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In vitro genotoxicity studies of chrysotile asbestos fibers dispersed in simulated pulmonary surfactant

J. Lu [a], M.J. Keane [b], T. Ong [a,b] and W.E. Wallace [a,b,*]

[a] *West Virginia University and* [b] *Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, 944 Chestnut Ridge Road, Morgantown, WV 26505-2808, USA*

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Summary

Micronucleus (MN) formation and sister-chromatid exchange (SCE) assays were performed for asbestos in cultured Chinese hamster lung (V79) cells to determine the effect of surfactant treatment on the genotoxicity of two chrysotile asbestos samples of different fiber lengths. The cells were challenged in vitro with NIEHS intermediate- and short-length chrysotile fibers in both their native state and with surfactant pretreatment. For the surfactant pretreatment, the fibers were incubated in a simulated pulmonary surfactant which was prepared by ultrasonically dispersing dipalmitoyl lecithin (DPL), a primary component of pulmonary surfactant, in minimal essential medium (MEM). Chrysotile asbestos was ultrasonically mixed into the prepared surfactant dispersion or into MEM. V79 cells were exposed to DPL-treated intermediate-length chrysotile (TICA), intermediate-length chrysotile (ICA), DPL-treated short-length chrysotile (TSCA) or short-length chrysotile (SCA) fibers for 48 h. For each treatment, 2000 mononucleated cells were scored for MN formation, and 30 M_2 metaphase cells were scored for SCE induction. The results showed that all samples, TICA, ICA, TSCA and SCA, caused significant elevation in the frequency of cells with micronuclei and of cells with two or more nuclei. The increase in micronucleus frequency was greatest in cells challenged with untreated intermediate-length fibers, and was greater for untreated than for DPL-treated short-length fibers. For the short-length fiber samples, DPL surfactant treatment decreased activity for multiple nucleus formation, while DPL treatment did not result in consistent changes in that activity for intermediate-length fibers. Results of SCE assays were either negative or inconclusive. Cells were more viable following TICA and TSCA than following ICA and SCA challenge as measured by cell counts after 48 h of incubation.

* Corresponding author.

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Asbestos is known to induce plural mesothelioma and carcinoma in the lungs of humans and rats (Davis, 1970; Stanton and Wrench, 1972; Wagner et al., 1973, 1982; Harington et al., 1975; Selikoff, 1979). In vitro, asbestos induces chromosomal aberrations and micronuclei in cultured mammalian cells (Sincock and Seabright, 1975; Brown et al., 1979; Babu et al., 1980, 1981; Oshimura et al., 1984; Hesterberg et al., 1985, 1986a,b). The biological effects of asbestos are influenced by several factors, which include size, durability, chemistry, and physiochemistry of fibers. Jaurand et al. (1980, 1988) and Vallyathan et al. (1987) indicated that the surface of asbestos fibers is one of the critical factors in determining their in vitro toxicity. Surface modifications, therefore, may affect the genotoxicity or cytotoxicity of native asbestos fibers.

When inhaled into the deep lung airspaces, fibers first contact the pulmonary surfactant on the inner surfaces of the lung alveoli or bronchioles. Studies have shown that the initial response of the rat lung to chrysotile asbestos is a progressive increase in the amount of pulmonary surfactant generated (Tetley et al., 1977; Oblin et al., 1978). It was reported that chrysotile asbestos can absorb dipalmitoyl lecithin (DPL) which is a principal constituer surfactant, and that the absorpt pholipid changes the surface prope e asbestos, resulting in the suppre emolytic activity (Light and Wei, 1977a,b; Jaurand et al., 1980). It has also been reported that DPL surfactant-dispersion reduces the in vitro cytotoxicity of diesel exhaust particles (Wallace et al., 1987; Keane et al., 1991). Therefore, the question arises as to whether pulmonary surfactant can affect the genotoxicity of chrysotile asbestos.

In this study, DPL was ultrasonically dispersed in minimal essential medium (MEM) to model phospholipid surfactant dispersion in the pulmonary alveolar hypophase. Cultured Chinese hamster lung cells, V79, were challenged with untreated chrysotile asbestos and with chrysotile asbestos treated by incubation in DPL surfactant dispersion. Micronucleus (MN) formation and sister-chromatid exchange (SCE) were used as in vitro genotoxicity assay systems.

Material and methods

DPL (1 mg / ml) dispersion

Dipalmitoyl lecithin was obtained from Calbiochem (La Jolla, CA). Minimal essential medium (Gibco, Grand Island, NY) without fetal bovine serum (FBS; Gibco) was added to DPL that had been weighed into a sterile centrifuge tube, and the contents were ultrasonicated for 10 min at 30 W (Heat Systems Inc., Plainview, NY).

