Role of Phagocytosis in Syrian Hamster Cell Transformation and Cytogenetic Effects Induced by Asbestos and Short and Long Glass Fibers

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ABSTRACT

We have shown previously that asbestos and other mineral dusts, including glass fibers, induce cell transformation and chromosomal mutations in Syrian hamster embryo cells in culture. In the present study, we observed that both asbestos and glass fibers were phagocytized by these cells and accumulated in the perinuclear region of the cytoplasm. In order to understand the mechanism of fiber length-dependent cellular effects, we examined the phagocytosis and intracellular distribution of glass fibers of differing lengths in cells at various times after treatment. Glass fiber length was decreased by milling with a mortar and pestle. Cells treated with an equal dose of milled glass fibers (on a weight per surface area basis) were exposed to 7-fold more fibers since milling of glass fibers resulted in a 7-fold decrease in length with little change in diameter. However, cells exposed to milled glass fibers phagocytized a similar number of fibers as cells exposed to an equal mass of unmilled glass fibers, indicating that milled fibers were less readily phagocytized. In cells treated with either unmilled or milled glass fibers, the length of the intracellular fibers was more than 2-fold greater than the length of the fibers on the surface, suggesting that cells selectively internalized longer fibers. Fiber length, however, did not appear to affect the migration of intracellular fibers to the perinuclear region of the cytoplasm. Even though cells treated with milled glass fibers contained a similar number of fibers as those treated with unmilled glass fibers, the resulting cytotoxicity, transformation frequency, and frequency of micronuclei were greatly reduced in the cultures treated with milled glass fibers. Thus, fiber length appears to affect the phagocytosis of fibers as well as the ability of intracellular fibers to induce cytogenetic damage and the resultant transformation.

INTRODUCTION

Occupational exposure to asbestos has been associated with an increased incidence of pleural mesothelioma and carcinoma of the lung (1–3). In addition, asbestos and other mineral dusts, including glass fibers, cause pleural mesothelioma in rats following intrapleural injection (1, 4, 5) and pulmonary carcinoma following inhalation exposure (6, 7). Epidemiological studies (2) as well as experimental animal studies (8) have shown that asbestos can act synergistically with other carcinogens. In vitro, asbestos has been shown to have both solitary or direct carcinogenic activity (9–13) as well as promoting and cocarcinogenic activities (13–15). These observations suggest that the mechanisms by which mineral dusts induce cancer are probably diverse. The in vitro findings provide experimental models to examine some of the mechanisms of asbestos effects on cellular alterations associated with neoplastic transformation.

Morphological transformation of SHE2 cells in culture is induced by asbestos and other mineral dusts (9, 13), and fiber length is more important than chemical composition in the induction of transformation (9); long fibers are more potent than short fibers. Since others have shown similar fiber dimension dependence for the induction of mesotheliomas in rats (4, 16), these observations support the use of this model for studying some aspects of asbestos carcinogenicity (9).

The cellular targets for asbestos-induced carcinogenicity are unknown. One approach to this problem is to determine the cellular targets for cell transformation and extrapolate these findings to the in vivo situation. Phenotypic alterations of cells in culture such as morphological or growth changes theoretically can occur by either genetic or epigenetic mechanisms (17). In order to explore the role of genetic events in asbestos carcinogenicity, we have examined whether mutational changes, at the gene or chromosomal level, are involved in asbestos-induced cell transformation using the Syrian hamster embryo cell culture model. Transforming doses of asbestos fail to induce mutations at two specific genetic loci (18) and induce only a slight elevation in sister chromatid exchange at the highest dose (10). However, the same doses of mineral dusts induce a dose-dependent increase in micronuclei and chromosome aberrations, especially numerical chromosome changes (18). Furthermore, when fiber length is reduced, the potency in inducing chromosome changes is also reduced (18). Thus, the induction of chromosome changes correlates with the induction of cell transformation by mineral dusts, suggesting a mechanistic relationship (10, 18).

Recently, we have shown that asbestos induces an almost 20-fold increase in the incidence of cells with anaphase abnormalities, including lagging chromosomes, bridges, and sticky chromosomes (19). Fibers are observed in the mitotic cells shortly after treatment and, in some cases, appear to interact directly with the chromosomes. These findings suggest that the target(s) for asbestos effects are intracellular and support our hypothesis that asbestos-induced aneuploidy is important in the induction of transformation of cells by mineral dusts.

In the present study, we have examined in detail the kinetics of phagocytosis and the perinuclear accumulation of asbestos and glass fibers in SHE cells in culture. We have also examined the effect of milling glass fibers on the uptake of the fibers. Our primary interest in studying the phagocytosis and perinuclear accumulation of unmilled and milled glass fibers was to understand why short fibers are less potent on a per weight basis than long fibers in the induction of cell transformation (9) and cytogenetic effects (18) in these cells. We considered the following hypotheses: (a) short fibers were less readily phagocytized by the cells; (b) short fibers were not as efficiently translocated to the perinuclear region of the cytoplasm; or (c) short and long fibers were phagocytized and translocated to the perinuclear region with the same efficiency but perhaps, once inside the cells, the short fibers were less effective in causing the cytogenetic changes that resulted in transformation. Our results suggest that explanations a and b both contribute to the decreased activity of short fibers.

