ENVIRONMENTAL RESEARCH 52, 164–177 (1990)

On the Mechanism of Cell Internalization of Chrysotile Fibers: An Immunocytochemical and Ultrastructural Study

W. MALORNI, F. IOSI, M. FALCHI, AND G. DONELLI

Department of Ultrastructures, Istituto Superiore di Sanità, Viale Regina Elena, 299-00161 Rome, Italy

Received October 6, 1989

Human breast carcinoma cells (CG5) and human laryngeal carcinoma cells (HEp-2) were exposed to 10 and 50 μg/ml of small (about 5 μm) chrysotile asbestos fibers. Morphological and ultrastructural changes were evaluated by means of immunocytochemistry and by scanning and transmission electron microscopy. Our attention was focused on the mechanisms of cell internalization and on transport of chrysotile fibers. The fibers appeared to penetrate the cell cytoplasm and to be translocated in proximity of the nucleus. Small chrysotile fibers could also be found inside the nucleus of interphase cells. Involvement of the main cytoskeletal components, i.e., microfilaments, intermediate filaments, and microtubles, in the cytotoxicity of chrysotile fibers was also evaluated. Our findings suggest that after fiber penetration, a rearrangement of the cytoskeletal apparatus occurs. It has also been observed that small fibers remain associated with the cytoskeletal framework, which can thus play a role in asbestos intracytoplasmic translocation in epithelial cells. Furthermore, after the cell has completely recovered its morphology, fiber internalization ultimately seems to lead to the formation of giant multinucleated cells. These data could be indicative of an interaction occurring between asbestos fibers and the normal mitotic process. The disturbance of the cell cytoskeleton and the close morphologic contact between asbestos fibers and the cell's nuclear region may be of importance in explaining the well-known carcinogenic effects of asbestos mineral fibers.

INTRODUCTION

The chrysotile fiber, the main component of mineral asbestos, is a hydrated magnesium silicate which forms a soft flexible and tensile fiber particularly resistent to abrasion and heat (Allison *et al.*, 1975). In recent years, the mechanism of asbestos dust cytotoxicity and tumorigenicity has been widely investigated. General morphological characteristics such as size, shape, and diameter were considered to play an essential role in the fibrogenic and carcinogenic potential of asbestos dusts (Kaw *et al.*, 1982).

The association between exposure to asbestos and several human diseases, including lung and gastrointestinal malignancies, has been suggested (Neuberger *et al.*, 1984). Mesotheliomas and carcinomas seem to be the main forms of neoplasia related to the exposure to breathable asbestos fibers (Environmental Health Criteria, 1986). Some of these *in vivo* studies have been confirmed *in vitro* using different cultured cell systems. In particular, the hemolytic capacities (Harington *et al.*, 1971; Allison *et al.*, 1975; Beck and Bignon, 1980; Hunt *et al.*, 1981), the possible interaction with extracellular matrix and plasma membrane (Brody *et al.*, 1983), and the mechanisms of endocytosis and putative relationships with chromosomes (Hesterberg *et al.*, 1985; Jaurand *et al.*, 1986; Wang *et al.*, 1987) have

0013-9351/90 $3.00

ysotile Fibers:
ructural Study

G. DONELLI

à, Viale Regina Elena,

rcinoma cells (HEp-2) were
estos fibers. Morphological
cytochemistry and by scan-
focused on the mechanisms
fibers appeared to penetrate
leus. Small chrysotile fibers
olvement of the main cyto-
nts, and microtubles, in the
ngs suggest that after fiber
rs. It has also been observed
work, which can thus play a
. Furthermore, after the cell
ultimately seems to lead to
indicative of an interaction
ess. The disturbance of the
sbestos fibers and the cell's
own carcinogenic effects of

stos, is a hydrated
nsile fiber particularly re-
cent years, the mechanism
been widely investigated.
shape, and diameter were
d carcinogenic potential of

