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Dependence of Asbestos- and Mineral Dust-induced Transformation of Mammalian Cells in Culture on Fiber Dimension

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ABSTRACT

The abilities of chrysotile and crocidolite asbestos, glass fibers of differing dimensions, and nonfibrous mineral particulates to induce morphological transformation of Syrian hamster embryo cells in culture were compared. Chrysotile and crocidolite asbestos induced morphologically transformed colonies which were indistinguishable from transformed colonies observed following treatment with known chemical carcinogens. A linear, dose-dependent increase in the frequency of transformed colonies was observed. The slope of the dose-response curve on a log-log scale was ~1, which is consistent with a one-hit mechanism for their induction. The transforming potency of chrysotile asbestos was decreased by milling of the fibers but not by extraction with an organic solvent. Chrysotile asbestos was nearly twice as potent in inducing morphological transformation as crocidolite asbestos. Glass fibers were also very active in this assay. Thin glass fibers with an average diameter of 0.1 to 0.2 μm were as active as asbestos. In contrast, two nonfibrous particulates, α-quartz and Min-U-Sil, were inactive over the same concentration range used for the fibrous dusts; however, both were active at higher doses.

The effect of varying fiber dimension on induction of morphological transformation was examined with glass fibers. When compared on a per-weight basis, thick glass fibers [average diameter, 0.8 ± 0.06 μm (S.E.)] were 20-fold less potent than thin fibers [average diameter, 0.13 ± 0.005 μm] in inducing cell transformation. When the average fiber length of thin glass fibers was reduced from 9.5 to 1.7 μm by milling the fibers in a mortar and pestle, a 10-fold decrease in transforming activity resulted. When the average fiber length was reduced to 0.95 μm, transforming ability was totally absent. The cytotoxic potencies of the various mineral dusts correlated with their transforming potencies. The varying abilities of the mineral dusts to induce cell transformation *in vitro* are similar to their abilities to induce mesotheliomas *in vivo*. Thus, this system provides a unique model for studying the mechanism of mineral fiber tumorigenesis and for comparing the relative risks of mineral dusts.

INTRODUCTION

Asbestos is a family of naturally occurring hydrated silicates having a fibrous crystalline structure (30). The 2 major classes of asbestos are the serpentines, of which chrysotile is the only commercially important variety, and the amphiboles, of which crocidolite and amosite are the important varieties (30).

Asbestos exposure causes lung and other forms of cancer in humans (30, 48, 58, 73), even after very brief (57) or low-level (2, 76) exposure. However, it is unclear whether certain types of asbestos are more tumorigenic than others (40, 44, 50). Some studies have indicated that crocidolite is more carcinogenic in humans than is chrysotile (37, 40, 44, 74), but there is evidence that chrysotile is as potent, if not more potent, than crocidolite (35, 49, 50). It is also unclear whether chrysotile and crocidolite asbestos differ in their tumorigenic effects in animals (63, 64, 69, 70). Most animal studies have used only a single dose of asbestos. If that dose were at the high end of the tumorigenic dose response for the dusts being compared, then all dusts could appear to be of equal tumorigenicity, especially if there were only a small difference in their actual tumorigenic potencies. Comparisons of the relative carcinogenicity of dusts by reconstruction of the retrospective data on human exposures is even more complex. For instance, it is difficult to determine the dose of asbestos to which a group of workers was exposed 30 years ago and to determine whether the doses were comparable in 2 different work environments where 2 different asbestos types were used.

It is also uncertain whether man-made mineral fibers, such as fiberglass (9, 24, 27, 56) or nonfibrous particulates such as quartz (26), pose a significant carcinogenic hazard to humans. Stanton *et al.* (61–64) have shown that mesotheliomas are induced in rats by intrapleural injection of asbestos or other mineral fibers of diverse chemical composition, including fiberglass. Furthermore, Stanton's studies have demonstrated that chemical composition was not as important in the induction of mesotheliomas as fiber length and diameter. It has been postulated that fiber dimension, especially length, might influence tumorigenicity by affecting the clearance of mineral fibers from the lung (29).

The mechanism(s) by which asbestos causes cancer is unknown. Because of its lack of activity in gene mutation assays, it has been proposed that asbestos acts by epigenetic mechanisms (77). Others have specifically proposed that asbestos acts primarily as a promoter or cocarcinogen (45, 46, 66), or that cancer occurs secondarily to the induction of inflammation or fibrosis (30) by asbestos. One of the difficulties in determining the mechanism of asbestos carcinogenesis is the lack of a suitable *in vitro* model to experimentally address this question.

In the present study, we have demonstrated that asbestos and other mineral dusts can directly induce morphological transformation of SHE[2] cells in culture. Transformation of these cells is a preneoplastic change known to result from exposure to a wide variety of chemical and physical carcinogens (7, 10, 21–23). We have also shown that fiber dimension influences the transforming potency of mineral fibers in this *in vitro* system. Our results suggest that *in vitro* induction of cell transformation is related to fiber length and diameter in a manner similar to mesothelioma induction *in vivo*. This cell culture model is well

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[2] The abbreviation used is: SHE, Syrian hamster embryo.

suited for mechanistic studies, because the dosage-dependent increase in the frequency of transformation can be related to other effects induced by mineral fibers. In addition, this system can be used as a short-term assay for potentially hazardous mineral dusts and for comparative risk analysis of these substances.

MATERIALS AND METHODS

Cells and Culture Conditions. SHE 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 (4). 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 of sodium bicarbonate per liter, 20% (v/v) Hy-Clone fetal bovine serum (Sterile Systems Inc., Logan, UT), 100 units of penicillin per ml, and 100 μg of 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°. Cultures were routinely tested (52) and found to be free of *Mycoplasma*.

Preparation of Asbestos and the Other Mineral Dusts. International Union Against Cancer chrysotile and crocidolite asbestos were obtained from V. Timbrell (Medical Research Council, Great Britain). Code 100 and Code 110 glass fibers were generously provided by Dr. James Leineweber, Johns Manville Corporation (Denver, CO). α-Quartz and Min-U-Sil (Pennsylvania Glass Sand Co.) were obtained from Dr. A. R. Brody, National Institute of Environmental Health Sciences (Research Triangle Park, NC). Electron micrographs of the mineral dusts used in this study are shown in Figs. 1 and 2.

The glass fibers (Codes 100 and 110) were processed to reduce their length and to remove large amorphous nonfibrous particulates by placing 1 g of raw fibers in a Waring blender containing 500 ml of distilled water and blending at maximum speed for 10 min. The suspension was centrifuged at 1000 × *g* for 10 min, and the fibers from the supernatant were collected on a 0.4-μm filter (Nalge Co., Rochester, NY). The fibers were then rinsed with 95% ethanol, dried, and stored in a desiccator. For some experiments, the length of the Code 100 fibers was further reduced by milling in a mortar and pestle.

To extract organic material from chrysotile asbestos, 1 g of asbestos was placed in the thimble of a soxlet apparatus and refluxed with 500 ml of chloroform (Fisher Scientific Co., Fairlawn, NJ) for 24 hr. The asbestos was then collected on a 0.45-μm Millipore filter and dried in a vacuum desiccator.

