A STUDY OF THE EFFECT OF CHRYSOTILE FIBER SURFACE COMPOSITION ON GENOTOXICITY IN VITRO

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Chrysotile fibers (NIEHS intermediate length) were treated with ultrapure HCl to alter the fiber surface chemistry without substantially changing fiber morphology or dimensions. The objective of the study was to determine whether fiber surface chemistry is an important variable in fiber genotoxicity in vitro. The modified fibers, along with native chrysotile fibers, were used to challenge Chinese hamster lung fibroblasts (V79) in vitro using the micronucleus induction genotoxicity assay. Fiber dimensions were assessed using scanning electron microscopy by measuring the distribution of fiber lengths in 3 length ranges: less than 3 μm, 3–10 μm, and greater than 10 μm. For both treated and native fiber samples, 500 fibers were examined. Results indicate that acid-treated fibers were about 20% shorter than untreated chrysotile. Surface chemistry alterations were verified by zeta-potential reversal, x-ray photoelectron spectroscopy (XPS), and scanning electron microscopy/energy-dispersive x-ray spectroscopy (SEM-EDS) elemental analysis. Scanning Auger spectrometry indicated the presence of Mg, O, and Si in both treated and native chrysotile samples, which confirmed the surface purity of both fiber samples. Both XPS and SEM-EDS analysis demonstrated substantial depletion of Mg from fiber surfaces. Results of the micronucleus assay showed a positive concentration-related response for both samples, with toxicity evident only at the highest concentration. No significant difference was found for the treated and untreated chrysotile samples. These results indicate that the surface chemistry is not an important variable in the in vitro genotoxicity of chrysotile asbestos in V79 cells as detected by the micronucleus assay under the conditions used in this study, and support a model of chemically nonspecific chromosomal and spindle damage effects.

The risks of exposure to airborne asbestos are well understood and documented (Mossman et al., 1996; Steenland & Stayner, 1997; Vu & Lai, 1997; Nishimura & Broaddus, 1998). Asbestosis (a form of diffuse pulmonary fibrosis), lung cancers, and mesothelioma have all been linked to asbestos exposure in humans. Likewise, animal models have been success-

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fully developed to study exposure–disease relationships. However, the cellular mechanisms by which asbestos fibers initiate disease remain unclear. A mechanistic understanding of asbestos cytotoxic and genotoxic events may permit the prediction of disease potential for new fibrous materials of respirable dimensions that enter the workplace and the environment.

There are several hypotheses that attempt to explain what the important variables are in relating fibers and disease; these include morphology, durability, surface chemistry, reactive oxygen species, and others. Most of these hypotheses have some support, and most are not mutually exclusive of one or more of the alternative hypotheses. To date, there is no simple in vitro test that will predict disease for any given fiber, although certain cytotoxicity assays seem to correlate well with cellular transformation assays, at least in vitro (Hesterberg & Barrett, 1984).

There have been several studies that link disease with fiber dimension. In studies by Wagner et al. (1973), Pott et al. (1974), and Stanton et al. (1977), fiber dimension is the dominant factor in disease (mesothelioma) potential. Results from those studies showed that fibers longer than 8 μm and less than 0.25 μm in diameter were highly effective in inducing mesotheliomas after injection into the pleural or peritoneal spaces. The conclusions of these studies were that sufficient fiber length (>4 μm) and sufficiently small diameter (<0.25 μm) were the only important factors in disease potential. However, it is possible that other factors may be important that are not evident in these studies. Other studies (Bonneau et al., 1986; Kane, 1991; Jaurand, 1991; Fubini, 1993) have suggested that fiber surface composition and surface chemistry or other factors may also contribute to disease potential of different fiber types.

Reactive oxygen species and free radicals have been implicated in disease processes in many studies, including cancer, fibrosis, and other diseases. A review by Brody (1993) examines reactive oxygen species as related to disease processes, as well as other factors.

Another enigma related to fibers and disease relates to the fact that although asbestos and other fibers are carcinogens, they fail to yield consistent positive results in most genetic toxicology tests, especially gene mutation and some DNA damage assays. In the few assays that yield positive results, the dominant effect seems to be chromosomal damage; genotoxicity findings were reviewed by Jaurand (1991).