l several human diseases,
een suggested (Neuberger
be the main forms of neo-
ers (Environmental Health
n confirmed *in vitro* using
lytic capacities (Harington
80; Hunt *et al.*, 1981), the
ia membrane (Brody *et al.*,
/e relationships with chro-
6; Wang *et al.*, 1987) have

been extensively studied in several cell models. Notwithstanding, complex mechanisms underlying fiber cytopathology are far from having been elucidated. A possible involvement of free radicals causing lipid peroxidation and DNA breakages has been hypothesized as a result of toxic injury caused by intracellular chrysotile fibers (Sincock and Seabright, 1975; Mossman *et al.*, 1983b). Furthermore, cytoskeletal elements, which have been shown to play a pivotal role in cytoplasmic traffic, could also represent important carriers of small intracellular fibers (Rüttner *et al.*, 1987). Mesothelial cell cytoskeleton has, in fact, been found to be associated with asbestos fibers, and a close contact between the nuclear membrane and chrysotile fibers has also been detected (Rüttner *et al.*, 1987). However, the mechanisms of chrysotile fiber internalization and transport seem to need further investigations. The purpose of this study was thus to try to better elucidate these peculiar mechanisms of cytotoxicity.

MATERIALS AND METHODS

Cell Cultures

Human breast carcinoma cells (CG5), kindly provided by Dr. G. Sica (Università Cattolica, Rome, Italy), and human laryngeal carcinoma cells (HEp-2), were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with nonessential amino acids, L-glutamine, vitamins, 10% fetal calf serum plus 100 IU/ml penicillin and 100 μg/ml streptomycin.

For light and scanning electron microscopy (SEM) both control and treated cells were grown on 13-mm-diam glass coverslips in separate wells and seeded at a density of approximately 10^4 cells/ml.

Cells were subcultured in 25-cm^2 Falcon plastic flasks at a density of approximately 10^5 cells/ml for transmission electron microscopy (TEM). For the observation of cytoskeleton by TEM, gold grids (200 mesh) were coated and adhered to plastic flasks using 0.2% Formvar, in clorophorm. The flasks were seeded with cells at a density of 10^4 cells/ml.

UICC (Union International Contre le Cancer) standard reference samples of chrysotile fibers were suspended in culture medium (DMEM) dispersed by sonication and sterilized by autoclaving. Granulometric SEM studies showed that after 20 min of sonication more than 80% of fibers were less than 5 μm in length. The resulting suspension was added to a final volume of 5 ml at a concentration of 50 μg/ml. Immunocytochemical and electron microscopic observations were performed after 3 and 6 hr. For long-term treatment, chrysotile fibers were added to the cultures at a concentration of 10 μg/ml. After 24 hr, cells were washed with phosphate-buffered saline (pH 7.3) and incubated in fresh medium. Cells were then observed daily by phase contrast microscopy for at least a week. Cytochalasin B (CB) (Sigma) treatment was performed as follows: CB was dissolved in dimethyl sulfoxide (DMSO) and then added to the culture medium to obtain a final concentration of 1 μ*M*. After a 1-hr incubation with CB, chrysotile treatment was performed. Cultured cells treated with equal amounts of DMSO alone were considered as controls. Finally, immunocytochemical observations were performed.

Fluorescence Microscopy

Cells grown on coverslips were fixed in 3.7% formaldehyde in phosphate buffer (pH 7.4) for 10 min at room temperature. After washing in the same buffer, for the detection of cytoskeletal elements, cells were permeabilized with 0.5% Triton X-100 (Sigma, T-6878) for 5 min at room temperature. For nuclei detection, the cells were incubated with 20 μg/ml Hoechst solution (Sigma) at 37°C for 10 min. Once through washing, coverslips were mounted with 50% glycerol–phosphate-buffered saline.

For tubulin labeling, incubations with monoclonal antibody directed against α-tubulin (Amersham International) were carried out at 37°C for 30 min. After washing, cells were incubated with a sheep anti-mouse IgG fluorescein-linked whole antibody (Amersham International). For actin detection, cells were stained with fluorescein–phalloidin (NBD-phallacidin, Molecular Probes) at 37°C for 30 min.