For measurement of fiber size, the 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 previously been coated with 100 μl of 95% ethanol to promote spreading. 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 a scanning electron microscope.

Quantitation of Cytotoxicity and Morphological Transformation. The *in vitro* transformation procedure was a modification of the earlier work of Berwald and Sachs (10), DiPaolo *et al.* (20), and Pienta *et al.* (51). Target cells (250 cells) were seeded on a layer of 2×10^4 lethally irradiated (5000 R) homologous feeder cells (52) in 60-mm dishes (Falcon Plastics, Oxnard, CA) in complete medium. Asbestos and quartz dusts were weighed and mixed directly with complete medium (1 mg/ml). Glass fibers were prepared in double distilled water (1 mg/ml) and mixed in a Polytron tissue grinder (PT10ST; Brinkmann Inst., Inc., Westbury, NY) at the highest setting for 2 min to break up any remaining clumps of fibers and then diluted 1:1 in medium (2×) and serum before further diluting and adding to the dishes containing cells. This step was necessary to produce a homogenous suspension of glass fibers but was unnecessary for chrysotile or crocidolite asbestos or the quartz dusts, which could be readily suspended by pipeting back and forth using a 10-ml pipet. Appropriate dilutions were made, and 2 ml of the suspension were added to dishes containing feeder cells, target cells, and 6 ml of complete medium. Mineral dust was left in the dishes for 7 days. The dosages of the mineral dusts were expressed in μg/sq cm of culture dish, because the dusts settled to the bottom of the culture dish within 1 hr after treatment.

Following treatment with mineral dust, the cultures were incubated in a 37° humidified incubator with 12% CO_2 in air for 7 days. The dishes were then fixed in absolute methanol (Fisher) and stained with 10% aqueous Giemsa (Fisher). The colony-forming efficiency was determined by dividing the number of colonies formed 7 days after treatment with asbestos by the number of target cells seeded (250 cells). 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. Colonies that were morphologically transformed were scored according to criteria that have been described previously (10, 20). The transformation frequency was calculated by dividing the total number of transformed colonies by the total number of colonies surviving treatment. Each experiment was repeated at least once unless otherwise indicated.

RESULTS

Asbestos-induced Cytotoxicity and Morphological Transformation. The colony-forming efficiency of the untreated cells was about 25%, and the relative survival of the cells decreased logarithmically with a linear increase in asbestos dose over the dose range of 0.25 to 2 μg/sq cm (Chart 1*A*). Chrysotile asbestos was nearly twice as cytotoxic as crocidolite asbestos at equal doses; the doses resulting in 50% survival were 0.9 and 1.7 μg/sq cm, respectively.

Treatment with either asbestos type resulted in morphological transformation, an early preneoplastic change in SHE cells (5, 8, 19). The morphology of the colonies transformed by asbestos or any other mineral dust, including glass fibers (Fig. 3), was indistinguishable from that observed after treatment with chemical carcinogens. A linear dose-dependent increase in the transformation frequency was observed when log-log plots of the data were made (Chart 1*B*). Chrysotile asbestos was more potent than crocidolite asbestos, but the dose-response curves of both asbestos types had slopes of ~1.2.

To determine whether the transforming properties of asbestos were due to contamination by organic carcinogens, chrysotile asbestos was extracted with chloroform to remove organic contaminants. Chloroform extraction of chrysotile asbestos enhanced its cytotoxic and transforming potency (Table 1). At a dosage of 0.25 μg/sq cm, the extracted chrysotile was almost twice as cytotoxic and nearly 3 times as potent in inducing transformation as the unextracted chrysotile.

Comparison of the Cytotoxic and Transforming Potencies of Chrysotile Asbestos and Quartz Dusts. To determine whether cell transformation was induced by only fibrous mineral dusts, 2 quartz dusts (Min-U-Sil and α-quartz) were examined. Electron-microscopic examination of both dusts (Fig. 2, *C* and *D*) revealed the presence of only nonfibrous particulates in these samples. At doses comparable by weight to transforming doses of chrysotile asbestos, Min-U-Sil and α-quartz were neither cytotoxic nor transforming in this system (Chart 2). In fact, at doses up to 10 μg/sq cm, both quartz dusts reproducibly enhanced the colony-forming efficiency of cells relative to untreated controls, although all increases in dose resulted in decreases in relative

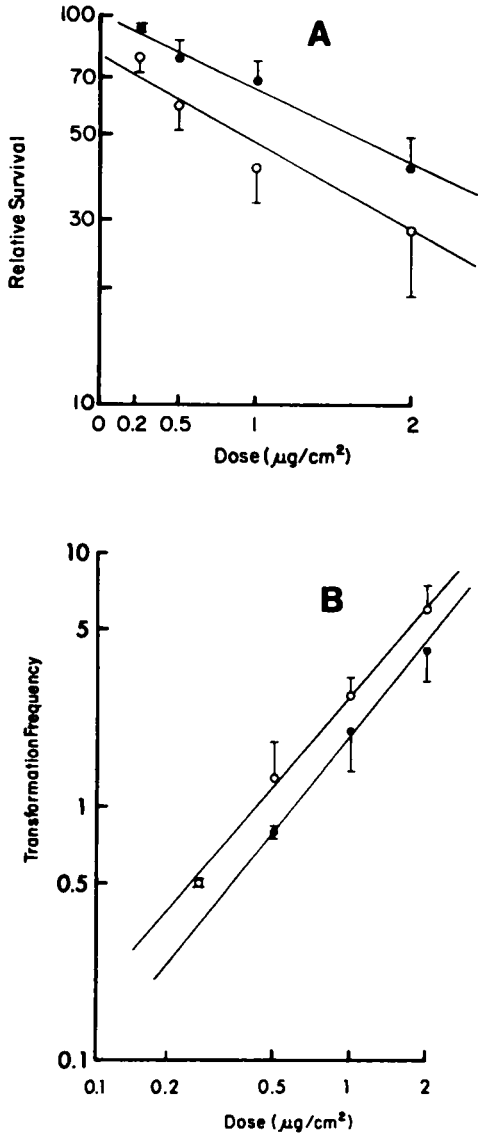

Chart 1. Effects of different doses of chrysotile (○) and crocidolite (●) asbestos on the relative survival (*A*) and the transformation frequency (*B*) of SHE cells in culture (*bars*, S.E.). These results represent the compilation of 8 independent experiments, and at least 2000 colonies were examined for each data point.

survival. Both Min-U-Sil and α-quartz induced a dose-dependent increase in transformation frequency at doses greater than 2 and 10 μg/sq cm, respectively. Min-U-Sil was more potent than α-quartz at all doses tested, and the slopes of the dose-response curves for these quartz dusts were 0.7 and 0.5, respectively.