A number of studies indicate that chemical surface modification of asbestos fibers can affect cytotoxicity (Light & Wei, 1977a, 1977b; Vallyathan et al., 1985), perhaps due to a change in the electrostatic attraction between the fibers and cell membranes (Jaurand et al., 1983). Asbestos surface chemistry has also been shown to affect the in vitro production of oxygen metabolites, which may play a role in fibrosis and carcinogenesis (Zalma et al., 1987; Pezerat, 1991). Since a single study by Hesterberg and Barrett (1984) successfully related cytotoxicity with in vitro transformation, this raises the question of whether surface chemical or physical properties of asbestos fibers might also be important in relation to in vitro

genotoxic activities. In this study, samples of chrysotile fibers were prepared with similar morphologies but differing surface chemistries. The hypothesis to be tested is that surface properties of chrysotile fibers affect genotoxicity in V79 cells using an in vitro micronucleus assay successfully used previously in this laboratory (Lu et al., 1994).

MATERIALS AND METHODS

Preparation of Chrysotile Asbestos

Intermediate-length chrysotile, obtained from the National Institute of Environmental Health Sciences, was used because this material has been physically and chemically characterized in great detail (Campbell et al., 1980). Samples of these fibers were suspended in deionized H_2O (18.2 MΩ-cm, Millipore) and sonicated 10 min at 72 W using a probe sonicator (Mysonics, Farmingdale, NY). It has been shown that acid treatment of chrysotile fibers results in a rapid dissolution of the outer, magnesium-containing layer of the fiber, followed by a much slower leaching of the interior magnesium (Atkinson, 1973; Ozeki et al., 1989). Therefore, fibers to be surface modified were treated with 1 *N* HCl (Baker ULTREX) for 2 h to remove surface magnesium. Both the treated and untreated samples were then filtered through 0.4-μm pore size polycarbonate filters and washed with deionized H_2O, followed by vacuum drying. Fibers were resuspended and centrifuged 20 min at 2500 × g to remove small fragments.

The fiber samples were analyzed by scanning Auger spectroscopy to determine the surface purity of the samples; only silicon, oxygen, and magnesium peaks were observed on the Auger spectra. However, due to sample charging, it was not possible to obtain quantitative results from the Auger spectra. Therefore, the samples were also analyzed using x-ray photoelectron spectroscopy (XPS) to determine elemental surface composition. The XPS spectra are shown in Figure 1. Results indicate substantial reduction, about 21%, in the Mg to Si ratio, which is consistent with acid depletion of surface Mg on the chrysotile fibers. The native and surface-altered chrysotile samples were also analyzed using scanning electron microscopy/energy-dispersive x-ray spectroscopy (SEM-EDS) to determine the change in the magnesium concentration upon acid treatment. Since the goal of the acid treatment was to remove magnesium from the fiber surface without changing the silicon content, the change in the ratio of magnesium counts to silicon counts was used as a measure of the magnesium depletion (Figures 2 and 3). This ratio decreased by 15 ± 5% upon acid treatment, consistent with removal of the outer layer of magnesium for fibers in the 0.1 μm diameter range.

A change in surface chemistry would likely affect the surface charge and thus the zeta potential of the samples. Zeta potential measurements were made at pH 5.0 using a Zeta-Meter ZM-80; zeta potential measurement at this pH is relatively insensitive to small changes in pH. The untreated fibers