For the detection of keratin filaments, cells were fixed with methanol for 5 min at room temperature and for 5 sec with acetone at −20°C. Cells were then incubated with a polyclonal antibody directed against keratin (Ortho Diagnostic) and with a sheep anti-rabbit IgG fluorescein-linked whole antibody (Amersham International).

Electron Microscopy

For transmission electron microscopy, cells grown on 25-cm^2 flasks were fixed in 1.5% glutaraldehyde in 0.05 M cacodylate buffer, postfixed in 1% OsO_4, dehydrated in ascending grades of ethanols, and embedded in Agar 100 (Agar AIDS). Ultrathin sections were stained with uranyl acetate and lead citrate and were examined with a Zeiss EM 10 C electron microscope.

Cytoskeleton Preparation for Electron Microscopy

The procedure followed was an adaptation of the detergent extraction method previously described (Schliwa and Van Blerkom, 1981). All of the steps were carried out at 37°C. Before detergent extraction, the cells were rinsed briefly in PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM $MgCl_2$), pH 6.9[15] They were then dipped in PHEM buffer containing 0.75% Triton X-100 for 2 min. The detergent-permeabilized cells (cytoskeletons) were washed briefly in PHEM and then fixed for 10 min in a 10 mM sodium phosphate buffer containing 1% glutaraldehyde–0.2% tannic acid, pH 7.0. After fixation, salt was removed from the specimens by washing the cytoskeletons in distilled water. The cytoskeleton preparation was then dehydrated by increasing concentrations of ethanol and critical-point-dried in CO_2. For transmission electron microscopy the specimens were freeze-dried in a Balzers BAF 300 freeze-etch unit at a stage temperature of −80°C for 30 min and then rotary-coated with platinum at a 45° angle and with carbon at a 90° angle. They were then examined with a Philips 430 electron microscope at 300 kV. For scanning electron microscopy the cells were gold-coated (<10-nm film thickness) by sputtering and examined with a Philips 515 scanning electron microscope. Both SEM and TEM microscopes are equipped

with energy-despersive X-ray detection system (EDAX PV-9900). X-ray spectra were collected over 60 sec, using a 0.5-μm-diam circular probe. The accelerating voltage was 300 kV for TEM and 30 kV for SEM.

RESULTS

Chrysotile fiber internalization seems to occur by a sequence of events that can be analyzed by light microscopy and thin section TEM. In our experiments only small fibers, less than 5 μm in length, were considered. The longer ones (>10 μm) did not appear to be able to be actively internalized in the cell cytoplasm, but they seemed to easily induce cell degeneration and death. In fact, a quantitative evaluation performed by the trypan blue exclusion test showed that most of the nonviable cells (90%) included the longer fibers, whereas only viable cells were to be considered for this study.

When cultured epithelial cells were analyzed by TEM after treatment with chrysotile fibers at a concentration of 10 μg/ml for 3 hr, small fibers could be found on the cell surface (Fig. 1a). However, after 6 hr of treatment, asbestos fibers were found in the cytoplasm of 70% of the cell sections examined. These fibers appeared to have no membrane envelope (Fig. 1b) whereas cell plasma membrane and organelle integrity seemed to be maintained. Furthermore, when the nuclear region ultrastructure of interphase cells was examined, asbestos fibers were also detected. Fibers were found in the perinuclear region (Fig. 1c), and smaller fibers of about 0.5 μm were observed inside the nuclear envelope (Fig. 1d) in 20% of the thin sections examined. Moreover, fibers were also found in the cell nucleoli (Fig. 1e). Nevertheless, nuclear envelope ultrastructure seemed to be maintained.