Effect of Milling on the Cytotoxic and Transforming Potency of Chrysotile Asbestos. A number of *in vitro* studies have shown that milling of asbestos results in a loss of its cytotoxicity (14, 16). In addition to changing its physical dimensions, milling changes the crystallinity of asbestos (38). When we milled chrysotile asbestos in a mortar and pestle, its cytotoxic potency was greatly reduced (Chart 3*A*). A dosage of 0.5 μg/sq cm of unmilled chrysotile asbestos resulted in a relative survival of 30%, whereas the same dose of the milled asbestos resulted in a relative survival of 90%. More importantly, milling of chrysotile asbestos completely eliminated its transforming potency at all doses tested (Chart 3*B*). Electron-microscopic examination of

Table 1

Effect of chloroform extraction on the cytotoxicity and transformation induced by chrysotile asbestos

SHE cells were seeded at clonal density (250 cells/dish) onto 2 × 10^4 lethally irradiated feeder cells in 60-mm dishes in complete medium. Cells were treated 24 hr later with either chloroform-extracted or unextracted chrysotile asbestos (either 0.25 or 0.5 μg/sq cm). Seven days following asbestos treatment, the cultures were fixed, stained, and scored for the total colony number and the number of morphologically transformed colonies.

Treatment	Dose[a] (μg/sq cm)	Relative[b] survival	Transformation frequency[c]
Control	0	100	0
Chrysotile	0.25	67	0.2
Extracted chrysotile[d]	0.25	35	0.7
Chrysotile	0.5	33	1.6
Extracted chrysotile	0.5	25	1.9

[a] Dose was expressed in μg/sq cm since all of the asbestos precipitated to the bottom of the culture dish within 1 hr after treatment.

[b] Relative survival = $\frac{\text{No. of colonies in 10 treated dishes}}{\text{No. of colonies in 10 control dishes}}$

[c] Transformation frequency = $\frac{\text{No. of transformed colonies in 10 dishes}}{\text{Total no. of colonies in 10 dishes}}$

[d] Chrysotile asbestos was refluxed for 24 hr with chloroform in a soxlet apparatus.

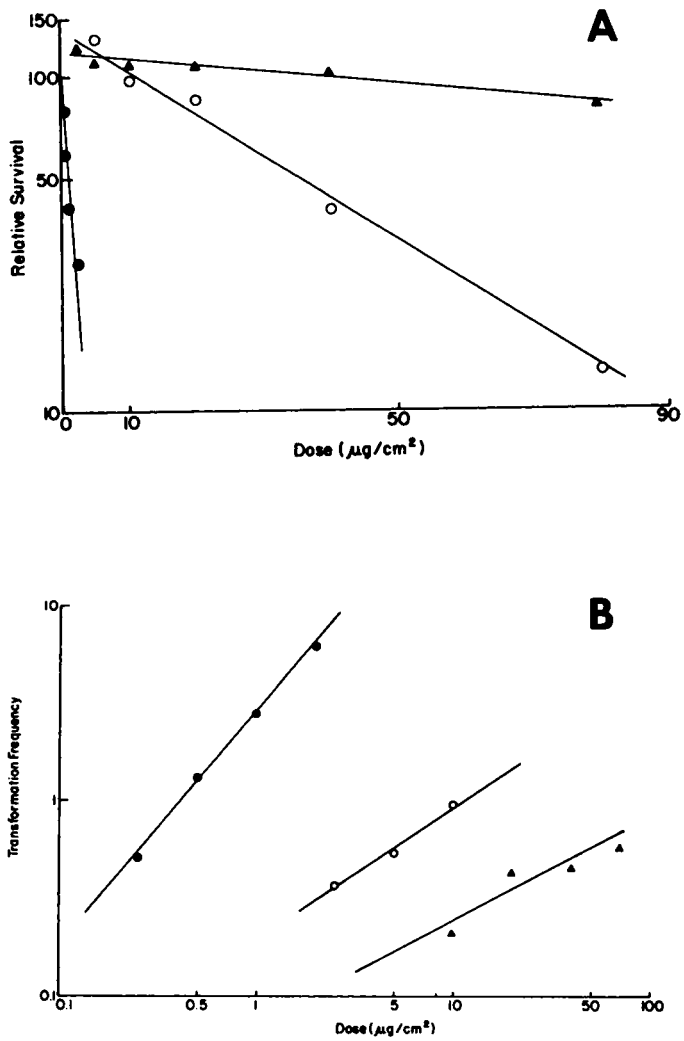

Chart 2. Effects of different doses of chrysotile asbestos (●), Min-U-Sil (○), and α-quartz (▲) on the relative survival (*A*) and the transformation frequency (*B*) of SHE cells in culture.

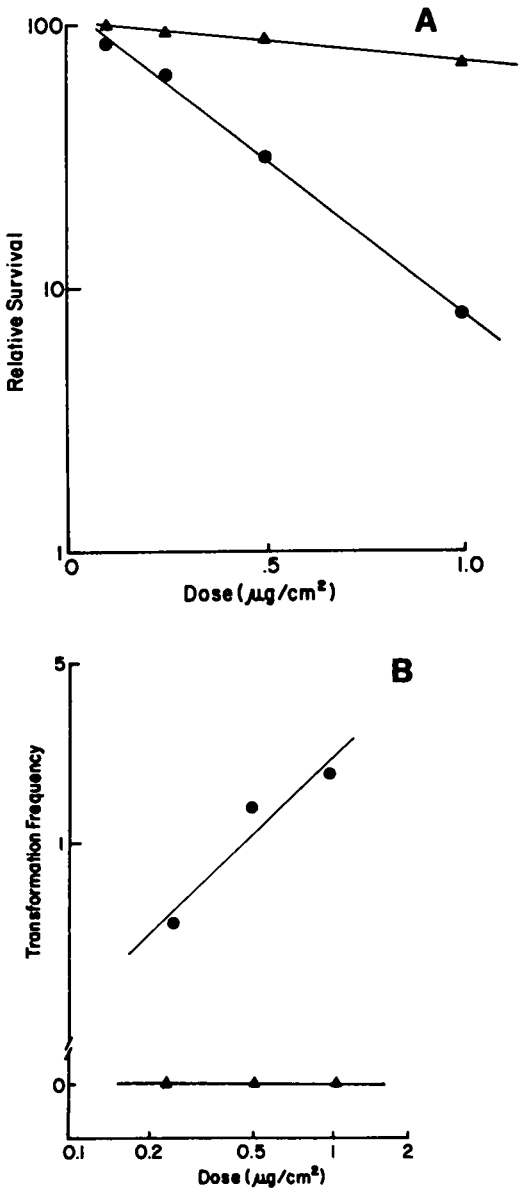

Chart 3. Effects of different doses of unmilled chrysotile (●) and milled chrysotile (▲) asbestos on the relative survival (*A*) and the transformation frequency (*B*) of SHE cells in culture.

the milled asbestos revealed that much of it was clumped together in aggregates rather than being dispersed into individual fibers as seen when the unmilled asbestos was examined (Fig. 2, *A* and *B*).