MATERIALS AND METHODS

Cells and Culture Conditions. Syrian hamster embryo cell cultures were established from 13-day-gestation fetuses collected aseptically by
cesarean section from inbred Syrian hamsters, strain LSH/ss LAK (Lakeview Hamster Colony, Newfield, NJ). Pools of primary cultures from littermates were cryopreserved in liquid nitrogen (20). Secondary cultures were initiated from the frozen stocks and all experiments were performed with tertiary cultures. The culture medium was IBR-modified Dulbecco's Eagle's reinforced medium (Grand Island Biological Co., Grand Island, NY) supplemented with 3.7 g sodium bicarbonate per liter, 20% (v/v) Hy-Clone fetal bovine serum (Sterile Systems, Inc., Logan, UT), 100 units penicillin per ml, and 100 µg streptomycin (Grand Island Biological Co.) per ml. Cells were gently trypsinized with 0.1% trypsin solution (1:250; Grand Island Biological Co.) in phosphate-buffered saline (pH 7.4) for 5 min at 37°C. Cultures were routinely tested (21) and were found to be free of Mycoplasma.

Mineral Dust Exposures. International Union Against Cancer crocidolite and chrysotile asbestos were obtained from V. Timbrell (MRC, Great Britain). Code 100 glass fibers were generously provided by Dr. James Lineweber, Johns Manville Corp. (Denver, CO). The glass fibers were further processed to remove clumps of fibers and large amorphous nonfibrous particulates as has been described previously (9). The length of the code 100 glass fibers were decreased by milling with a mortar and pestle. Fiber lengths and diameters were determined as described previously (9).

For ultrastructural studies of cells exposed to mineral dusts, 10th SHE cells were seeded in 35-mm dishes (Falcon Plastics, Oxnard, CA) which contained 25-mm Thermonox discs (Lux Scientific Corp., Newbury Park, CA). On the next day, approximately 15 h after plating, the cells were treated with mineral dust prepared as described previously (14).

Measurement of Phagocytosis and Perinuclear Accumulation of Crocidolite Asbestos and Glass Fibers. Before examining the kinetics of phagocytosis, it was necessary to determine how rapidly fibers settled to the bottom of the culture dish after treatment. To accomplish this, 1-ml aliquots of culture media were taken from treated cultures at 0, 1, 2, and 4 h after asbestos addition. Each aliquot was diluted with 10 ml distilled water and centrifuged at 40,000 × g for 1 h. The pellet was resuspended in 0.1 ml absolute ethanol and the suspension was spread on a carbon disc that had been wetted with 0.1 ml ethanol to promote spreading. Discs were then coated with 100 Å of gold and examined using SEM (×10,000), and the number per aliquot of fibers was quantitated.

For studies on the kinetics of phagocytosis, crocidolite asbestos and glass fibers were chosen because they are more electron dense than chrysotile asbestos and therefore they can be more readily observed using backscatter electron imaging. The latter technique was used to prevent dust or debris or even folds in the cell membrane from being mistaken for mineral fibers.

The kinetics of the phagocytosis and perinuclear accumulation of crocidolite asbestos and glass fibers was determined as follows. At various times after treatment with crocidolite or glass fibers (1, 2, 6, 24, or 48 h), media were removed from dishes containing fiber-treated cells; cells were rinsed with phosphate-buffered saline and then fixed with 1.5% glutaraldehyde-parafomaldehyde for at least 2 h. The cells were then dehydrated in ethyl alcohol and immersed in liquid CO2 for critical point drying. Dehydrated cultures were coated with 250 Å of gold for final viewing in a JEOL JSM-35 scanning electron microscope. Electron backscatter imaging at ×6000 and ×10,000 was used to observe intracellular fibers.

To show that mineral fibers were indeed inside and not under the cells, cells that had been exposed to crocidolite asbestos for 48 h were sectioned and examined using TEM. Briefly, cells on Thermonox discs were fixed in Karnovsky's fixative, postfixed in 1% osmium tetroxide, and stained en bloc in 2% uranyl acetate overnight at 4°C. Cells were then dehydrated through an ethanol series, embedded in a low viscosity epoxy resin, sectioned (60–80 nm), and poststained in uranyl acetate and lead citrate. Using TEM, examination of sections from 25 different cells were made and fibers were categorized as being on top of, inside, or under the cells.