Preparation of chrysotile asbestos

NIEHS intermediate length and short length chrysotile asbestos samples (15 mg), autoclaved 15 min at 121°C and separately mixed in either a DPL dispersion or in their native state in MEM without FBS, were sonicated for 10 min at 30 W. The samples were incubated 60 min at 37°C in a rotary drum incubator, then centrifuged for 10 min at 1200 *g*. After centrifugation, the supernatant was discarded, and the pellet resuspended in MEM without FBS. The mixing ratio for treatment in DPL dispersion was 500 mg DPL/g asbestos, which has been shown in this laboratory previously to supress membranolytic activity in the hemolysis assay (unpublished data). The mean fiber length for the short length sample was 11.6 μm, with 98% of fibers less than 10 μm. The intermediate length sample mean length was 101 μm, with 65% of the fibers greater than 10 μm. Additional physical and chemical characterizations of the fiber samples have been given by Campbell ,et al. (1980). Both asbestos samples were analyzed for specific surface area by N_2 adsorption using the BET method with a multipoint analyzer (Micromeritics, Norcross, GA). The short chrysotile measured 41.4 m^2/g and the intermediate 22.6 m^2/g. Examination of dispersed fibers by scanning electron microscopy indicated no aggregation of fibers.

Treatments

4.0×10^5 V79 cells were seeded into each 100-mm dish in 10 ml MEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine. After overnight incubation at 37°C and 5% CO_2, DPL-treated intermediate- (TICA), DPL-treated short- (TSCA), untreated intermedi-

ate- (ICA) and untreated short-length (SCA) chrysotile asbestos fibers were added into the culture cell dishes to give concentrations of 3.3–100 μg/ml, respectively. The concentrations selected were based on other studies reported in the literature (Lavappa et al., 1975; Sincock and Sebright, 1975; Livingston et al., 1980; Babu et al., 1981; Casey, 1983). Blank and DPL controls were included in each experiment.

Micronucleus assay

After 48-h treatment, cells were rinsed 5 times with phosphate-buffered saline, left to incubate an additional 24 h, then harvested by trypsinization. The contents of each dish were transferred into a 15-ml centrifuge tube and centrifuged at 285 *g* for 6 min. The supernatant was removed and the pellet resuspended in MEM to the desired dilution. Slides were prepared on a Cytospin and stained with Diff-Quik solution (American Scientific Products, McGaw Park, IL). The slides were coded before scoring. 2000 mononucleated cells from each group were scored at random for MN, and another 2000 cells were scored from each group for determining the percentage of cells with one nucleus and with two or more nuclei.

Sister-chromatid exchange assay

After 17 h of asbestos challenge, 15 μM bromodeoxyuridine (BrdU; Gibco) was added to each dish. Colcemid (Gibco) was added (0.15 μg/ml) 3 h before the end of culturing. Cells were harvested and slides prepared 48 h after the initial asbestos challenge. The slides were allowed to air dry and were stained by the modified fluorescence plus Giemsa method of Perry and Wolff (1974) and Goto et al. (1978). Slides were stained for 15 min with 10% Hoechst 33258, rinsed with distilled water, then placed in a pool of Sorenson's buffer (pH 6.8) at 56–60°C on a slide warmer and exposed to "black" light of approximate 355 nm at a distance of less than 1.0 cm for 15 min. Slides were rinsed in distilled water and stained with 5% Giemsa for 15–20 min. After a rinse, they were allowed to air dry and then were coded. 30 well-differentiated second division (M2) cells, with 20–22 chromosomes, from each dose and control, were scored for SCE. Cell replicative index (RI) was obtained by determining the numbers of M1, M2 and M3 cells in the first 100 mitotic cells observed.

Results

Cells with MN and multinucleated cells

Table 1 and Fig. 1 show that all doses of TICA, ICA, TSCA and SCA induced a statistically significant elevated percentage of micronucleated cell (MNC) over the DPL or blank control after a 48-h treatment ($p < 0.01$). Untreated short-length fibers induced greater frequencies of

TABLE 1

FREQUENCIES OF MICRONUCLEATED AND MULTINUCLEATED V79 CELLS AFTER TREATMENT WITH CHRYSOTILE ASBESTOS [a]