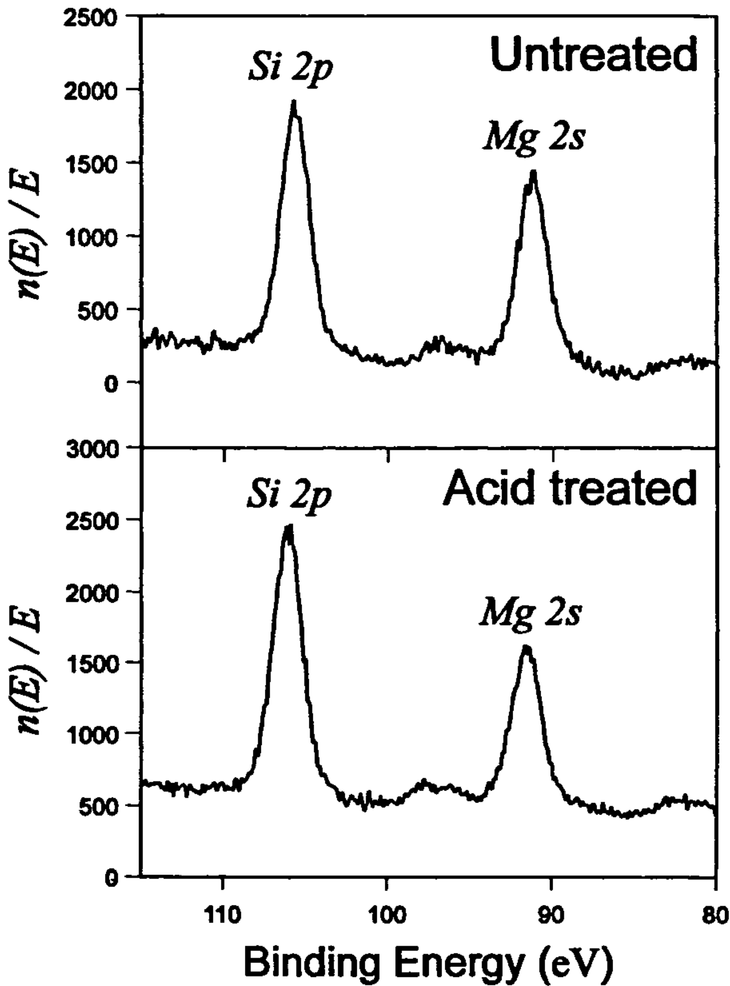

FIGURE 1. X-ray photoelectron spectra of acid-treated and untreated chrysotile asbestos.

demonstrated a strongly positive zeta potential (approximately +0.02 V), while the treated fibers showed a negative potential of similar magnitude (approximately −0.02 V). Moreover, a difference in surface charge was evident by macroscopic behavior of the dry fibers. The untreated fibers showed a much greater tendency to adhere to the side of the polystyrene containers, while the treated fibers formed large clumps in the middle of the vial.

Fiber length measurements were made by resuspending a small amount (<100 μg) of each sample in deionized H_2O and then filtering an aliquot of the suspension through a 0.4-μm pore size polycarbonate filter. A section of the filter was mounted on a brass stub for scanning electron micros-

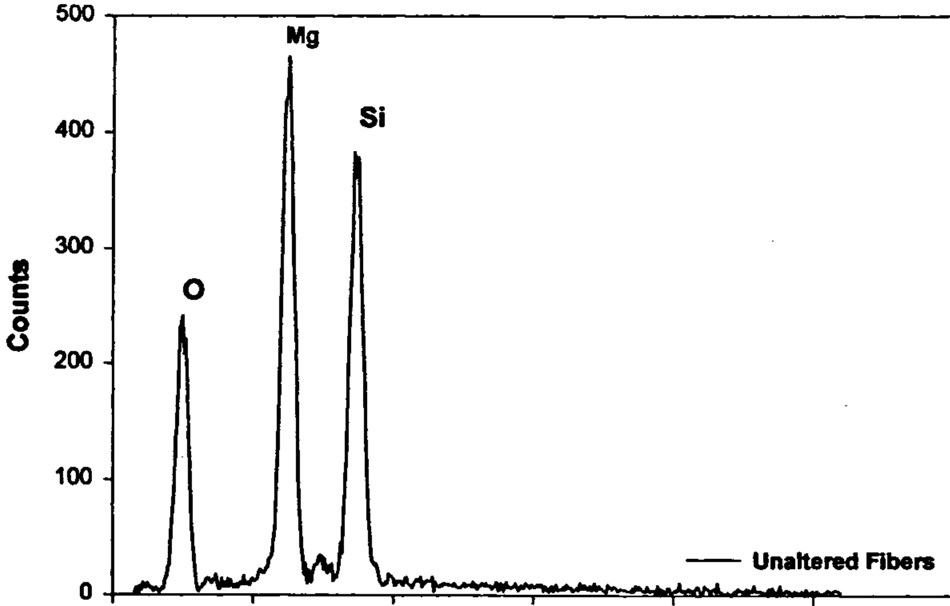

FIGURE 2. Energy-dispersive x-ray spectrum of acid-treated chrysotile asbestos.