To detect whether chrysotile fibers could interact with some cytoskeletal elements to be transported through epithelial cell cytoplasm, an immunocytochemical and a parallel electron microscopic analysis was performed. After treatment with detergents, as described under Materials and Methods, cytoplasmic and plasma membrane soluble proteins and phospholipids were extracted; only the cytoskeletal elements and the cell nucleus were detectable. Thus, the main cytoskeletal components were available for microscopic examination. In particular, actin microfilaments, microtubules, and keratin intermediate filaments were considered. Monolayer cultures of control epithelial cells revealed characteristic features in actin (Fig. 2a), microtubule (Fig. 2b), and keratin (Fig. 2c) arrangement which appeared to be a well-represented network of fibers and filaments organized throughout the cell cytoplasm. When the same cells were observed after 3 hr of chrysotile treatment, remarkable changes in the arrangement of cytoskeletal elements could be observed. In particular, wide breakages within the actin stress fiber network due to the penetration of fibers in the cells were detected (Fig. 3a). Likewise, the microtubular apparatus and keratin intermediate filament network underwent alterations in their arrangements (Figs. 3b and 3d, respectively); such alterations were clearly related to the presence of asbestos fibers in the cytoplasm. In fact, when the same cells in Figs. 3b and 3d were observed by phase contrast microscopy, several asbestos fibers were detectable (Figs. 3c and 3e, respectively). On the other hand, when CB-treated cells were exposed to chryso-

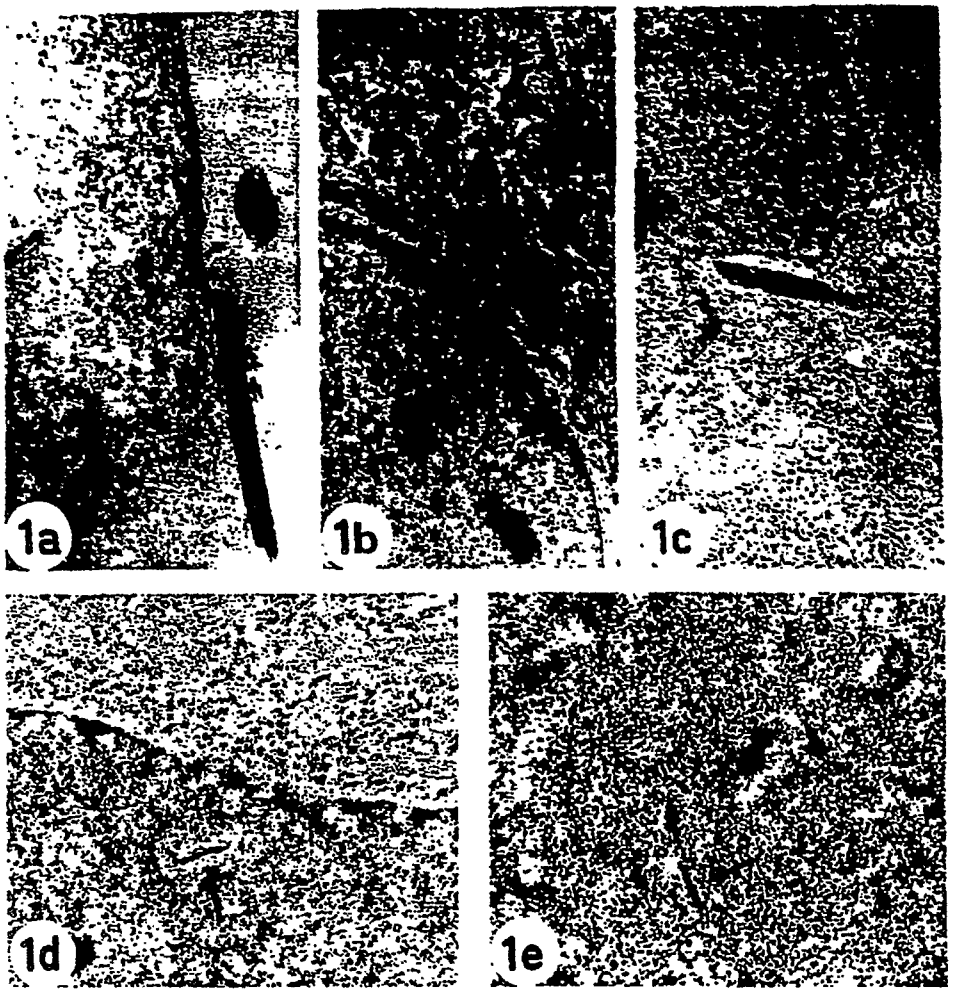
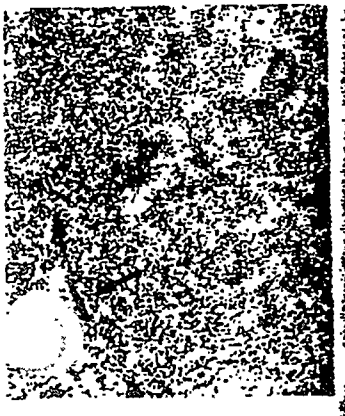


