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INDUCTION OF MICRONUCLEATED AND MULTINUCLEATED CELLS BY MAN-MADE FIBERS IN VITRO IN MAMMALIAN CELLS

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Many workers as well as the general public are exposed to glass fibers, which are among the most common man-made fibers. Information related to their genotoxicity and potential carcinogenicity is still limited. In this study, we investigated the ability of glass fibers to induce micronucleated and multinucleated cells in cultured Chinese hamster lung fibroblasts, the V79 cells. The induced micronuclei were further analyzed to determine the mechanism of micronucleus formation by staining the kinetochore with anti-kinetochore and fluoresceinated goat anti-human immunoglobulin G (IgG) antibodies. Three types of glass fibers (Manville 100 microfiber, Owens Corning AAA-10 microfiber, and Owens Corning general building insulation fiber) were studied. The results show that the two microfibers induced significant numbers of multinucleated and micronucleated cells in a concentration-related manner. Immunofluorescent staining demonstrated a significant dose-related increase in the proportion of kinetochore-positive micronuclei in cells treated with the two microfibers. These results indicate that the two microfibers are capable of inhibiting cytokinesis and are principally aneuploidogens. Unlike the two microfibers, the larger fibers neither induced micronuclei nor inhibited cytokinesis in V79 cells. Thus, the genotoxic potential of glass fibers in V79 cells may be related to their size.

Glass fibers are among the most common man-made fibers. They are widely used for building materials, air and liquid filtration, fire protection, insulation, textiles, and light transmission (IARC, 1988). Because of their widespread use, a large number of workers, as well as the general population, are likely to be exposed to glass fibers. It is important, therefore, to know whether glass fibers pose genotoxic and potential carcinogenic hazards to exposed individuals.

A limited number of studies on the genotoxicity of glass fibers have been reported in the literature. Manville code 100 (JM 100) and Manville code 110 coarse glass fibers (JM 110) did not induce mutation in *Escherichia coli* and *Salmonella typhimurium* (Chamberlain & Tarmy, 1977). Studies conducted with eukaryotic cells showed that neither JM 100 nor JM 110 increased sister chromatid exchange in Chinese hamster ovary (CHO) cells, in human fibroblasts, or human lymphoblastoid cells in vitro (Casey, 1983). However, JM 100, but not JM 110, induced chromosomal breaks and rearrangements in CHO

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cells (Sincock et al., 1982). The respirable fraction of JM 110 has also been shown to cause chromosomal breaks and fragments in Chinese hamster lung fibroblasts, the V79 cells (Brown et al., 1979).

The micronucleus assay is one of the most frequently used short-term assay systems for the detection of genotoxic agents and potential carcinogens (Mavournin et al., 1990). The system is designed to detect micronuclei in cells exposed to genotoxic agents *in vivo* or *in vitro*. Micronuclei are formed either by acentric chromosomal fragments due to chromosomal breakage or by centric chromosomes lagging behind in mitosis due to spindle damage. Therefore, an increase in the incidence of micronuclei following treatment indicates that the substance to which cells were exposed is capable of breaking chromosomes or inducing aneuploidy. Both cytogenetic changes may be involved in the initiation and/or progression of carcinogenesis (Bishop, 1989).

In our laboratory, studies have been performed to (1) determine the micronucleus- and multinucleus-inducing activities of glass fibers *in vitro* in mammalian cells and (2) characterize the possible mechanism of glass-fiber-induced micronuclei. Results of these studies are presented here.

MATERIALS AND METHODS

Cell Line and Culture

The Chinese hamster lung fibroblast cell line (V79 cells) was kindly supplied by Dr. C. C. Chang (Michigan State University, East Lansing, MI). Cells were grown exponentially in minimum essential medium (MEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma), 1 mM L-glutamine (Sigma), 100 units penicillin/ml, and 100 μ g streptomycin/ml (Sigma). Cultures were maintained in 75-cm² Falcon tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO₂. They were subcultured twice a week using 0.25% trypsin solution (Sigma) in phosphate-buffered saline (PBS).