Cell Transformation by Code 100 Glass Fibers: Effect of Altering Fiber Length on Cytotoxicity and Transforming Potency. When prepared as a well-dispersed suspension, Code 100 glass fibers were as active in transforming cells as chrysotile asbestos (Chart 6). Because a number of problems associated with milling asbestos fibers, such as clumping (also see "Discussion"), could be avoided by using glass fibers, we examined the importance of fiber length on fiber-induced cytotoxicity and transformation using Code 100 glass fibers. Milling of glass fibers in a mortar and pestle shortened the fibers without changing their diameter (Charts 4 and 5). For example, in Experiment 1, the average fiber diameter was 0.13 ± 0.005 μm (S.E) before milling and 0.11 ± 0.005 μm after milling. Milling, however, resulted in a decrease in the average length of the glass fibers from 9.5 ± 0.7 μm to an average length of 1.7 ± 0.1 μm after milling (see frequency histograms in Chart 4, *C* to *F*). In Experiment 2, the average diameters of the glass fibers before and after milling were 0.18 ± 0.01 and 0.19 ± 0.01 μm, respectively. In this experiment, the average fiber length was reduced from 16.0 ± 1.7 μm before milling to 0.95 ± 0.12 μm after milling (see frequency histograms in Chart 5, *A* to *D*). Thus, in both experiments, milling shortened the fibers without affecting their average diameters.

The effect of decreasing the length of Code 100 glass fibers on cytotoxicity and transformation in 2 different experiments is

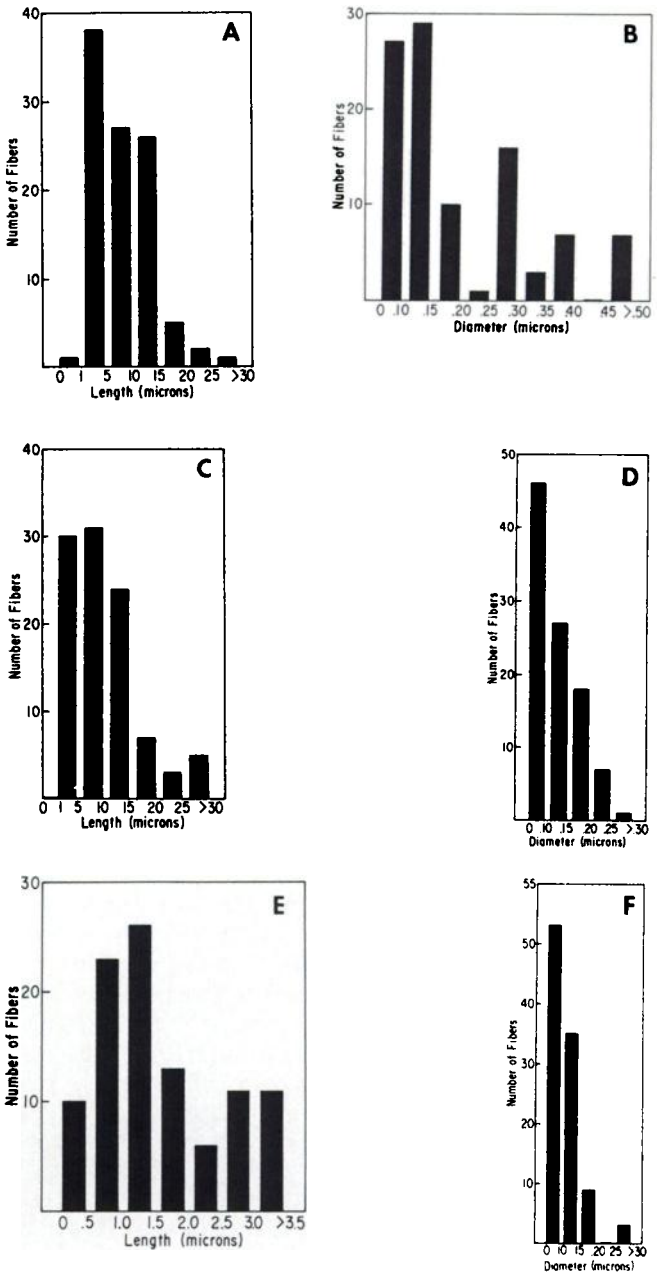

Chart 4. Frequency distributions of lengths and diameters of chrysotile asbestos (*A* and *B*), unmilled Code 100 glass fibers from Experiment 1 (*C* and *D*), and milled Code 100 glass fibers from Experiment 1 (*E* and *F*); 100 fibers of each mineral fiber type were randomly selected, and their diameters and lengths were measured at × 10,000 and × 4000, respectively.

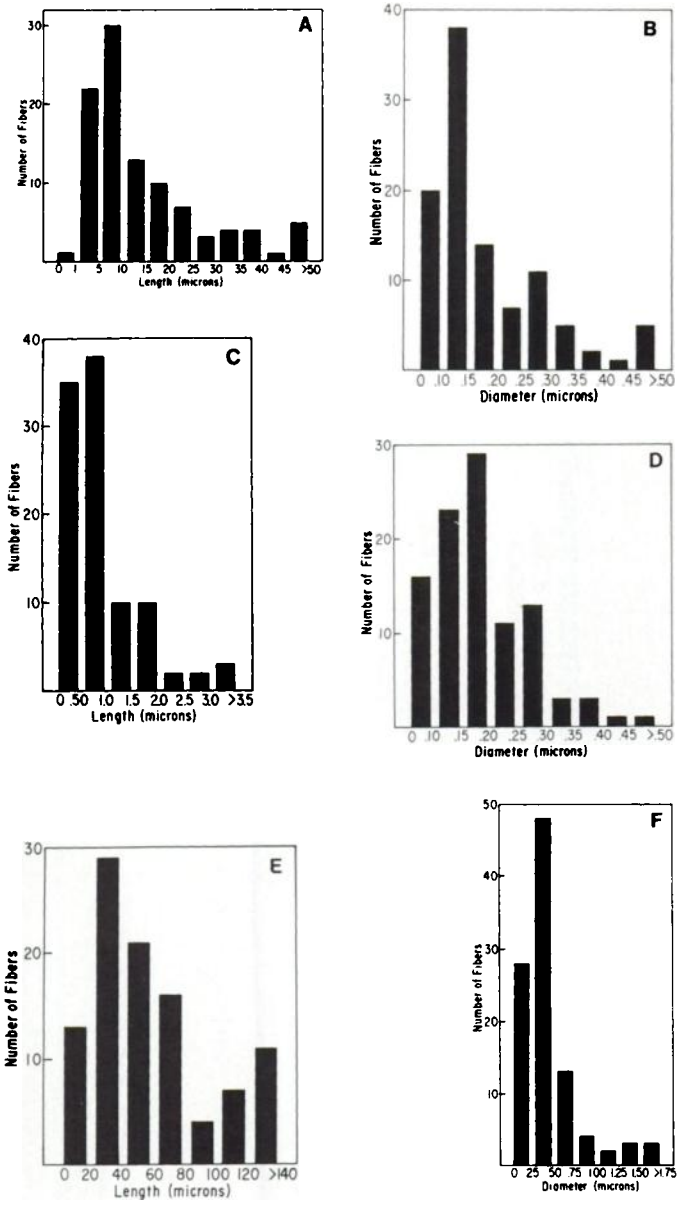

Chart 5. Frequency distributions of lengths and diameters of unmilled Code 100 glass fibers from Experiment 2 (*A* and *B*), milled Code 100 glass fibers from Experiment 2 (*C* and *D*), and Code 110 glass fibers (*E* and *F*).