Treatment [b]	Concentration [c]		Micronucleated cells		% ≥ 2 Nuclei
	μg/cm^2	mm^2/cm^2	Number	%	
TICA	1.27	28.7	78 [e,g]	3.9	3.5 [f]
	4.2	95	158 [e]	7.9	26.4
	12.7	287	178 [e]	8.9	36.5 [f]
ICA	1.27	28.7	157 [e]	7.9	19.1
	4.2	95	178 [e]	8.9	26.5
	12.7	287	211 [e]	10.6	20.4
TSCA	0.42	17.5	38 [e]	1.9	2.6 [f]
	1.27	53	65 [e,g]	3.3	4.6 [f]
	4.2	175	73 [e,g]	3.7	7.7 [f]
	12.7	527	79 [e,g]	4.0	8.3 [f]
SCA	0.42	17.5	36 [d]	1.8	1.8
	1.27	53	92 [e]	4.6	12.9
	4.2	175	143 [e]	7.2	15.9
	12.7	527	155 [e]	7.8	19.5
DPL	1.27		16	0.8	0.4
Blank	–		18	0.9	1.3

[a] Based on 2000 mononucleated cells scored.

[b] TICA, DPL-treated intermediate-length chrysotile asbestos.
ICA, intermediate-length chrysotile asbestos.
TSCA, DPL-treated short-length chrysotile asbestos.
SCA, short-length chrysotile asbestos.
DPL, dipalmitoyl lecithin.

[c] To convert doses to μg/ml or mm^2 ml, multiply dose figures by 7.85.

[d] $p < 0.05$, compared to proper control.

[e] $p < 0.01$, compared to proper control.

[f] $p < 0.05$, compared to native chrysotile at the same dose.

[g] $p < 0.01$, compared to native chrysotile at the same dose.

MNC than short-length chrysotile treated with DPL. For example, at 4.2 $\mu g/cm^2$ and 12.7 $\mu g/cm^2$ the number of MNC in SCA-challenged culture was about 2 times greater than that of TSCA-challenged culture. The frequencies of MNC induced by DPL-treated and untreated intermediate-length fibers, however, were not significantly different. Intermediate-length chrysotile asbestos induced a greater frequency of MNC than did short-length fibers on a mass or surface area basis. The frequency of MNC in SCA-challenged cultures at 12.7 $\mu g/cm^2$ was of the same order as that in ICA-challenged cultures at 1.3 $\mu g/cm^2$ (7.8% and 7.9% respectively). Results are expressed in both $\mu g/cm^2$ and mm^2/cm^2 to allow comparison from both a mass and surface area basis.

The TICA, ICA, TSCA and SCA treatments all caused a significant increase in the number of cells with 2 or more nuclei over the DPL or blank control ($p < 0.01$, Table 1). The intermediate-length chrysotile samples induced higher frequencies of multinucleated cells than the respective short samples, and treated fibers were generally less active than untreated fibers,of respective size, with the exception of the highest-concentration ICA sample. Increases in TICA, TSCA and SCA concentrations, with the exception of 12.7 μg ICA/cm^2, increased the number of multinucleated cells, while monocleated cells became less frequent with an increase in concentration (trend analysis, $p < 0.01$).

Several fiber samples that responded positively

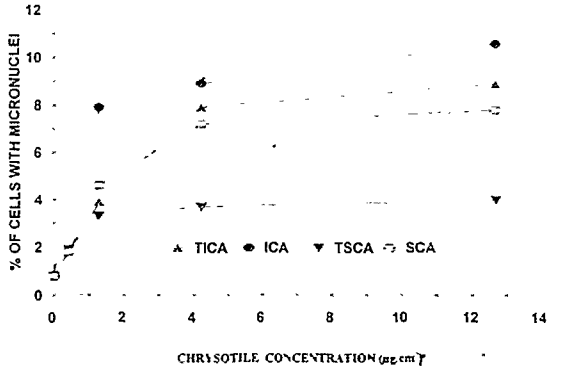

Fig. 1. Frequencies of micronucleated cells after asbestos treatments.

TABLE 2

SISTER-CHROMATID EXCHANGE FREQUENCIES AFTER ASBESTOS TREATMENTS [a]

Treatment [b]	Concentration		SCEs/cell (mean ± SD)	RI
	$\mu g/cm^2$	mm^2/cm^2		
TICA	1.3	29	7.33 ± 2.48	1.99
	4.2	95	7.43 ± 2.43	1.97
	12.7	287	7.77 ± 2.65	1.92
ICA	1.3	29	7.13 ± 2.32	1.93
	4.2	95	8.17 ± 2.96 [c]	1.97
	12.7	287	6.93 ± 1.98	1.93
TSCA	0.4	18	7.30 ± 2.15	1.97
	1.3	53	6.90 ± 1.86	1.97
	4.2	175	6.50 ± 1.89	1.94
	12.7	515	7.20 ± 1.61	1.98
SCA	0.4	18	6.27 ± 2.42	1.99
	1.3	53	7.03 ± 1.65	2.00
	4.2	175	6.50 ± 2.52	1.93
	12.7	527	7.43 ± 2.50	1.93
Blank			6.47 ± 2.35	1.99
DPL	10.0	–	7.13 ± 1.50	2.00