At each time point, the following parameters were measured on each of 25 to 50 randomly selected cells: (a) total number of fibers associated with a cell (cell-associated fibers); (b) number of fibers on the cell surface; (c) number of fibers partially phagocytized by the cell; (d) number of fibers totally phagocytized by the cell; (e) number of cell-associated fibers not located in the perinuclear region (>1 µm from the nucleus); (f) number of cell-associated fibers located in the perinuclear region (≤1 µm from the nucleus); and (g) for the cells treated with glass fibers of different lengths, the length (at ×6,000) and diameter (at ×10,000) of each cell-associated fiber were determined and its location on or within the cell was noted.

Quantitation of Cytotoxicity, Morphological Transformation, and Micronucleus Induction. The in vitro transformation procedure has been described in detail elsewhere (9). Target cells were seeded on a layer of lethally irradiated homologous feeder cells in 60-mm dishes in complete medium. Appropriate dilutions of mineral dusts were made and 2 ml of the suspension were added to dishes containing feeder cells, target cells, and 6 ml of complete medium. The dose was expressed in µg/cm2 of culture dish because the dusts settled to the bottom of the culture dish within 1 h after treatment. Following treatment with mineral dust, the cultures were incubated at 37°C in 12% CO2 in air for 7 days. The cells were then fixed and stained with Giemsa. The colony-forming efficiency was determined by dividing the number of colonies by the number of cells seeded. The cytotoxic response to asbestos was expressed as relative survival, which was determined by dividing the colony-forming efficiency of the treated cells by the colony-forming efficiency of the untreated controls (×100). Morphological transformation was scored according to criteria that has been described previously (9). The transformation frequency was calculated by dividing the total number of transformed colonies by the total number of colonies surviving treatment (×100).

The incidence of micronuclei in glass fiber-treated cultures was determined as described previously (18). Briefly, tertiary passage cultures were inoculated into 75-cm2 flasks (Falcon Plastics) at 5 × 105 cells/flask. After overnight incubation the culture medium was removed, 10 ml of either culture medium or medium containing suspended glass fibers (milled or unmilled) were added to the flasks, and the cultures were incubated for 48 h. The cells were then trypsinized, collected by centrifugation, and fixed in methanol:acetic acid (3:1). The suspension of cells in fixative was dropped on a glass slide wetted with 100% ethanol and air dried. This procedure allowed nuclei to remain intact in the cell cytoplasm. The slides were stained with Giemsa and 1000 cells were scored for each experiment.

RESULTS

Kinetics of Crocidolite Fiber Phagocytosis and Perinuclear Accumulation. Using phase contrast light microscopy, it appeared that virtually all of the asbestos fibers had settled to the bottom of the culture dish by 1 h after treatment with crocidolite asbestos, (1 µg/cm2). Since submicron particles cannot be observed using light microscopy, we decided to examine aliquots of the culture medium using electron microscopy at various times after the asbestos suspension was added. Aliquots of the culture medium were collected and centrifuged, and the number of fibers remaining in the medium was quantitated using SEM at 0, 1, 2, and 4 h after treatment with crocidolite asbestos. At the zero time point, an average of 399 fibers were observed in each of the 48 fields examined. At 1, 2, and 4 h after treatment, an average of 49 (12%), 14 (3.5%), and 5 (1.3%) fibers/field, respectively, were observed. Thus, it is unlikely that settling of fibers to the bottom of the cultures influenced the phagocytosis of fibers, especially at time points beyond 2 h after asbestos treatment.

The interaction of asbestos and glass fibers with Syrian hamster embryo cells in culture was examined after treating the cells in culture with crocidolite asbestos (1 µg/cm2). At various times after treatment (1 to 48 h), the cells were rinsed, fixed, and examined by SEM and electron backscatter imaging. Fibers on the surface were easy to distinguish from partially or totally phagocytized fibers using SEM. Any internalized fibers not

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seen using SEM were observed using electron backscatter imaging. A scanning electron micrograph (Fig. 1) with the corresponding electron backscatter image of a cell after asbestos treatment shows a surface fiber, a partially phagocytized fiber, and a totally phagocytized fiber. We quantitated the fibers per cell and divided them into three categories: the fibers remaining on the surface, partially phagocytized fibers; and totally phagocytized fibers. The total number of cell-associated fibers, which includes all three categories listed above, increased from 1 to 48 h (Table 1). Each cell had an average of 5.9 ± 0.3 (SE) crocidolite asbestos fibers at 1 h after treatment and 21.6 ± 0.9 fibers at 48 h after treatment (Table 1).

To show that mineral fibers were intracellular and not merely under the cells, cells exposed to crocidolite asbestos for 48 h were sectioned and examined using TEM. Examination of sections from 25 different cells demonstrated that, of the 725 fibers observed, all were either on top of the cells or inside the cells; none of the fibers were under the cells. Thus, cells are internalizing the fibers, not merely migrating over them. A transmission electron micrograph of a cell containing crocidolite asbestos fibers is shown in Fig. 2.