Fiber Samples

Three types of glass fibers—Owens Corning AAA-10 microfiber (AAA-10, Owens Corning Corp., Toledo, OH), Manville code 100 (JM 100, Manville Corp., Denver, CO), and Owens Corning general building insulation (INS, Owens Corning Corp.) were used in this study. These bulk samples consisted of matts of fibers several centimeters in length. In order to produce samples of a size suitable for study, the bulk material was milled for approximately 30 min using a Tekmar A-10 analytical knife mill operating at 20,000 rpm (Tekmar Company, Cincinnati, OH). For the insulation sample, measurements of length and width were made using a Zeiss Axioskop light microscope under

brightfield conditions. Size measurements for the JM 100 and the AAA-10 samples were made using a JEOL 100CX transmission electron microscope (JEOL USA, Inc., Peabody, MO). The median lengths and widths were 98 and 7.3 μm for INS, 3.5 and 0.2 μm for JM 100, and 2 and 0.18 μm for AAA-10, respectively.

Micronucleus and Immunofluorescent Staining Assays

The procedure used for glass fiber treatment and slide preparation was similar to that reported by Channarayappa et al. (1992) except that cytochalasin-B was not used. Exponentially growing cells were transferred to precleaned and sterile glass slides (0.5×10^5 cells in 1 ml of growth medium per slide) in $100 \times 100 \times 15$ mm square petri dishes and allowed to adhere for 2 h at 37°C before 15 ml growth medium was added to each dish. The cells were incubated at 37°C for 24 h in a humidified atmosphere containing 5% CO_2 and were then exposed to glass fibers for 24 h. Two sets of slides were used for each treatment group. After the end of treatment, slides were rinsed 3 times in PBS, treated with hypotonic solution (0.075 M KCl) for 10 min, and fixed in absolute methanol for 20 min. One set of slides was stained with Diff-Quik stain (Fisher, Pittsburgh, PA) and was used for the micronucleus (MN) assay. For each treatment, 4000 cells were scored for the presence of micronucleated cells (MNCs) using criteria described by Countryman and Heddle (1976). The frequency of multinucleated (more than one nucleus) cells (MTC), based on 500 cells scored, was also determined. For the immunofluorescence staining assay, the remaining slides were stained with human antikinetochore and fluoresceinated goat anti-human immunoglobulin G (IgG) antibodies (Eastmond & Tucker, 1989). Slides from each experiment were randomized and coded prior to scoring. The numbers of kinetochore-positive (KC+) and kinetochore-negative (KC-) MN in 4000 cells were determined for each treatment. Statistical analyses were performed using trend and chi-square tests.

RESULTS

Results as summarized in Table 1 demonstrate the micronucleus-inducing activity of glass fibers. Both AAA-10 and JM 100 microfibers induced micronucleus formation in V79 cells. The increase in the numbers of MNC was similar in both fiber types and was concentration related. At the highest concentration (80 $\mu\text{g}/\text{ml}$) tested, both types of fiber caused an approximately fivefold increase in the frequency of MNC over the medium control. Both AAA-10 and JM 100 were particularly effective for the induction of MTCs in V79 cells. At the highest concentration tested, the increase in the frequency of MTC over that of the medium control was 15- to 20-fold. On the other hand,

TABLE 1. Frequencies of micronucleated and multinucleated cells in V79 cell cultures treated with glass fibers

Fiber	Concentration (µg/ml)	Total micronucleated cells ^b	Total multinucleated cells ^c	Percent of micronucleated cells	Percent of multinucleated cells
AAA-10	0	58	12	1.5	2.4
	10	92 ^d	63 ^d	2.3	12.5
	20	133 ^d	112 ^d	3.3	22.3
	40	191 ^d	155 ^d	4.8	31.0
	80	223 ^d	178 ^d	5.6	35.5
JM 100	0	49	12	1.2	2.4
	10	102 ^d	77 ^d	2.6	15.4
	20	173 ^d	120 ^d	4.3	24.0
	40	216 ^d	171 ^d	5.4	34.1
	80	272 ^d	248 ^d	6.8	49.5
INS	0	59	12	1.5	2.3
	10	56	13	1.4	2.5
	20	59	18	1.5	3.5
	40	52	14	1.3	2.8
	80	62	21	1.6	4.2
	160	53	20	1.3	4.0
VS ^a	0.04	204 ^d	24	5.1	4.7

^aVincristine sulfate was used as a positive control.

^bBased on 4000 cells scored.

^cBased on 500 cells scored.

^dDifferent from the respective control at $p < .01$ by trend and chi-square tests.