[a] 30 M_2 metaphases containing 21 ± 1 chromosomes were scored for each group.
[b] Abbreviations, see Table 1.
[c] $p < 0.05$, compared to proper control.

to the micronucleus assay were studied by the immunofluorescent kinetochore staining method of Channarayappa et al. (1992) to investigate whether clastogenic or aneuploidogenic effects were associated with the micronuclei in this study. The limited results indicated that both effects were present in similar proportions (data not shown) in the samples studied.

SCE results

Table 2 shows that there was no dose-related increase in SCE frequency induced by any of the 4 chrysotile asbestos samples. Nor was the cell replicative index affected by the chrysotile asbestos samples used in the experiment.

Relative viability of cells

When harvested, viable cells in each of the cultures were counted with a hemacytometer under the microscope. The relative viability of cells was calculated from the number of cells that excluded trypan blue after 48 h of incubation

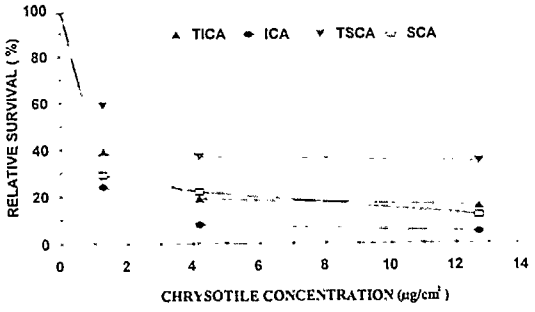

Fig. 2. Viability of V79 cells exposed to chrysotile fibers suspended in medium and simulated surfactant.

with chrysotile fibers divided by the number of cells in the medium control or DPL control. As shown in Fig. 2, the relative viabilities of cells at 12.7 $\mu g/cm^2$ were 15.1 and 34.4% for TICA and TSCA challenge, and were 3.8 and 12.1% for ICA and SCA challenge, respectively. At the higher doses of untreated chrysotile treatment, many cells were morphologically distorted, and the cell membrane was often broken in cells with 2 or more nuclei.

Discussion

Chrysotile asbestos has been shown to induce chromosomal aberrations and micronuclei formation in the Syrian hamster cell, the Chinese hamster ovary cell, and the rat pleural mesothelial cell (Sincock and Seabright, 1975; Oshimura et al., 1984; Hesterberg et al., 1986a,b; Jaurand et al., 1988). In this study, we used the Chinese hamster lung cell line, V79, since it has a rapid growth rate, high plating efficiency and stable karyotype (Bradley et al., 1981). The intermediate-length fiber sample appears to be more active for micronucleus induction than the shorter fiber sample. However, only two sets of fibers were used, and they were not size separated fractions from a common sample, thus the role of other structural or compositional factors cannot be ruled out. In this study, both DPL-treated and untreated chrysotile asbestos caused a significant increase in the frequency of MNC in V79 cells over the controls in a dose-related manner for samples of both lengths: Treatment with DPL reduced activity of the short-length chrysotile asbestos for induction of MN.

It has been suggested that the surface properties of asbestos, especially the surface charge, affect the in vitro toxicity (Jaurand et al., 1980, 1988; Vallyathan et al., 1985). Chrysotile asbestos can adsorb phospholipids on its surface, masking or reducing the surface charge of the fibers (Light and Wei, 1970a,b; Jaurand et al., 1980). The decrease in clastogenic activity of DPL-treated fibers seen here may be due to DPL masking fiber surface sites or due to DPL modification of the fiber surface area or composition. To determine if the latter occurs over more extended times in vivo, the surface composition of chrysotile asbestos could be analyzed after a long period of retention in the lungs of exposed animals during the course of an animal carcinogen study.

Recent data have indicated that long fibers have the higher genotoxic and carcinogenic potency as compared with shorter fibers (Stanton et al., 1981; Pott et al., 1984; Hesterberg et al., 1986a). The results of this study are not inconsistent with those findings. The frequencies of MNC in ICA- and TICA-challenged cultures at a dose of 1.3 $\mu g/cm^2$ or 29 mm^2/cm^2 were comparable to those in SCA- and TSCA-challenged cultures at a dose of 12.7 $\mu g/cm^2$ or 527 mm^2/cm^2, respectively (Table 1 and Fig. 1). Hesterberg (1986a) indicated that cells may selectively internalize longer fibers, and that the surface area per fiber could be an important factor in phagocytosis, whereas the mass or the length of fiber could be a critical factor affecting the induction of cytogenetic damage.