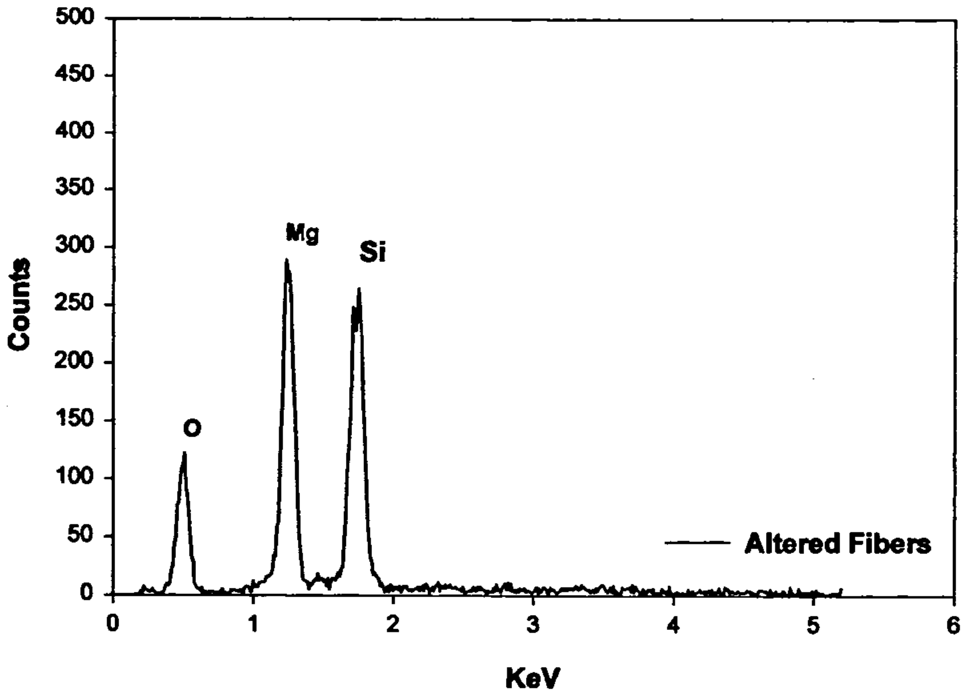

FIGURE 3. Energy-dispersive x-ray spectrum of untreated chrysotile asbestos.

copy. Analysis fields were randomly selected across the filter section by using the random point feature of the automated sample stage controller. Within each analysis field, all fibers with midpoints lying within the field were measured for length until 500 fibers had been measured for each sample; field limits were temporarily adjusted to allow measurement of fibers that exceeded the field width.

Figure 4 includes side-by-side histograms of the number of fibers in specific size ranges, for both unmodified and acid-treated chrysotile samples. Detailed statistical analysis indicates that the ratios of the median lengths of the untreated fibers are, on average, about 25% longer than the treated ones. The bootstrap method was used to calculate the 95% confidence interval, which suggests that the median length is probably between 10% and 40% longer for the untreated fibers.

Micronucleus Assay

V79 cells (4.0×10^5) in 10 ml minimal essential medium (MEM, Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco), and 2 m*M* L-glutamine (Gibco) were added to each

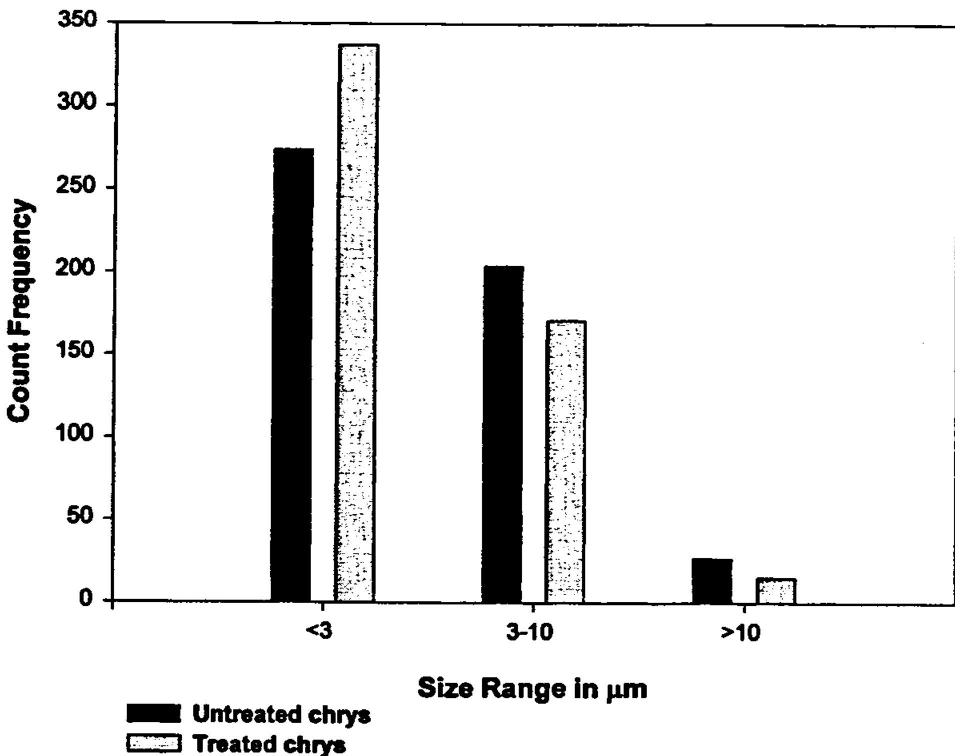

FIGURE 4. Fiber length distributions in three ranges for native and surface-altered chrysotile fibers.