FIG. 1. Transmission electron microscopy. Micrograph sequence showing the pattern of internalization of small chrysotile fibers in CG5 cultured cells. Fibers were found to adhere to the cell surface (a), inside the cytoplasm devoid of a membrane envelope (b), in the perinuclear region close to the nuclear envelope (c), inside the nucleus (arrow) (d), and, finally, small fibers can be detected in the nuclear matrix (arrow) (e). (a, ×55,200; b and c, ×14,720; d, ×18,400; e, ×23,000).

tile fibers for 3 hr, actin depolimerization was observed and no fibers could be detected inside the cell cytoplasm (Fig. 5). However, the cytoskeletal element network appeared to have fully recovered after 6 hr treatment (Figs. 4a and 4b). Furthermore, when recovery experiments were carried out in fiber-free culture medium for a week after the beginning of exposure to asbestos, a remarkable increase in the number of giant multinucleated cells could be observed (Figs. 6, 7a). These polynucleated cells displayed large areas of cytoplasm with no nuclei, and some regions filled with several nuclei (Fig. 7a). Small nuclei, probably deriving from nuclear segmentation, were also observed (Fig. 6). When analyzed by



ce showing the pattern of internal-
: found to adhere to the cell surface
the perinuclear region close to the
small fibers can be detected in the
:,400; e, ×23,000).

rved and no fibers could be
er, the cytoskeletal element
treatment (Figs. 4a and 4b).
ried out in fiber-free culture
e to asbestos, a remarkable
could be observed (Figs. 6,
of cytoplasm with no nuclei,
. Small nuclei, probably de-
d (Fig. 6). When analyzed by