Kinetic analysis of interaction of fibers with cells and subsequent phagocytosis revealed that at 1 h after treatment, 88% of the fibers were located on the cell surface (Table 1). By 48 h, only 24% of the total number of fibers were on the cell surface and 57% of the fibers were totally phagocytized. Interestingly, the actual number of fibers on the cell surface was relatively unchanged over 48 h (Table 1), suggesting that additional fibers were being picked up by the cells and replacing those that were phagocytized.

It was also apparent that, in addition to being phagocytized, fibers were also being accumulated in the perinuclear region of the cells (Fig. 3). In order to quantitate this process, a fiber was classified as perinuclear if any portion of the fiber was within 1 μm of the nucleus. Kinetic analysis of perinuclear accumulation of fibers (Table 1) showed that 1 h after treatment only 1.1 ± 0.1 fibers (19%) of a total of 5.9 ± 0.3 cell-associated fibers/cell were located in the perinuclear region, while at 48 h 13.6 ± 0.7 (63%) of 21.6 ± 0.9 fibers/cell were located in the perinuclear region. Even though the percentage of nonperinuclear fibers decreased with time, the absolute number of nonperinuclear fibers actually increased (Table 1), which was similar to what was observed with cell surface fibers. Thus, throughout the 48-h period of observation, cells appear to be interacting with new fibers, phagocytizing them, and moving them to the perinuclear region of the cytoplasm.

It was important to show that the ability of cells to phagocytize fibers was not saturated at the concentration of fibers used for these studies, i.e., 1 μg/cm². To accomplish this, cells were plated on Thermanox discs, treated with either 1- or 2-μg/cm² samples of crocidolite asbestos for 48 h, and fixed for SEM as described above. The total number of cell-associated fibers were then determined in 10 cells at each concentration of asbestos. As expected, cells treated with crocidolite asbestos (2 μg/cm²) had 93% more cell-associated fibers (nearly double) than cells treated with 1 μg/cm². The proportions of fibers that were surface, partially phagocytized, and totally phagocytized after treatment with 1 μg/cm² were similar to what was observed after treatment with 2 μg/cm². Thus, phagocytosis was not saturated at the mineral fiber concentration used in these studies.

Phagocytosis and Perinuclear Accumulation of Unmilled and Milled Glass Fibers. To determine the role of fiber length in fiber uptake, two preparations of glass fibers (milled and unmilled) of differing lengths were studied. The dimensions of unmilled and milled glass fibers were determined after the fibers were spread on carbon discs and examined by scanning electron microscopy (frequency distributions in Table 2). The average length of unmilled glass fibers (15.5 ± 1.5 μm) was 7 times that of the milled glass fibers (2.2 ± 0.2 μm). Milling resulted in relatively little change in fiber diameter. Light microscopic observation showed, as expected, a greater than 6-fold increase in the number of particles per unit surface area in dishes treated with milled glass fibers compared to dishes treated with an equal weight of unmilled glass fibers.

Syrian hamster embryo cells were treated with an equal concentration (1 μg/cm²) on a weight basis of either unmilled or milled glass fibers, incubated at 37°C for varying times, and then processed and analyzed by scanning electron microscopy. As with crocidolite asbestos, the phagocytosis of unmilled and milled glass fibers had leveled off between 24 and 48 h after...
**PHAGOCYTOSIS AND TRANSFORMATION BY ASBESTOS AND GLASS FIBERS**

Table 1  Average number of fibers per cell for each cellular compartment at various times after treatment of Syrian hamster embryo cells in culture with crocidolite asbestos (1 μg/cm²)

<table>
<thead>
<tr>
<th>Time After treatment (h)*</th>
<th>Total cell-associated fibers/cell</th>
<th>Cell surface fibers/cell</th>
<th>Partially phagocytized fibers/cell</th>
<th>Totally phagocytized fibers/cell</th>
<th>Perinuclear fibers/cell</th>
<th>Non-perinuclear fibers/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.9 ± 0.3</td>
<td>5.2 ± 0.3 (88)*</td>
<td>0.1 ± 0.03 (1.7)</td>
<td>0.6 ± 0.1 (10)</td>
<td>1.1 ± 0.1 (19)</td>
<td>4.8 ± 0.3 (81)</td>
</tr>
<tr>
<td>2</td>
<td>5.2 ± 0.4</td>
<td>3.9 ± 0.4 (75)*</td>
<td>0.4 ± 0.1 (7.7)</td>
<td>1.0 ± 0.1 (19)</td>
<td>1.1 ± 0.1 (21)</td>
<td>4.1 ± 0.4 (79)</td>
</tr>
<tr>
<td>6</td>
<td>8.9 ± 0.3</td>
<td>3.4 ± 0.1 (38)</td>
<td>2.1 ± 0.1 (23)</td>
<td>3.5 ± 0.2 (39)</td>
<td>3.1 ± 0.1 (35)</td>
<td>5.8 ± 0.2 (65)</td>
</tr>
<tr>
<td>24</td>
<td>18.7 ± 0.4</td>
<td>3.2 ± 0.1 (17)</td>
<td>2.7 ± 0.1 (14)</td>
<td>12.8 ± 0.3 (68)</td>
<td>12.1 ± 0.3 (65)</td>
<td>6.6 ± 0.2 (35)</td>
</tr>
<tr>
<td>48</td>
<td>21.6 ± 0.9</td>
<td>5.2 ± 0.3 (24)</td>
<td>4.0 ± 0.2 (19)</td>
<td>12.4 ± 0.5 (57)</td>
<td>13.6 ± 0.7 (63)</td>
<td>8.0 ± 0.4 (37)</td>
</tr>
</tbody>
</table>