shown in Chart 6. Milling the Code 100 glass fibers used in Experiment 1 resulted in a decrease in the average fiber length from 9.5 to 1.7 μm and more than a 10-fold decrease in the transforming potency of the glass fibers (Chart 6*B*). The effects of milling could not be explained on the basis of fiber aggregation, since the milled Code 100 glass fibers used in these experiments were mixed vigorously using a polytron tissue grinder before addition to the cells, and they were not aggregated (Fig. 2*B*). A significant decrease was observed in the cytotoxic effects of the glass fibers milled in Experiment 2 (Chart 6*C*), in which the average length of the fibers was reduced to 0.95 μm. Cells treated with 2 μg/sq cm of the unmilled glass fibers had a relative survival of 19% compared to a 91% relative survival of cells treated with an equal dose of milled glass fibers. In this same experiment, milling resulted in a complete loss of transforming potency at all of the doses tested (Chart 6*D*). In comparing Experiments 1 and 2, it should be noted that the average diameters of the fibers in the 2 experiments differed slightly. This may account for the small differences in the potencies of the unmilled Code 100 glass fibers in the 2 experiments. However, the partial loss or complete elimination of the transforming ability of the fibers is not associated with an alteration of fiber diameter but is directly related to the degree of reduction in fiber length.

Comparison of the Cytotoxic and Transforming Potencies of Thick Glass Fibers *versus* Thin Glass Fibers. The importance of fiber diameter on the cytotoxic and transforming potency of glass fibers was also examined. The thick glass fibers (Code 110; John Mansville) were mixed in a Waring blender, centrifuged, and further processed in the same manner as the thin glass fibers (Code 100; John Mansville; see "Materials and Methods"). A scanning electron micrograph of Code 110 fibers is shown in Fig. 2*A*. The average diameter of the thick glass fibers was 0.8 ± 0.06 μm, (Chart 5, *E* and *F*), compared to an average diameter of 0.13 ± 0.005 μm of the thin glass fibers. The thin glass fibers used in this experiment were the same Code 100 glass fibers used in Experiment 1 above (Chart 4, *C* and *D*).

The thin glass fibers were much more cytotoxic than thick glass fibers when doses based on weight were compared (Chart 7*A*). A dose of 1 μg/sq cm of the thin glass fibers resulted in 5% relative survival, while the same dose of thick glass fibers resulted in 86% relative survival. In addition to being more cytotoxic on a per-weight basis, the thin glass fibers were also 20-fold more potent in the induction of transformation than were the thick glass fibers (Chart 7*B*).

DISCUSSION

In the present study, we have compared the cytotoxic and transforming effects of various mineral dusts on SHE cells in culture. A number of studies have attempted to correlate cytotoxicity of mineral dusts *in vitro* with induction of mesothelioma *in vivo* (14, 16, 17, 42, 72). Since we can correlate cytotoxicity and cell transformation in the same system, it is interesting to compare our results with previous findings on cytotoxicity, which has been a major assay for the cellular effects on mineral dusts in the past. In our study, chrysotile asbestos was twice as potent as crocidolite in reducing the colony-forming efficiency and inducing cell transformation of SHE cells. The cytotoxicity results are in agreement with the data of other investigators which showed that chrysotile was more effective in the inhibition of the growth of cell populations than crocidolite using macrophage-like cell lines (11, 16, 67), epithelial cells (16, 32, 47), mesothelial cells (36, 39), and fibroblasts (18, 28, 32, 54).

One of the difficulties in comparing cytotoxicity studies is that few investigations have used colony-forming efficiency as a measure of cytotoxicity, as we have done in the present study. Reiss *et al.* (53) found that chrysotile was about 10-fold more potent than crocidolite in the inhibition of colony formation of intestinal cells from human embryos, adult rat liver cells, and mouse colon cells. However, the cell lines used in that study varied considerably in their sensitivities to asbestos. DiPaolo *et al.* (18), using an *in vitro* system similar to ours, also found that chrysotile more effectively inhibits colony formation than does crocidolite. On the other hand, Chamberlain and Brown (16) showed that crocidolite was more potent than chrysotile in the inhibition of colony formation of V79-4 Chinese hamster lung cells when asbestos and the cells were added to the culture dish

