to 850°C can exhibit a lower cytotoxicity (10) and a reduced carcinogenic potential (11), these two effects may not be directly related. The carcinogenic potential may be reduced after a level of heating which still leaves the dust with the ability to damage cells *in vitro*. An alternative explanation may be that the heat cleaning process alters the solubility of the chrysotile.

The apparent enhancement of both cytotoxicity and mesothelioma production resulting from the

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treatment of chrysotile by the wet dispersion process is difficult to explain. It does, however, seem possible that the bonding between the chrysotile fibrils, while strong under normal conditions, becomes weak in the presence of biological fluids. Thus, within the body or in tissue culture medium, the bundles of fibrils split up into their individual components more rapidly than bundles of conventionally treated chrysotile. The number of individual fibrils available for reaction with cells either *in vivo* or *in vitro* might, therefore, be far greater than with the same mass of conventional chrysotile.

There is evidence to suggest that the manufacture and use of wet dispersed chrysotile products may be associated with a relatively low level of respirable dust. They are, therefore, considered to pose a reduced health hazard when compared to standard chrysotile materials. Evidence from the present studies suggests, however, that if dust is produced from wet dispersed chrysotile, then it may be more dangerous than similar amounts of standard chrysotile dust.

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