* Fifty randomly selected cells were analyzed for the 1-, 6-, and 24-h time points and 25 cells for the 2- and 48-h time points. This experiment was repeated with similar findings.

* Cell-associated fibers were all fibers interacting with the cell, including cell surface and those partially and totally phagocytized. Values followed by ± SE.

* Any portion of the fiber was <1 μm from the nucleus.

* No portion of the fiber was ≤1 μm from the nucleus.

* Data also expressed as percentage of the total number of cell-associated fibers/cell (in parentheses) for a given time point.

* Significantly different (P < 0.05) from the 1-h time point by Student's t test.

* Significantly different (P < 0.05) from the number of cell surface fibers/cell at 48 h (Student's t test).

* Significantly different (P < 0.05) from the number of nonperinuclear fibers/cell at 48 h (Student's t test).

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**Fig. 2.** Transmission electron micrograph of a 60–80-nm section of a Syrian hamster embryo cell 48 h after treatment with crocidolite asbestos (1 μg/cm²). The nuclear region is demarcated by the dark-staining chromatin (Ch). The electron-dense, transected crocidolite asbestos fibers (arrowheads), located in the cytoplasm of the cell, appear black in the micrograph. Note that each asbestos fiber is composed of smaller fibrils. × 17,000.

**Fig. 3.** Scanning electron micrograph (A) and the corresponding backscatter electron image (B), showing the perinuclear accumulation of crocidolite asbestos fibers by a Syrian hamster embryo cell 24 h after treatment with 1 μg/cm². N, nuclear region, which is demarcated by prominent nucleoli (asterisks); c, cytoplasm. × 1,780.
Table 2. Length and diameter distributions of unmilled and milled code 100 glass fibers

Fiber suspensions were diluted to 100 µg/ml in 95% ethanol, and 10 µl of the suspension were spread on a carbon disc that had been wetted with 95% ethanol. The discs were dried in a desiccator and then coated with 100 Å of gold. The length and diameter of 100 fibers/sample were measured using scanning electron microscopy at ×6,000 and ×10,000, respectively.

<table>
<thead>
<tr>
<th>Length (µm)</th>
<th>% of total fibers (%)</th>
<th>Unmilled</th>
<th>Milled</th>
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<tbody>
<tr>
<td>0-1</td>
<td>0</td>
<td>29</td>
<td>26</td>
</tr>
<tr>
<td>1-5</td>
<td>23</td>
<td>14</td>
<td>14</td>
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<tr>
<td>5-10</td>
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<td>10-15</td>
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<td>&gt;50</td>
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<td>0</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Diameter (µm)</th>
<th>% of total fibers (%)</th>
<th>Unmilled</th>
<th>Milled</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-0.1</td>
<td>0-0.10</td>
<td>34</td>
<td>26</td>
</tr>
<tr>
<td>0.1-0.15</td>
<td>0.15-0.20</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>0.2-0.25</td>
<td>0.25-0.30</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>0.3-0.35</td>
<td>0.35-0.40</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>0.4-0.45</td>
<td>0.45-0.50</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>0.5-0.55</td>
<td>0.55-0.60</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>0.6-0.65</td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

The number of unmilled glass fibers that were totally phagocytized was 8.9 ± 0.4 fibers/cell 24 h after treatment and 10.6 ± 0.3 fibers/cell 48 h after treatment. The number of milled glass fibers that were totally phagocytized was 10.4 ± 0.4 fibers/cell at 24 h and 14.8 ± 0.5 fibers/cell at 48 h after treatment. Thus we chose 48 h after treatment to compare the phagocytosis of unmilled versus milled glass fibers.

Scanning and electron backscatter micrographs of cells 24 h after treatment with milled glass fibers (1 µg/cm²) showed that most of the fibers were intracellular and located in the perinuclear region of the cytoplasm (Fig. 4). This was also true for cells treated with unmilled glass fibers.