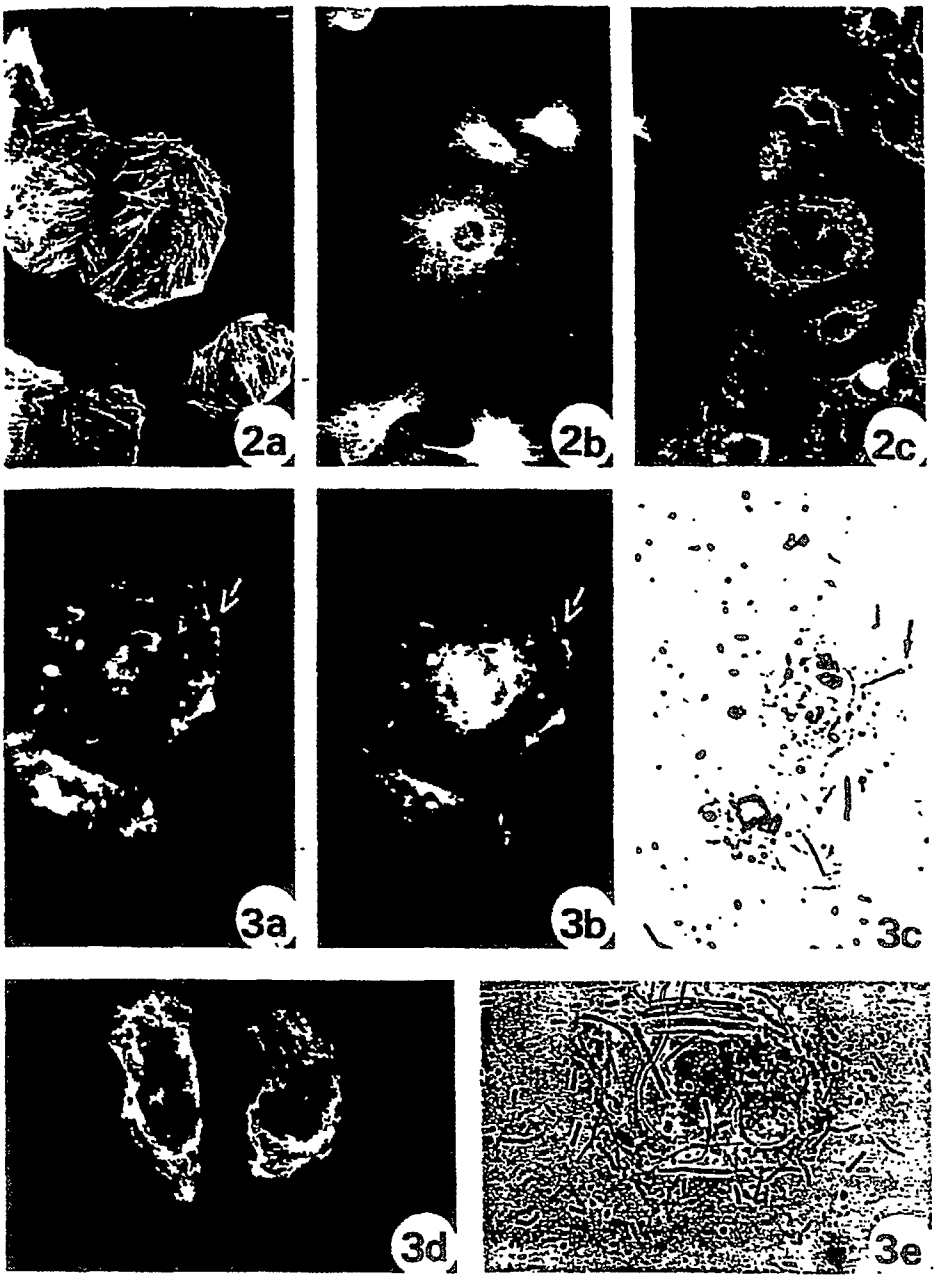
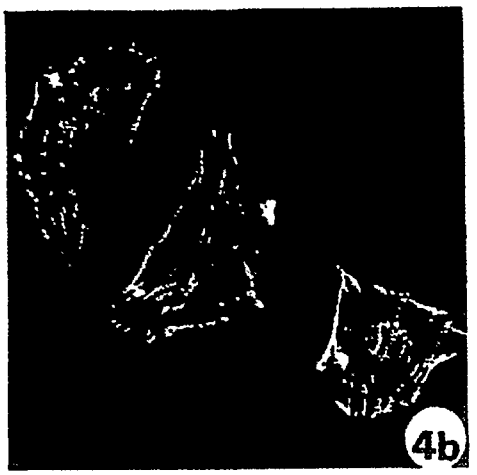


FIG. 2. Fluorescence microscopy. CG5 control cells. Staining (a) or immunostaining (b, c) showing the normal arrangement of actin fibers (a), microtubules (b), and cytokeratin filaments (c) in flat adhering cells (a, b, and c, ×860).

FIG. 3. Fluorescence microscopy. CG5 cultured cells treated for 3 hr with chrysotile fibers. Actin staining (a) and α-tubulin immunostaining (b) of the same cells shown in (c) by brightfield. Morphological modifications in these cytoskeletal components, characterized by rearrangements and breakages in the asbestos-inserting regions is well evidenced when long fibers are considered (arrows). A cytokeratin rearrangement was also detectable in chrysotile-treated CG5 cells (d, cytokeratin immunostaining; e, the same cells observed by brightfield) (a–e, ×1720).