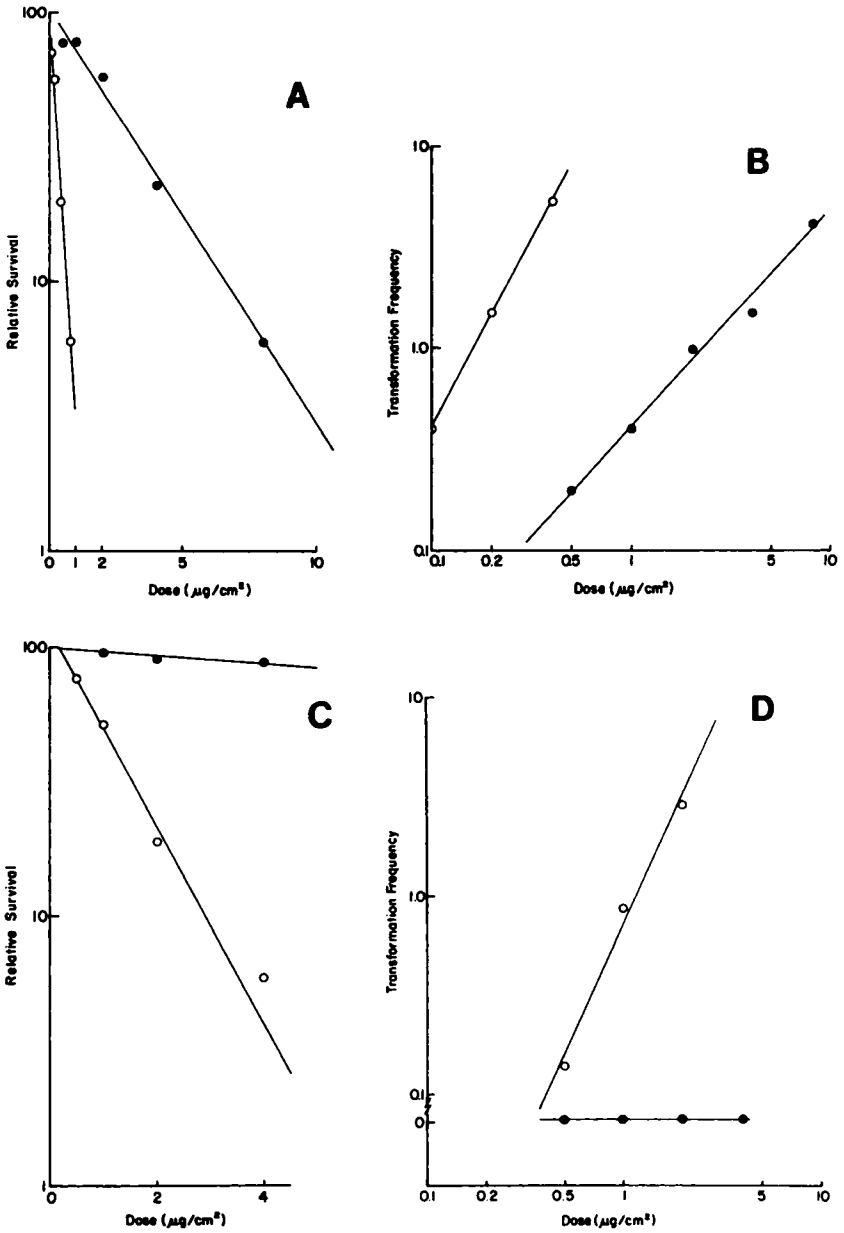

Chart 6. Effects of different doses of unmilled Code 100 glass fibers (○) and milled Code 100 glass fibers (●) on the relative survival (*A* and *C*) and the transformation frequency (*B* and *D*) of SHE cells in culture in 2 separate experiments. *A* and *B*, data from Experiment 1; *C* and *D*, data from Experiment 2. In Experiment 1, milling decreased the average length of the fibers from 9.5 ± 0.1 μm (S.E.) to an average length of 1.7 ± 0.1 μm. In Experiment 2, milling decreased the average length of the fibers from 16.0 ± 1.7 μm to 0.95 ± 0.12 μm. The average fiber diameters (0.13 ± 0.005 μm in Experiment 1 and 0.18 ± 0.01 μm in Experiment 2) were not significantly changed by milling. See Charts 4 and 5 for frequency histograms of the dimensions of the glass fibers used in Experiments 1 and 2, respectively.

at the same time. This apparent reversal of the cytotoxic potencies of chrysotile and crocidolite may have been due to the greater inhibition of cell attachment by crocidolite than chrysotile as shown by Reiss *et al.* (53). In the present study, since we treated the cells 24 hr after plating, inhibition of cell attachment by asbestos is not a component of the observed decrease in colony formation.

One of the major findings of our studies is that mineral fibers can induce cell transformation in a dose-dependent manner. A slope of ~1 for the dose-response curves is consistent with one-hit kinetics for the induction of morphological transformation as has been proposed for other carcinogens in this assay (25). This implies that mineral fibers have the ability to directly affect cellular phenotypes which may be important in their carcinogenic activity.

Few other studies have examined the effects of mineral fibers on cell transformation. Morphological transformation of murine 3T3 cells by chrysotile and crocidolite asbestos has been reported (59), but no difference in the potencies of the 2 asbestos types was mentioned in that study. In another study, it was shown that asbestos did not transform C3H 10T½ murine fibroblasts (15). DiPaolo *et al.* (18), using an *in vitro* transformation system similar to ours, showed that 4 different types of asbestos, including chrysotile and crocidolite, induced only rare transformations in SHE cells, even at doses higher than the ones used in the present study. The difference between their findings and ours may be related to differences in the preparation of the asbestos. DiPaolo *et al.* (18–22) heated the asbestos used in their study to 175° prior to use. Others have shown that heating

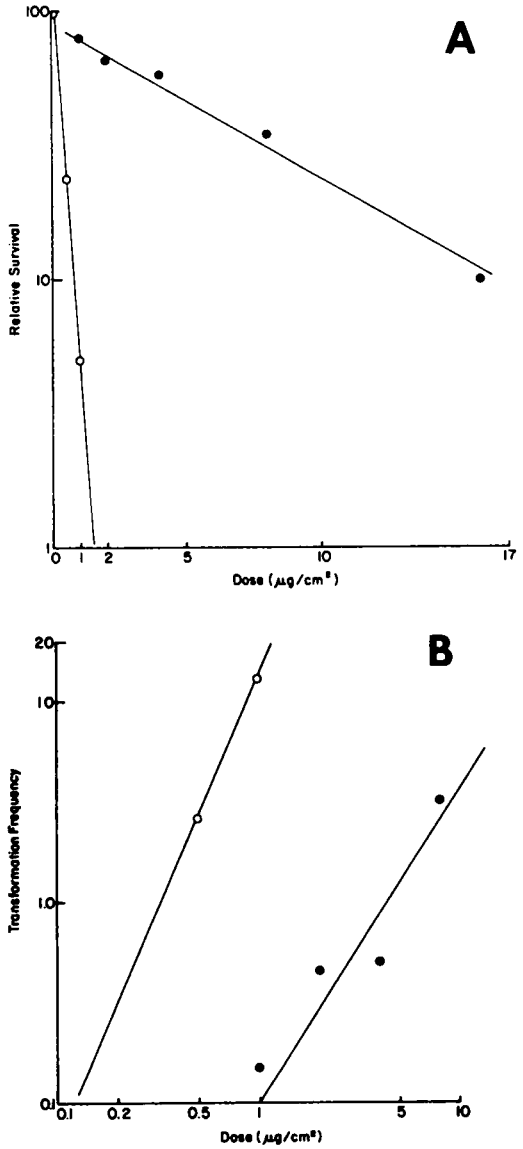

Chart 7. Effects of different doses of thin fibers, Code 100 glass fibers (○), and thick fibers, Code 110 glass fibers (●) on the relative survival (*A*) and the transformation frequency (*B*) of SHE cells in culture.

asbestos results in a decrease in its biological activity (31, 34).

It has been proposed that asbestos-induced tumors may be caused by polyaromatic hydrocarbon contaminants in the mineral fibers (30). In order to determine whether the asbestos-induced transformation was due to the asbestos fibers themselves or to some extractable organic contaminant, we refluxed chrysotile with chloroform. The observed increase, rather than decrease, in the cytotoxic and transforming potency of the chloroform-extracted chrysotile (Table 1) suggests that organic contaminants are not important in the induction of these effects. This increase in potency may have been due to a change in the physical structure of the chrysotile resulting from the refluxing procedure. We did not determine the physical dimensions of the extracted chrysotile. Others have observed that extraction with organic solvents does not influence chrysotile asbestos-induced cytotoxicity (47) or tumorigenicity (69, 71). Furthermore, milling of chrysotile or glass fibers, in the present study, also decreased their transforming potency. Since milling should not alter contaminant levels, contaminants do not appear to be playing a role in the induction of transformation.

High doses of nonfibrous quartz dusts such as α-quartz and Min-U-Sil also induced toxicity and transformation. Although toxic to macrophages *in vitro* (11, 30), nonfibrous silicates such as talc, Min-U-Sil, and South African silica are relatively nontoxic to mammalian cell lines in culture (16). Our results confirm those findings. Interestingly, the lower doses of Min-U-Sil and α-quartz appeared to enhance the colony-forming efficiency of SHE cells in the present study. This may be related to the finding by others that silica stimulates rabbit lung fibroblasts (55) and human lung fibroblasts (1) to proliferate *in vitro*.