INS, the bigger fiber, did not induce either micronuclei or multinuclei in V79 cells.

Results of the immunofluorescent staining assay are shown in Table 2. Again, both microfibers induced similar concentration-related increased frequencies of MNC. Almost all the MNCs induced by AAA-10 and JM 100 carried KC+ micronuclei. The frequency of KC+ micronuclei is similar to that induced by vincristine sulfate, which is a known aneuploidogen. The number of MNCs that carried KC- micronuclei was slightly higher in cultures treated with higher concentrations of glass fibers. However, the increase was not significantly different from the control.

DISCUSSION

The data reported here indicate that man-made microfibers such as AAA-10 and JM 100 are capable of inducing micronucleated and multinucleated cells in cultured Chinese hamster lung fibroblasts. It appears that both microfibers induced similar increased frequencies of MNC in a concentration-related manner. This may be attributed to

similarities in fiber size. Conversely, INS, comprised of thicker and longer fibers, did not affect either cytogenetic endpoint. It is not known whether V79 cells are able to phagocytize INS. INS induces morphological transformation in BALB/c-3T3 cells. However, the transforming activity of INS is much less than that of AAA-10 and JM 100 (Gao et al., 1995). The relative inactivity of INS in the in vitro system is consistent with the decreased in vivo carcinogenicity of fiber associated with these dimensional characteristics (Stanton et al., 1977).

In vitro induction of micro- and binucleated, aneuploid, and polyploid cells by JM 100 in Syrian hamster embryo cells (Oshimura et al., 1984) and polyploidy in CHO cells have also been reported (Sincock et al., 1982). Thin glass wool has recently been shown to induce multinucleated cells in human primary mesothelial cells (Pelin et al., 1995). If the induction is based on the number of fibers per culture area, then thin glass wool and chrysotile or crocidolite asbestos appear to be equally effective (Pelin et al., 1995). Our results, therefore, are consistent with the information available in the literature for man-made fibers.

Since MNCs induced by glass fibers, such as those induced by vincristine sulfate, carried KC+ micronuclei, the presence of centric chromosomes can be inferred. Thus, the induction of micronucleus formation by glass fibers is likely due to spindle damage. Surprisingly, very few, if any, micronuclei induced by AAA-10 or JM 100 are due

TABLE 2. Kinetochore-positive (KC+) and kinetochore-negative (KC-) micronucleated cells in V79 cell cultures treated with AAA-10 and JM 100

Fiber	Concentration (µg/ml)	Micronucleated cells ^b			Micronucleated cells (%)	
		Total ^c	KC+ ^c	KC- ^c	KC+	KC-
AAA-10	0	53 (1.3)	27 (0.7)	26 (0.7)	50.9	49.1
	10	103 (2.6) ^d	75 (1.9) ^d	28 (0.7)	72.8	27.2
	20	133 (3.3) ^d	102 (2.6) ^d	31 (0.8)	76.7	23.3
	40	157 (3.9) ^d	132 (3.3) ^d	25 (0.6)	84.1	15.9
	80	201 (5.0) ^d	169 (4.2) ^d	32 (0.8)	84.1	15.9
JM 100	0	52 (1.3)	28 (0.7)	24 (0.6)	53.8	46.2
	10	120 (3.0) ^d	95 (2.4) ^d	25 (0.6)	79.2	20.8
	20	162 (4.1) ^d	132 (3.3) ^d	30 (0.8)	81.5	18.5
	40	198 (5.0) ^d	166 (4.2) ^d	32 (0.8)	83.8	16.2
	80	228 (5.7) ^d	195 (4.9) ^d	33 (0.8)	85.5	14.5
VS ^a	0.04	197 (4.9) ^d	162 (4.1) ^d	35 (0.9)	82.2	17.8

^aVincristine sulfate was used as a positive control.

^bBased on 4000 cells scored.

^cPercent of cells in parentheses.

^dDifferent from the respective control at $p < .01$ by trend and chi-square tests.

to chromosomal breaks, although JM 100 and the respirable fraction of JM 110 have been reported to cause chromosomal breaks in cultured mammalian cells (Brown et al., 1979; Sincock et al., 1982). Nevertheless, glass fibers can be considered aneuploidogens and may pose genotoxic and potential carcinogenic hazards to exposed workers.

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