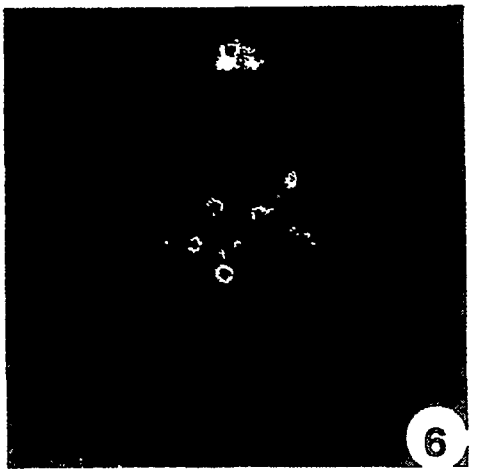


FIG. 4. Light microscopy. Brightfield (a) and actin staining by fluorescein–phalloidin (b) of CG5 cells. A complete recovery of the actin network is observed after 6 hr treatment with chrysotile fibers (×1680).

FIG. 5. F-actin fluorescence microscopy of CB-treated CG5 cells after asbestos exposure. Chrysotile fibers were not detectable in the cytoplasm (×1680).

FIG. 6. CG5 cell stained with Hoechst solution showing several clustered nuclei (×1680).

phase contrast microscopy, asbestos fibers were visible inside the giant cells, which were sometimes as much as 50 times larger than control cells. Furthermore, cytoskeletal elements appeared normal in shape, having recovered their arrangement (Figs. 7a–7d). After a week, the percentage of giant multinucleated cells was 30%, compared to approximately 5% in control cells.

To better elucidate the possible mechanisms of interaction between chrysotile fibers and the cytoskeletal apparatus in epithelial cells, cytoskeletal extracts, prepared as described above, were studied by transmission and scanning electron microscopy. Observations performed by TEM allowed us to visualize the cyto-

fluorescein–phalloidin (b) of CG5
hr treatment with chrysotile fibers

5 cells after asbestos exposure.

clustered nuclei (×1680).

sible inside the giant cells,
control cells. Furthermore,
ing recovered their arrange-
ant multinucleated cells was

eraction between chrysotile
cells, cytoskeletal extracts,
ission and scanning electron
ed us to visualize the cyto-

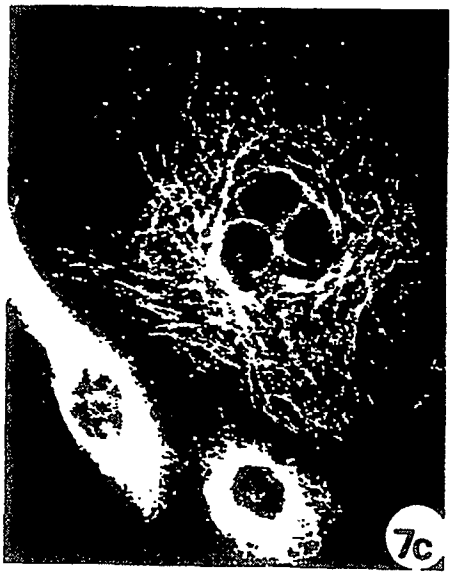


FIG. 7. The appearance of giant polynucleated cells is clearly evident in CG5 cells treated for 6 hr with chrysotile fibers, when observed by brightfield after a week in fiber-free medium (a). Parallel fluorescence microscopy observations showed the normal arrangement of actin filaments (b), microtubules (c), and cytokeratin intermediate filaments (d) in multinucleated flattened cells. A remarkable positivity for microtubules and cytokeratin filaments is visible in the perinuclear region (a, ×1260; b, c, and d, ×1680).

skeletal network arrangement after treatment with chrysotile fibers, as well as to detect possible signs of interaction between small fibers and cytoskeletal elements (Fig. 8). In fact, after Triton X-100 extraction, a morphologic relationship between fibers and the cytoskeleton appeared to be maintained. The observation that