The average number of fibers per cell 48 h after treatment was similar for cells treated with unmilled (20.7 ± 0.6 fibers/cell) and milled glass fibers (19.4 ± 0.6 fibers/cell) (Table 3). Furthermore, the total numbers of fibers partially or totally phagocytized were similar in cells treated with unmilled glass fibers (13.1 ± 0.3 fibers/cell) or milled glass fibers (15 ± 0.5 fibers/cell) (Table 2). Since the cells treated with the milled glass fibers were exposed to greater than 6-fold more particulates than the cells treated with the unmilled glass fibers, the finding of similar numbers of fibers per cell suggests that shorter fibers are less likely to be phagocytized.

Examination of fiber dimensions of cell-associated fibers, both surface and phagocytized, revealed several interesting findings (Table 4). First, in cells treated with unmilled glass fibers, the fibers associated with the cell had an average length (17.9 ± 1.4 µm) greater than that of fibers in the stock suspension to which the cells were exposed (15.5 ± 1.5 µm). This effect was more marked in cells treated with the milled glass fibers; the length of the cell-associated fibers (6.3 ± 0.4 µm) was nearly 3-fold greater than that of the fibers in the stock suspension (2.2 ± 0.2 µm). The average diameter also increased but this was probably due to the fact that longer fibers tend to be thicker (Table 4).

Cell-associated fibers were further categorized into surface-associated and phagocytized fibers. Phagocytized fibers (length, 25.5 ± 3.1 µm) were longer than cell surface-associated fibers (length, 9.6 ± 0.8 µm) in cells treated with unmilled glass fibers (Table 4). The preferential phagocytosis of longer fibers was also apparent when cells were treated with milled glass fibers (Table 4). Surface-associated milled fibers (2.9 ± 0.2 µm) were less than one-half as long as phagocytized fibers (7.1 ± 0.8 µm). Thus, in the cases of both unmilled and milled glass fibers there appears to be selective phagocytosis of long fibers.

When the percentage of the phagocytized fibers in the perinuclear region were compared at 48 h after treatment, it was observed that 51% of the fibers were in the perinuclear region of the cytoplasm of cells treated with unmilled glass fibers (Table 3). The preferential phagocytosis of longer fibers was also apparent when cells were treated with milled glass fibers (Table 4). Surface-associated milled fibers (2.9 ± 0.2 µm) were less than one-half as long as phagocytized fibers (7.1 ± 0.8 µm). Thus, in the cases of both unmilled and milled glass fibers there appears to be selective phagocytosis of long fibers.

The slight difference may have resulted from the greater probability of some portion of a long fiber being within 1 µm from the nucleus by virtue of its length rather than any inherent difference in the translocation of long and short fibers to the perinuclear region. Thus, fiber length does not appear to affect translocation of internalized fibers to the perinuclear region. This is further supported by the finding that there was no difference in average fiber length between fibers that were...
perinuclear and those that were not perinuclear in cells exposed to either milled or unmilled glass fibers (data not shown).

Effect of Altering Fiber Length on Cytotoxicity, Morphological Transformation, and Micronucleus Induction. We determined the potencies of the fibers used in the present study in the induction of cytotoxicity, transformation, and micronucleus induction, which reflects cytogenetic damage and aneuploidy. Cytotoxicity (relative survival) and transformation frequencies were measured 1 week after treatment of cells with unmilled or milled glass fibers (1 μg/cm²). As shown in Table 5, cells treated with milled glass fibers had more intracellular fibers at 48 h after treatment than cells treated with unmilled glass fibers, but the intracellular fiber length was shorter in the case of the cells treated with the milled glass fibers. Treatment of the cells with milled glass fibers resulted in less cytotoxicity (relative survival of 84 versus 14%), and in a lower transformation frequency (0.3 versus 12%) than treatment with unmilled glass fibers. The induction of micronuclei was determined 48 h after treatment with 1-μg/cm² samples of the two glass fibers. As shown in Table 5, milled glass fibers were nearly 7-fold less potent than unmilled glass fibers in the induction of micronuclei. Thus the intracellular "dose" of fibers (i.e., numbers of fibers per cell) is not the only factor important in determining induction of cytotoxicity, transformation, and cytogenetic damage; the length of the phagocytized fibers also appears important.

DISCUSSION

In the present study, we attempted to obtain a better understanding of the role of phagocytosis, fiber length, and dose of intracellular fibers in the induction of cell transformation by mineral dusts. Initial studies focused on phagocytosis of crocidolite asbestos. The number of crocidolite fibers associated with SHE cells in culture increased over a period of 24 h after asbestos treatment. Kinetic analysis of the phagocytosis of crocidolite and glass fibers showed that fibers were rapidly

<table>
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<tr>
<th>Table 5 Relationship between average fiber length of unmilled and milled glass fibers and the biological effects induced by treatment with 1 μg/cm²</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Glass fibers</th>
<th>Av. no. of phagocytized fibers/cell</th>
<th>Intracellular fiber length (μm)</th>
<th>Relative survival ( % )</th>
<th>Transformation frequency ( % )</th>
<th>Frequency of micronuclei ( % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmilled</td>
<td>13.1 ± 0.3</td>
<td>25.5 ± 1.3</td>
<td>14</td>
<td>12</td>
<td>5.0 ± 1.0</td>
</tr>
<tr>
<td>Milled</td>
<td>15.0 ± 0.4</td>
<td>7.1 ± 0.8</td>
<td>84</td>
<td>0.3</td>
<td>0.7 ± 0.05</td>
</tr>
</tbody>
</table>

* Fibers that were partially or totally phagocytized were counted (± SE) (from Table 3).