The presence of binucleated cells in asbestos-treated cultures have been reported (Sincock and Seabright, 1975; Casey, 1983; Hesterberg et al., 1985, 1986a). The initiation of cytokinesis is dependent on the successful completion of earlier events of mitosis. Thus possible interference of chrysotile asbestos with the structure or function of the mitotic apparatus could affect cytokinesis, resulting in binucleation or multinucleation. In this study, we found a common trend between the percentage of cells with micronuclei and the percentage of cells with ≥ 2 nuclei. Whether there is a relationship between the two effects is not known. It is known that micronuclei are formed either by acentric chromosomal fragments due to chromosomal breakage or by centric chromo-

somes lagging behind in mitosis due to spindle damage. However, it is not apparent as to how chrysotile asbestos induces multinucleated cells. Neither is it clear as to how DPL treatment reduces the activity of the short-length chrysotile asbestos sample to induce MNC and multinucleated cells observed in the present study. DPL treated intermediate-length fibers appeared to be as active as untreated fibers, but this could be due to cytotoxic effects of the untreated fibers.

The SCE results were different from those for micronucleus induction. No significant difference in the frequency of SCE was found after V79 cells were challenged with all 4 types of chrysotile asbestos samples. Conflicting data on SCE induction by asbestos fiber have been reported. Casey (1983) did not find SCE enhancement in asbestos-treated CHO cells, human fibroblasts or lymphoblastoid cells. In contrast, Babu et al. (1980) and Livingston et al. (1980) indicated that chrysotile asbestos caused an increase in SCE frequencies. Recent data have shown that in vitro SCE induction is either absent or very slight after chrysotile asbestos challenge (Jaurand et al., 1985; Rosin et al., 1985; Kelsey et al., 1986).

In conclusion, the present findings indicate that both short- and intermediate-length chrysotile asbestos fibers, with or without DPL surfactant treatment, induce micronuclei and multiple nuclei but not SCE in V79 cells. Treating chrysotile fibers with simulated pulmonary surfactant reduced their cytotoxicity. Intermediate-length chrysotile asbestos induced micronuclei and multiple nuclei in a greater percentage of cells than did the short-length chrysotile asbestos. The primary effect of treating chrysotile fibers with simulated pulmonary surfactant was to reduce cytotoxicity. DPL treatment had no consistent significant effect on micronucleus or multiple nucleus induction by intermediate length fibers, while DPL treatment significantly reduced micronucleus and multiple nucleus induction by short-length chrysotile fibers.

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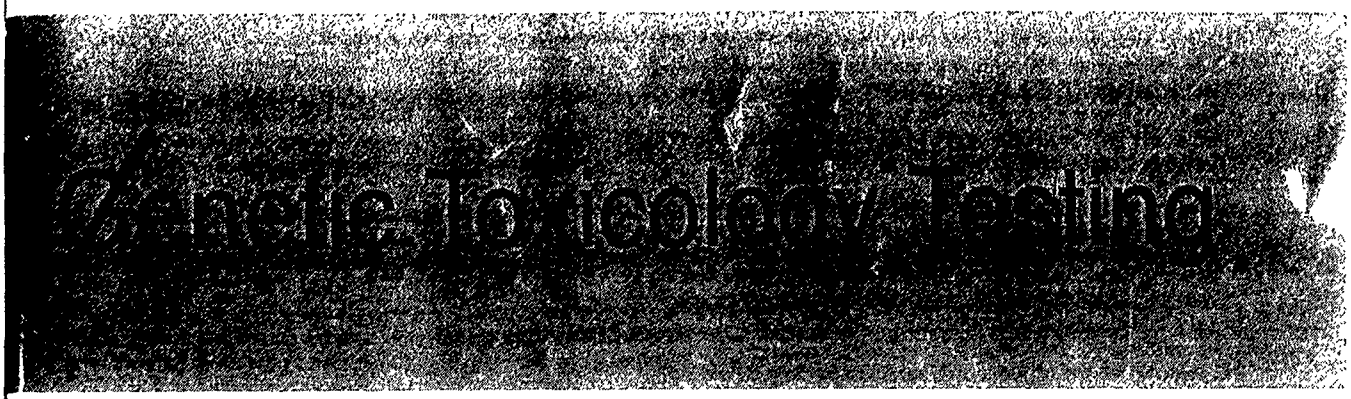

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