Although our data suggest that nonfibrous dusts can induce cell transformation, these dusts are much less potent than fibrous mineral dusts. These results are consistent with *in vivo* tumorigenicity studies of mineral dusts. For example, Wagner (68) showed that silica induced mesotheliomas in only one of 10 rats given intrapleural injections. Other nonfibrous particulates, such as barium sulfate, glass powder, and aluminum oxide, were also shown to induce a low, but detectable, incidence of mesotheliomas in rats (69). Furthermore, talc induces a low incidence of mesotheliomas in rats (62), and others have suggested that gastric carcinoma among Japanese may be related to their consumption of rice coated with talc (43). More recent studies showed that intrapleural injection of silica, although only weakly active in inducing mesotheliomas, induced malignant lymphomas of the histiocytic type in up to 50% of the rats inoculated (69, 75). Holland *et al.* (33) recently demonstrated a 30% tumor incidence in rats exposed by long-term inhalation to Min-U-Sil. These tumors were mostly carcinomas with a few adenomas. Goldsmith *et al.* (26), in a recent review, showed an association between occupational exposure to silica and lung cancer. They proposed that silica-induced lung cancer may result from fibrosis associated with silicosis, thus mimicking the processes involved in foreign-body tumorigenesis (12). Our results suggest that silica might be acting by a more direct mechanism to induce neoplasia.

Harington (29) and Stanton *et al.* (61–64), using a variety of different mineral fibers of diverse chemical composition, demonstrated a good correlation between the induction of pleural mesothelioma in rats and the number of fibers less than 0.25 μm in diameter and longer than 8 μm. Therefore, in the present study, we examined whether fiber dimension was important in the cytotoxicity and cell transformation induced by mineral fibers *in vitro*. We initially milled chrysotile asbestos in order to alter the physical dimensions of the fibers. Others have shown that milling of asbestos reduces its cytotoxicity *in vitro* (14, 16) and reduces its potency in inducing mesotheliomas in hamsters (60) and rats (64). Our observation that milling of chrysotile reduces toxicity and completely eliminates morphological transformation at the doses tested (Chart 3) is in agreement with those findings. However, the clumping of milled chrysotile fibers might account for these effects. It is not known if this problem occurred in other studies; however, an electron micrograph of partially pulverized International Union Against Cancer crocidolite used in the studies of Stanton and Wrench (64) also showed clumping of fibers.

In further studies on the role of fiber dimension in cell transformation, we used glass fibers having different ranges of diameters which were readily available from Johns Manville Corporation. Milling decreased the length but did not affect the diameter of glass fibers, whereas both dimensions are changed when asbestos fibers are milled (3). Milling of Code 100 glass fibers

decreased or completely eliminated their cytotoxic and transforming abilities (Chart 6). The extent of the decrease was associated with degree of reduction in fiber length. Fibers with an average length of 0.95 μm were inactive at the doses tested (Chart 6*D*). In previous *in vitro* studies (14, 16, 17, 42, 65, 72) which attempted to relate fiber dimension to cytotoxicity, neither the average length nor the average diameter of the fibers being compared was held constant.

We also examined whether fiber diameter was important in the induction of cytotoxicity and transformation. Thick fibers, on a per-weight basis, were less cytotoxic and transforming than thin fibers (Chart 7, *A* and *B*), which is in agreement with findings that thick fibers are less tumorigenic than thin fibers to the pleura of rats (29). However, there is a problem in the interpretation of the effect of varying fiber diameter on biological activity. When doses are based on weight, a decrease in fiber diameter is reflected as an increase in fiber number. Thus, the decreased potencies of thick fibers could be due, at least in part, to the smaller number of fibers in a given weight of thick fibers. Brown *et al.* (13) calculated the number of fibers in a given weight of Code 100 and 110 glass fibers and found that the Code 110 glass fibers, although much less cytotoxic than the Code 100 glass fibers on a per-weight basis, were more cytotoxic than the Code 100 glass fibers on a per-fiber basis. We performed similar calculations on our data and found that, on the average, each Code 110 fiber was 40-fold more potent than each Code 100 fiber, since there are 800-fold more fibers in a given weight of Code 100 glass fibers than in an equal weight of Code 110 glass fibers. We agree with Brown *et al.* (13) that, before any material is considered inactive, the number of fibers should be taken into account.

The problem of fiber number, however, does not affect the interpretation of the experiments in which we examined the effect of fiber length on cytotoxicity and transformation, since shortening the fibers actually increased the fiber number and yet decreased their cytotoxic and transforming potencies. When these parameters are compared on a per-fiber basis, the difference between potencies of the short and long Code 100 glass fibers is actually increased 5- to 16-fold.

Stanton *et al.* (62) postulated that long fibers were more tumorigenic than short fibers, because they were not as readily cleared from the lung as short fibers. He made no comment as to a possible mechanism by which fiber diameter might affect tumorigenicity. The present studies show that fiber length is important in the direct induction of cytotoxicity and preneoplastic transformation of cells in culture. This is not to say that indirect effects of fiber dimension, such as altering fiber deposition and clearance from the lung, are not also important in determining mineral fiber tumorigenicity *in vivo*.

In this study, we have shown that asbestos, fiberglass, and even high levels of nonfibrous particulates directly induce cytotoxicity and transformation of mammalian cells in culture. These effects do not appear to be due to carcinogenic contaminants in mineral dusts. In addition, the physical dimensions of the fibers, especially length, are important in their transforming potency. The SHE cell transformation system may be useful as a short-term assay for screening mineral dusts for their tumorigenic potentials. In addition, this system appears to be ideally suited for studies aimed at elucidating the cellular and molecular mechanism(s) of mineral dust-induced neoplastic transformation. Our recent studies (6) indicate that asbestos fibers are taken up by SHE cells and accumulated in the perinuclear region of the cells within 24 hr after exposure. In addition, although transforming doses of asbestos do not cause gene mutations, they do cause a dose-related increase in heteroploidy. The potential importance of chromosomal changes in asbestos-induced transformation has been discussed elsewhere (6).