TABLE 1. Numbers of Micronucleated Cells per 1000 V79 Cells Exposed to Various Concentrations of Treate and Untreated Chrysotile Asbestos Fibers

Reader		Experiment	Concentration				
			0	3.3 μg/ml	10 μg/ml	33 μg/ml	100 μg/ml
1	Treated	1	**7:** 8, 10, 4	**9:** 15, 6, 5	**18:** 19, 15, 19	**38:** 49, 37, 29	**39:** 41, 43, 34
	Untreated			**19:** 15, 16, 25	**17:** 20, 16, 15	**34:** 32, 40, 30	**33:** 47, 39, 14
	Treated	2	**4:** 3, 4	**9:** 8, 11, 9	**15:** 13, 15, 16	**31:** 28, 36, 29	**32:** 24, 37, 35
	Untreated			**6:** 3, 7, 9	**7:** 16, 16, 18	**31:** 27, 30, 35	**30:** 28, 31, 32
2	Treated	1	**11:** 7, 17, 9	**9:** 11, 7, 9	**23:** 29, 27, 12	**36:** 43, 32, 32	**29:** 36, 39, 12
	Untreated			**17:** 7, 14, 30	**14:** 8, 20, 15	**24:** 32, 21, 18	**26:** 26, 42, 9
	Treated	2	**5:** 5, 4	**6:** 8, 2, 7	**8:** 5, 8, 10	**20:** 21, 20, 20	**24:** 20, 31, 20
	Untreated			**8:** 9, 9, 6	**13:** 15, 11, 14	**17:** 16, 15, 20	**25:** 19, 21, 36
3	Treated		**6:** 2, 5, 7	**7:** 7, 5, 8	**21:** 19, 22, 22	**37:** 31, 52, 28	**43:** 55, 44, 30
	Untreated			**23:** 11, 11, 46	**18:** 20, 20, 14	**38:** 30, 40, 43	**25:** 30, 38, 7

Note. The number is based on 3000 cells scored; means of readings are in boldface. Each number in a tripl is the number of micronucleated cells on a single slide (1000 cells).

100-mm petri dish. After overnight incubation at 37°C, untreated and surface modified chrysotile asbestos fibers were added into the culture cell dishes to give concentrations of 0.42–12.7 μg/cm^2 or 3.3–100 μg/ml.

The cells were treated for 24 h and then rinsed 8–10 times with phosphate-buffered saline (PBS, pH 7.4) to remove fibers. After an additional 24-h incubation, the cells were harvested by rinsing with 2 ml of 0.02% ethylenediamine tetraacetic acid (EDTA, Sigma), removing the liquid with suction, adding 2 ml of 2.5 mg/ml of trypsin solution (Sigma), rocking the dishes for 2 min, and adding 2 ml of fresh complete medium to inhibit further digestion. The contents of each flask were transferred and then centrifuged at 285 × g for 6 min. The supernatant was removed, the remaining pellet was rinsed with PBS and resuspended in complete medium, and slides were prepared on a Cytospin (Shandon, Pittsburgh, PA). Cells were fixed in absolute methanol and stained with Diff-Quik (Baxter, McGaw Park, IL).

The entire micronucleus experiment was performed twice. For the first experiment, 3 different readers each scored 3000 cells from each group for micronucleated cells (MNC). For the second experiment, 2 readers each scored 3000 cells from each group. The slides were coded before scoring. The frequency of MNC for each treatment was based on the scoring criteria of French (1993).

RESULTS AND STATISTICS

Table 1 contains the outcomes for the five replications of the experiment. Three readers took part in the scoring of cells exposed to concen-

trations of chrysotile asbestos fibers. Each replication provided two data sets: (1) a reader's scores of cells exposed to concentrations of treated (surface-modified) chrysotile and (2) the same reader's scores of cells exposed to untreated chrysotile. The first two readers each scored two experiments, while the third reader scored one experiment. Figure 5 shows a typical data set for a single reader (Reader 1); Figure 6 displays the fitted curves for the paired data sets. The curves are determined using the nonlinear dose-response model

$$\text{Response} = [b_0 + \beta_1(\text{Dose})] \exp[-\beta_2(\text{Dose})] \quad (1)$$

where the response is the number of MNC per 1000 cells as determined by a reader for a dose concentration, which is measured in micrograms per squared centimeter. The two parameters are β_1, which represents the genotoxicity rate per dose, and β_2, the cytotoxicity parameter. The fixed value b_0 is the mean spontaneous MNC frequency, which is estimated