** Length was determined using electron backscatter imaging at X6000 (± SE).

° Relative survival = no. of colonies in 10 glass fiber-treated cultures / no. of colonies in 10 control cultures × 100

 Transformation frequency = no. of morphologically transformed colonies in 10 cultures / Total no. of surviving colonies in 10 cultures × 100

* There were 1000 cells examined per treatment group. A frequency of 5.0 ± 1.0% is significantly different but a frequency of 0.7 ± 0.05% is not significantly different from control (0.6 ± 0.05) (Fisher's exact test). Values are followed by ± SE.

Table 4 Dimensions of unmilled and milled glass fibers on the cell surface compared to those phagocytized by cells 48 h after treatment

<table>
<thead>
<tr>
<th>Fibers in stock suspension</th>
<th>Cell-associated fibers</th>
<th>Surface-associated fibers</th>
<th>Phagocytized fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmilled glass fibers</td>
<td>Length (μm)</td>
<td>15.5 ± 1.5</td>
<td>17.9 ± 1.4</td>
</tr>
<tr>
<td>Diameter (μm)</td>
<td>0.18 ± 0.01</td>
<td>0.30 ± 0.02</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>Milled glass fibers</td>
<td>Length (μm)</td>
<td>2.2 ± 0.2</td>
<td>6.3 ± 0.4</td>
</tr>
<tr>
<td>Diameter (μm)</td>
<td>0.27 ± 0.01</td>
<td>0.37 ± 0.02</td>
<td>0.27 ± 0.02</td>
</tr>
</tbody>
</table>

- Fibers were suspended in distilled water (1 mg/ml) and mixed in a polytron tissue grinder at high setting for 2 min. Then 10 μl were spread on a carbon disc that had been precoated with 100 μl of 95% ethanol and then dried. After coating with 200 Å of gold, the fiber diameter was measured at x 10,000 and the length at x 6000 using scanning electron microscopy. Values followed by ± SE.

- Fibers either on the cell surface or inside the cell.

- No part of the fiber below the cell surface.

- Fibers that were partially or totally phagocytized.

- Average length was significantly different (P < 0.05) from that of fibers in stock suspension by Student's t test.

- Average length was significantly different (P < 0.05) from that of fibers in stock suspension and also surface-associated fibers by Student's t test.
The increase in number of cell-associated fibers could have resulted from several factors. The settling of fibers onto the culture dish, which could have permitted interaction of the cells with additional fibers. However, we believe that the increase in the number of cell-associated fibers with time after exposure results because fibers laying on the cells become more firmly attached to the cell surface and phagocytized with time. Thus, the primary rate-limiting factor is the period of time required for uptake (phagocytosis) of the fibers.

Studies from a number of other laboratories have shown that cells phagocytize different types of particulates. Phagocytosis of carbon particles by Kupffer cells (22) and epithelial cells (23) and phagocytosis of latex beads (24) by epithelial cells have been described. Phagocytosis of asbestos by fibroblasts (25), macrophages (26, 27), lung epithelial cells (28–31), and mesothelial cells (32) have also been described.

Following phagocytosis, we observed perinuclear accumulation of the crocidolite asbestos after 24 h. Perinuclear accumulation of carbon particles by type 1 epithelial cells of mouse lung (33), nickel sulfide particles by Chinese hamster ovary cells (34), and fibronectin-coated heads by hamster cells have been reported (35).

The main purpose of these studies was to determine whether differences in phagocytosis and/or intracellular distribution could account for the previously observed differences in the transforming potencies of long versus short glass fibers (9). Glass fibers rather than asbestos were used in these studies because, as we have previously shown, milling glass fibers decreases the average length with little or no effect on the average diameter (8); asbestos milling results in changes in both dimensions (36). In the present studies, two lines of evidence support the conclusion that short fibers are less readily phagocytized by cells than long fibers: (a) the average length of intracellular fibers was always greater than the average length of the stock suspension fibers to which the cells were exposed; (b) although cells treated with an equal mass of milled versus unmilled glass fibers were exposed to 6-fold more milled fibers, the number of intracellular fibers was nearly the same. Thus, cells appear to be selectively phagocytizing longer fibers. However, we have not excluded the possibility of preferential elimination of short fibers by cells.