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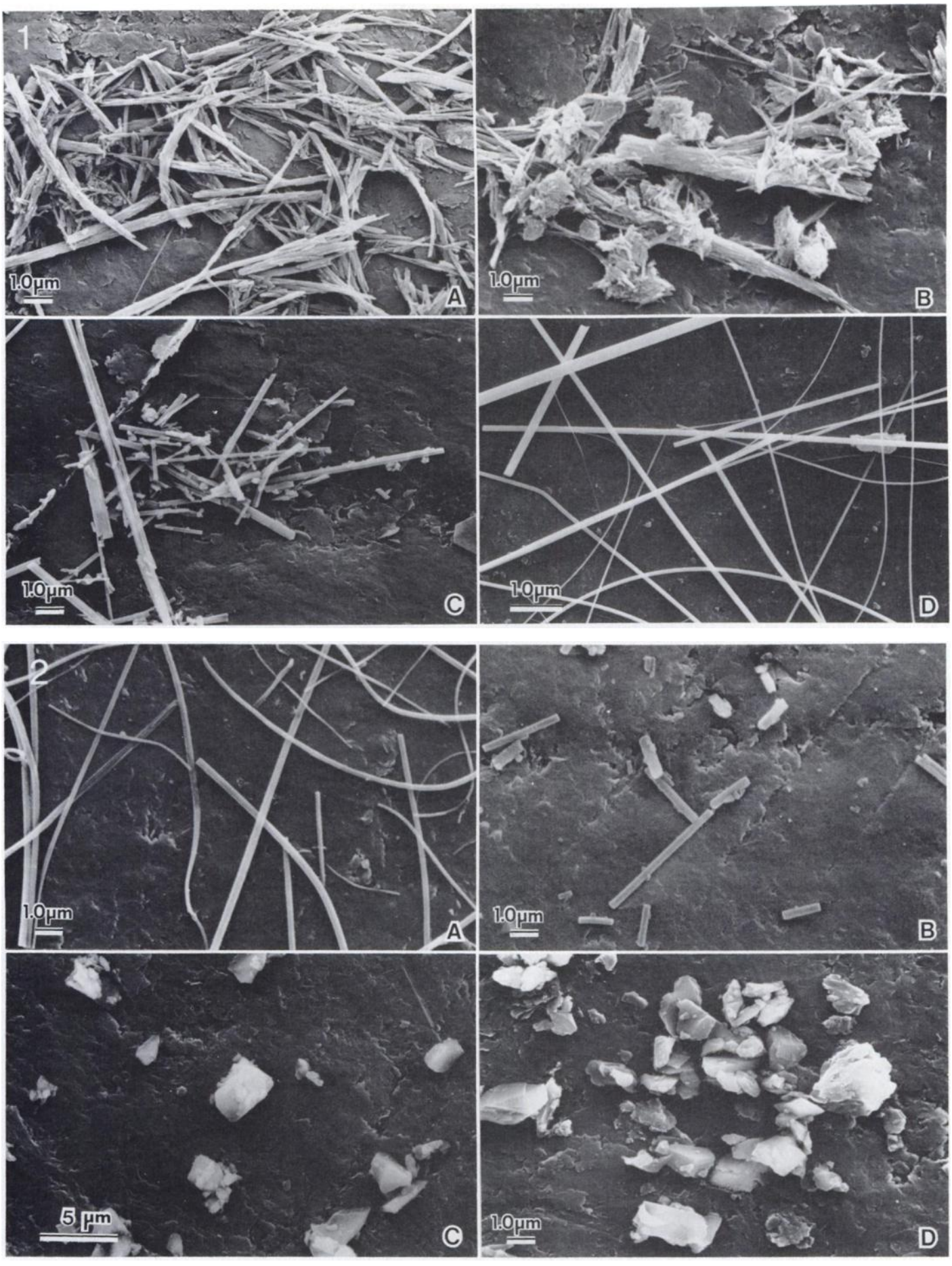
Fig. 1. Scanning electron micrographs of chrysotile asbestos (*A*), milled chrysotile asbestos (*B*), crocidolite asbestos (*C*), and Code 110 glass fibers (*D*). Suspensions of fibers in ethanol were spread on carbon discs, dried in a desiccator, and coated with 100 Å of gold before examination by scanning electron microscopy. Micrographs *A* to *C* were at × 6000 magnifications, and *D* was at × 1000.

Fig. 2. Scanning electron micrographs of unmilled Code 100 glass fibers (*A*), milled Code 100 glass fibers (*B*), α-quartz (*C*), and Min-U-Sil (*D*). Micrographs *A*, *B*, and *D* were at × 6000, and *C* was at × 3000.



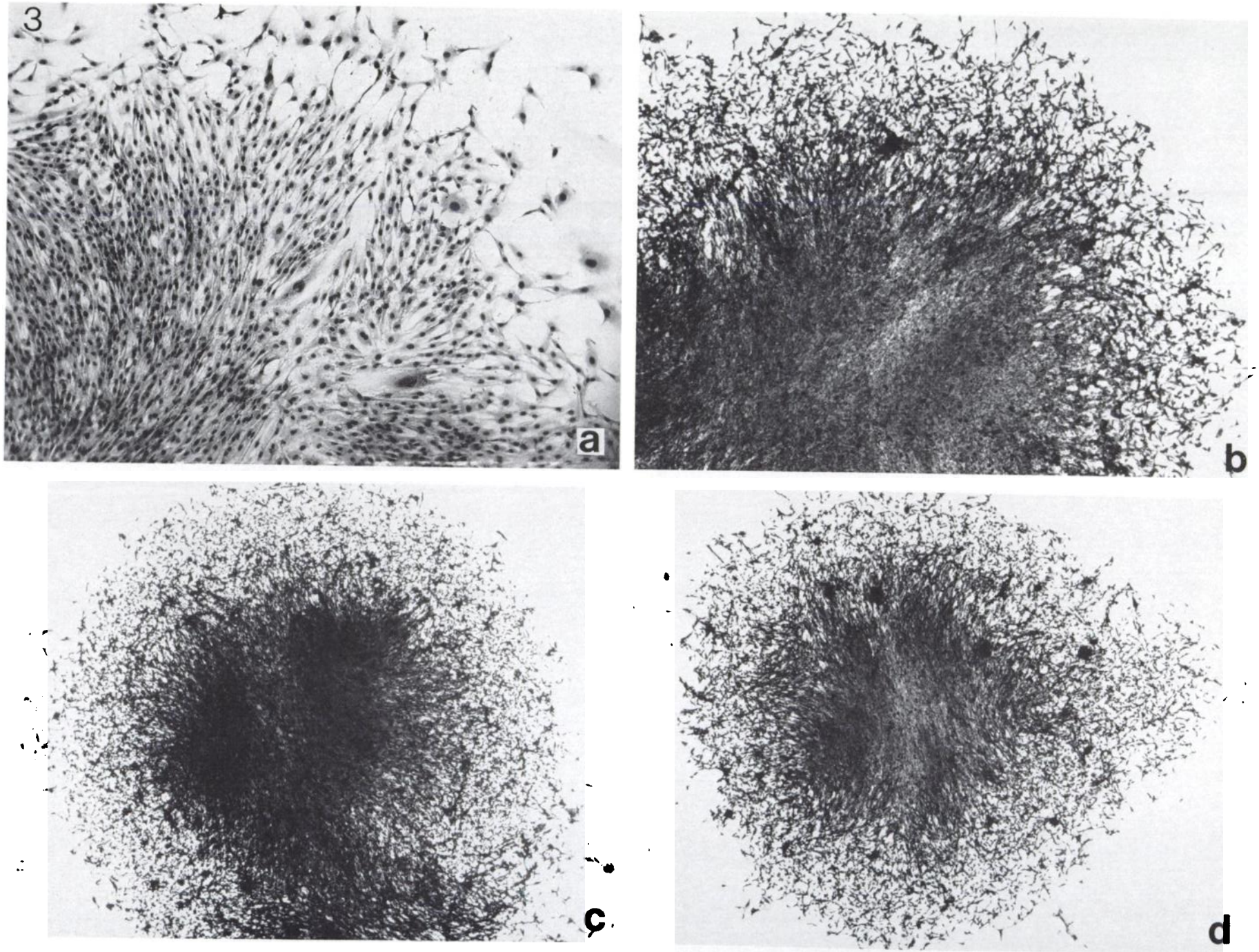


Fig. 3. Morphologies of colonies of SHE cells. Light microscope photographs show: *a*, a normal colony at × 50; *b*, a chrysotile asbestos-transformed colony at × 42; *c*, a crocidolite asbestos-transformed colony at × 27; and *d*, a Code 100 glass fiber-transformed colony at × 27 after fixation in absolute methanol and staining in 10% Giemsa 7 days after treatment.