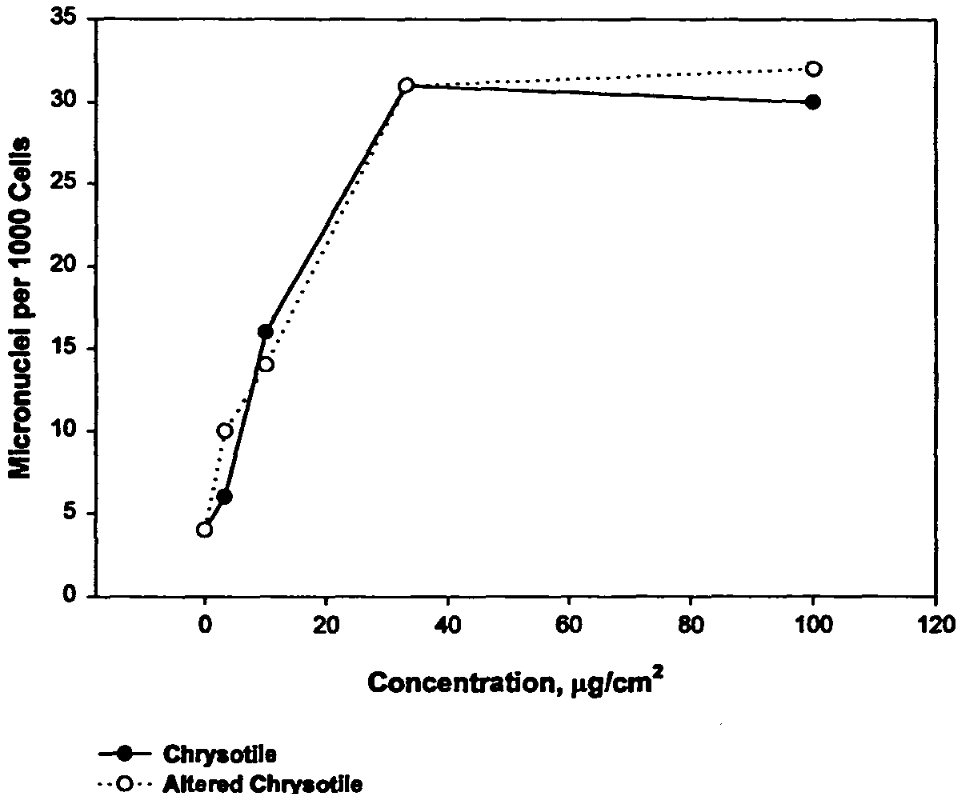

FIGURE 5. Micronucleated cell induction in 3000 scored cells after challenge with native and surface-modified chrysotile fibers, Reader 1, single experiment, average of 3 readings.