Valberg et al. (37) have shown that the phagocytic capacity of macrophages becomes rate limiting when exposed to a high concentration of colloidal gold. If 1 μg of fibers/cm² saturated the phagocytic capacity of cells in the present study, then this might provide an explanation for why an equal number of milled and unmilled glass fibers were phagocytized even though cells were exposed to a 6-fold difference in the number of milled versus unmilled fibers. However, our data show that 1 μg of mineral fibers/cm² does not saturate phagocytosis, since doubling the concentration of fibers to 2 μg/cm² resulted in an approximate doubling of the number of intracellular fibers. The latter finding also demonstrates that the intracellular particle number is important in determining cytotoxicity and transformation, since, as has been shown previously, doubling the concentration of asbestos from 1 to 2 μg/cm² resulted in concomitant increases in cytotoxicity and transformation (9).

Fiber length did not appear to affect the migration of phagocytized fibers within the cells to the perinuclear region of the cytoplasm. The percentage of phagocytized fibers associated with the nucleus was similar for cells treated with either unmilled or milled glass fibers. In addition, there appeared to be no difference between the length of phagocytized fibers located in the perinuclear region and those that were not located in the perinuclear region in cells treated with either unmilled or milled glass fibers. Thus, fiber length appeared to be important primarily in the attachment to the cell surface and/or subsequent internalization but not in the movement of the fibers to the perinuclear region.

Changes in fiber length result in changes in other characteristics of the fibers. For example, shorter fibers will have less mass, less surface area, fewer charges per fiber, etc. In addition, the characteristic of the fiber which is important for phagocytosis is not necessarily the same as that which is important for translocation or cytogenetic damage. For example, the surface area per fiber could be important for phagocytosis, whereas the mass of a fiber could be the critical factor affecting the induction of anaphase abnormalities once a fiber has been internalized. Unfortunately, it is not possible at this time to determine which, if any, of these characteristics are important in these biological processes; other studies are needed to specifically address these issues.

Studies which examined the phagocytosis of crystalline and amorphous metal sulfide particles by Chinese hamster ovary cells concluded that the negative surface charge of the particulates was the important determinant for phagocytosis (38, 39). Our observation that both chrysotile (positive surface charge) and crocidolite (negative surface charge) asbestos are readily phagocytized and similar in cytotoxic and transforming potencies (9) argues against surface charge playing a significant role in phagocytosis in the present studies. However, we cannot eliminate the possibility that milling of glass fibers alters the surface charge per unit surface area of the fibers in addition to fiber length. Unfortunately, to our knowledge, the technology for measuring surface charge of individual sub-light microscopic particles in complete serum-containing medium is not available.

The effect of fiber length on phagocytosis is not sufficient to completely explain the fiber length dependence of asbestos-induced cytogenetic effects or cell transformation. We observed that cells treated with unmilled glass fibers contained fewer intracellular fibers at 48 h than cells treated with milled glass fibers. Despite the lower intracellular fiber concentration, unmilled glass fibers induced a significantly higher transformation frequency and a higher frequency of micronuclei. Thus, the 3-fold greater fiber length of the intracellular fibers in the unmilled versus the milled glass fiber-treated cells must play a role in the difference in potencies of these samples of glass fibers. Based on our earlier studies which showed that intracellular asbestos fibers are found between the poles of the spindle and sometimes closely associated with displaced chromosomes in anaphases from asbestos-treated cells (19), we propose that long fibers are more easily entangled than short fibers in the chromosomes or in the microtubules of the spindle apparatus during the segregation of chromosomes which occurs during anaphase. Thus, in addition to being an important determinant of phagocytosis, fiber length may also affect the ability of fibers to disrupt the normal segregation of chromosomes during anaphase which results in aneuploidy.

In summary, we demonstrated that asbestos and glass fibers were phagocytized by SHE cells in culture and accumulated in

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Unpublished observations.
the perinuclear region of the cytoplasm. Milling of glass fibers, which resulted in a nearly 7-fold decrease in length, reduced phagocytosis. Phagocytized fibers were more than 2-fold longer than surface fibers in both the unmilled and milled glass fiber-treated cells, which suggested that cells selectively internalized longer fibers. Fiber length, however, did not appear to affect the migration of intracellular fibers to the perinuclear region of the cytoplasm. Even though cells treated with milled glass fibers contained a number of fibers similar to those treated with unmilled glass fibers, the resulting cytotoxicity, transformation frequency, and frequency of micronucleus induction was much less in the milled glass fiber-treated cells. Thus, fiber length appeared to affect not only the ability of fibers to be phagocytozed but also the ability of intracellular fibers to induce cytogenetic damage and cell transformation.

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REFERENCES


Role of Phagocytosis in Syrian Hamster Cell Transformation and Cytogenetic Effects Induced by Asbestos and Short and Long Glass Fibers

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