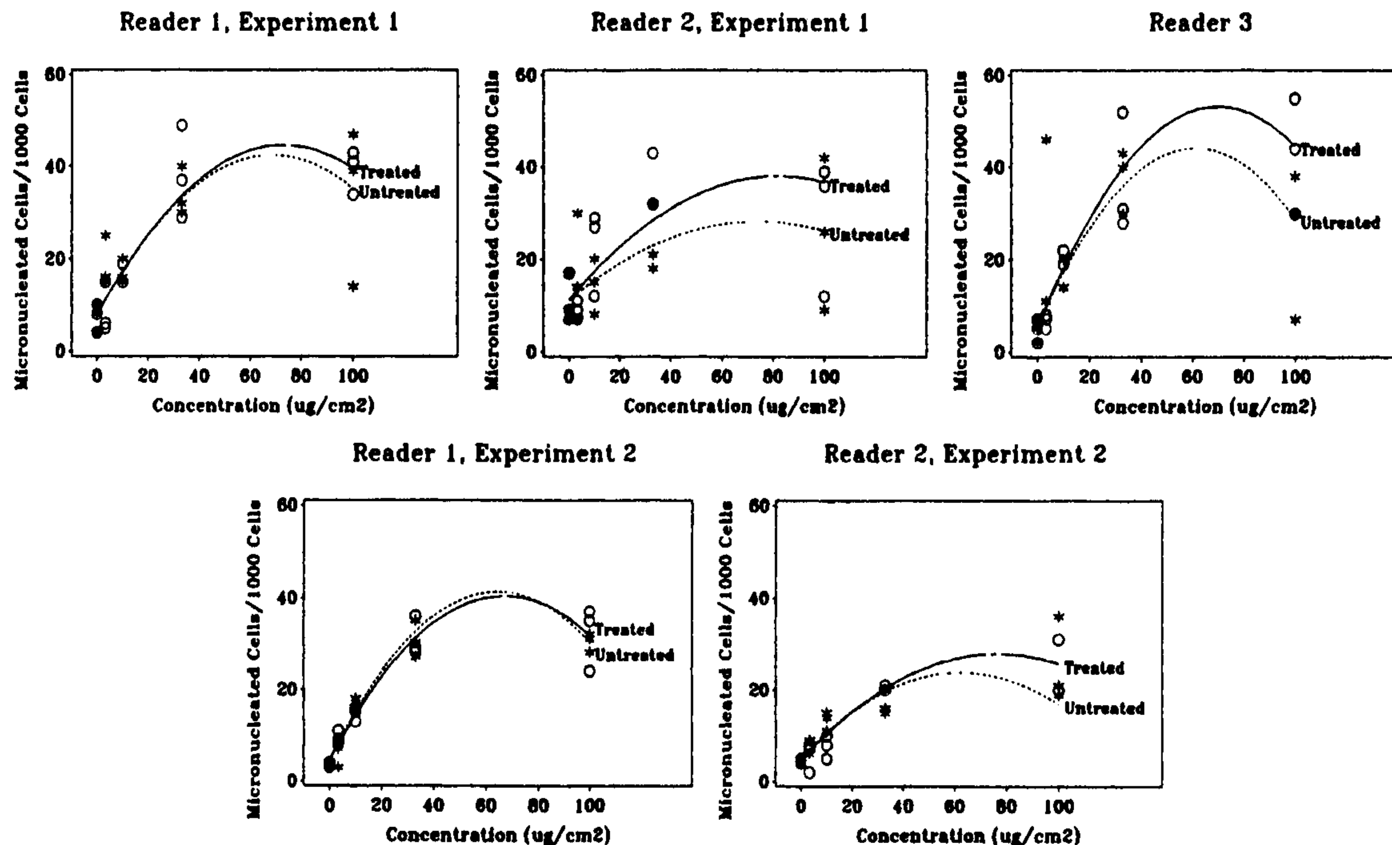

FIGURE 6. Fitted curves for the five paired readings. The response is the number of micronucleated cells per 1000 cells. Three of the plots show larger differences at higher dose levels, which suggest that the untreated fibers are more toxic. Open circles are treated fibers; asterisks are untreated fibers.

from the negative control data and then inserted into the model. The parameters are estimated using weighted nonlinear regression.

Lack-of-fit tests indicate that, in general, the model provides an adequate fit for the data. The paired data sets were compared with respect to the genotoxicity parameter β_1 using the extra sums-of-squares principle (Alvord et al., 1990). Initial comparisons showed that the genotoxicity parameter varied considerably between replications. This variation can be attributed to interreader, intrareader, or interassay differences. In general, the data sets are not combinable across replications with respect to the genotoxic response.

When comparisons are made within each experiment, the genotoxic responses are statistically indistinguishable for the treated (surface-modified) and untreated (unmodified) fibers. For the first experiment from the second reader, the genotoxicity parameter estimate is not statistically different from zero (p = .05) for the untreated fiber data. For the remaining readings from both experiments, the genotoxicity parameters are significantly different from zero for both types of fibers, and the ratios of the untreated to the treated parameter estimates are displayed in Table 2.

The fitted curves in Figure 6 result from the competing factors of genotoxicity and toxicity. The untreated fibers are associated with larger estimates for β_2, and greater toxicity can obscure the genotoxic response. As Table 2 shows for the four replications, the untreated fibers have slightly larger estimates of β_1, on average about 10% larger estimates, than the treated fibers. The 95% confidence interval is calculated for each of these ratios using a first-order Taylor series approximation. The upper bounds of the intervals indicate that the difference is probably no more than 32% to 76%, or approximately 50%, on average. Therefore, using the results of these 4 replications, we would expect the genotoxic response for the untreated fibers to be, on average, 10% greater, but probably no more than about 50% greater, than the genotoxic response for the treated fibers.

TABLE 2. Ratios of the Genotoxicity Parameter β_1 Estimates for the Untreated to the Treated Fibers for Four of the Five Readings

Reading	Ratio	Confidence interval
Reader 1, Experiment 1	1.04	(0.58–1.51)
Reader 3, Experiment 1	1.05	(0.64–1.47)
Reader 1, Experiment 2	1.10	(0.89–1.32)
Reader 2, Experiment 2	1.19	(0.63–1.76)
	Mean = 1.10	

Note. The first experiment reading for Reader 2 is excluded because the estimate for β_1 is not statistically significant from zero for the treated fibers. Confidence intervals are estimated using a first-order Taylor series approximation.

DISCUSSION

Multiple studies have demonstrated that asbestos is a carcinogen (Mossman et al., 1996; Steenland & Stayner, 1997; Vu & Lai, 1997; Nishimura & Broaddus, 1998), but studies aimed at understanding the underlying processes have been much more difficult and less illuminating than those of typical chemical carcinogens. Genotoxicity assays were generally negative in early studies (Butterworth, 1990; Ashby, 1991). More recent studies have changed this, but overall, very few studies have shown gene mutations in any of the common test cell types, either prokaryotic or eukaryotic (Jaurand, 1991). Assays of DNA damage have had mixed results. Cell-free studies measuring 8-hydroxyguanosine and similar endpoints of DNA damage have usually been positive, which suggests reactive oxygen species involvement. In vitro cellular assays of DNA damage, repair, and strand breakage provide mixed results (Jaurand, 1991). Most DNA damage assays using alkaline elution, in mammalian cells including human cells, have been negative (Jaurand, 1991).

Besides gene mutation and DNA damage assays, another broad class of genotoxicity testing involves chromosomal effects: chromosome breakage, chromosomal aberrations, sister chromatid exchange, aneuploidy and polyploidy, and assays detecting damage to the spindle apparatus. These assays provide consistently positive results, with relatively few negative studies (Jaurand, 1991).

Additionally, studies using microcinematography show major interferences of asbestos fibers in the process of chromosome segregation for long fibers, but not short fibers (Cole et al., 1991; Ault et al., 1995). A transmission electron microscopy (TEM) study by Wang et al. (1987) details some of the interactions of fibers with chromosomes, including apparent punctures, cuts, and attachments of fibers to chromosomes by repair processes.

A previous study in this laboratory used a fluorescent-labeled kinetochore assay to study a limited number of samples, which indicated that both clastogenic and aneuploidogenic effects were present in asbestos-challenged V79 cells, in similar proportions (Lu et al., 1994). The fiber samples of this study were a subset of the fibers in that study, with the addition of surface alteration. These fibers were well characterized for both surface and bulk properties by multiple methods and were clearly demonstrated to be substantially altered after acid treatment. The depletion of surface Mg was verified by both x-ray photoelectron spectroscopy and scanning electron microscopy/energy-dispersive x-ray spectroscopy. The reversal of zeta potential confirms substantial surface alteration. The conservation of fiber length after treatment was likewise successful; although the length distributions were not identical, there were comparable numbers of fibers in the three length groups. The results of this study are consistent with the general findings of chromosomal damage. The results

are very similar for the treated and nontreated fibers and support the hypothesis of a chemically nonspecific interaction with chromosomes that involves breakage and interference or damage to the mitotic spindle apparatus, leading to aneuploidy. The results also indicate that while the genotoxic responses are very similar for the two fiber types, the cytotoxicity responses are somewhat greater for the untreated fibers, indicating that either there may be different mechanisms of toxicity, or cytotoxicity may be very sensitive to fiber length. It is probably also true that genotoxicity is not a direct consequence of a cytotoxic process, or the response curves for the two materials would have tracked more closely at the highest concentrations.

In vitro testing has limitations in predicting in vivo effects; however, it is immensely useful in mechanistic studies. Experimental conditions may be controlled well, and exposures to target cells, including insoluble particles and fibers, are more uniform and predictable. The V79 cells used in this study and a previous study in this laboratory (Lu et al., 1994) are a lung-derived line and widely used in a number of different genotoxicity assays. The micronucleus assay used here is relatively sensitive and linear over a half order of magnitude with only moderate cytotoxicity. As a study tool, results are relatively rapid and easily interpretable. While in vivo studies would be required to absolutely confirm that surface-altered chrysotile fibers were equally carcinogenic, the assumption seems reasonable, noting the conclusions of the in vivo mesothelioma studies (Wagner et al., 1973; Pott et al., 1974; Stanton et al., 1977).

The results of our study indicate that the surface chemistry is not an important variable in the in vitro genotoxicity of chrysotile asbestos in V79 cells, as detected by the micronucleus assay under the conditions used in this study, and support a model of chemically nonspecific chromosomal and spindle damage effects.

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