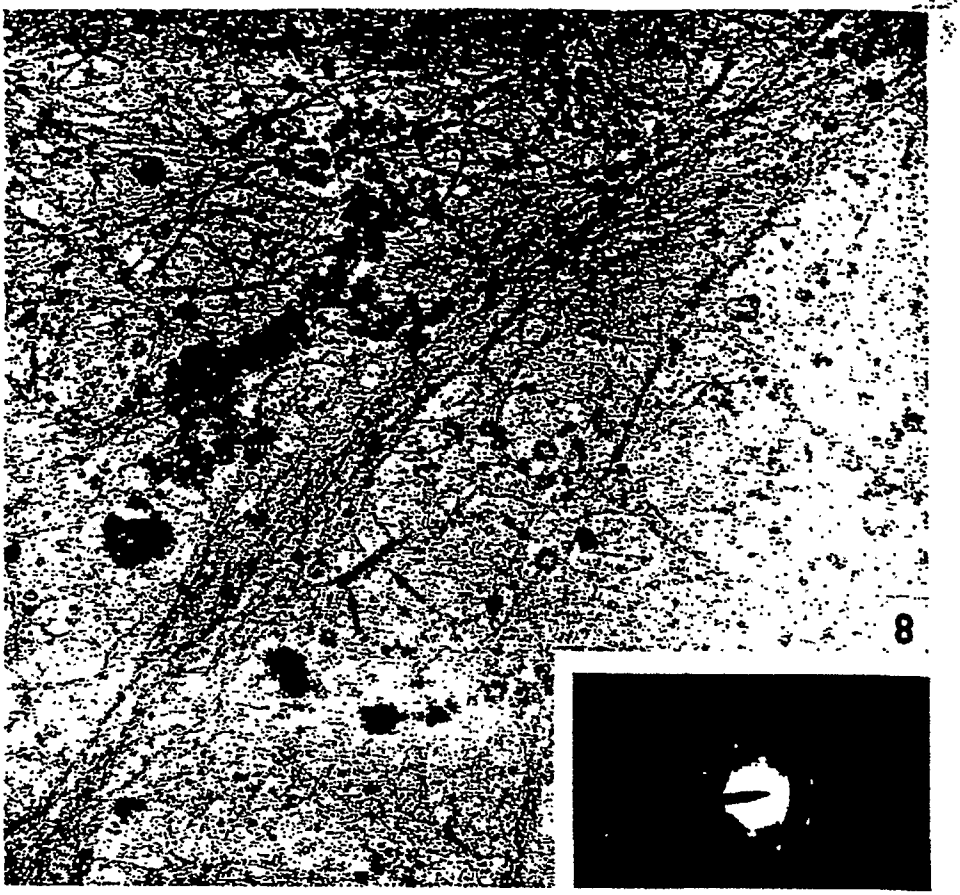


FIG. 8. Transmission electron microscopy. Triton-permeated CG5 cells observed after 6 hr of treatment with chrysotile fibers. Cytoskeletal elements are visualized. A small fiber, confirmed to be of chrysotile type by the diffraction pattern (inset), appears in close relationship with thin cytoskeletal elements (arrows) (×19,750).

internalized asbestos fibers are not free in the cytoplasm and are not removed from the cell by detergent extraction can shed some light on an intimate connection between fibers and the cytoskeleton. Energy-dispersive X-ray microanalysis and the diffraction pattern (Fig. 8, inset) confirmed that the electron-dense bodies were asbestos fibers of the chrysotile type in close relationship with cytoskeletal extracts. Parallel experiments performed using SEM enabled us to better visualize, along with the simple interlacing of fibers, a direct interaction between asbestos and the cytoskeleton (Figs. 9a, 9b). In fact, high magnification (Fig. 9c) reveals a close relationship between a chrysotile fiber and some cytoskeletal elements. In consideration of their diameter they could probably belong to the microfilament system.

DISCUSSION

The present results show that the small fibrogenic material added to epithelial

s observed after 6 hr of
all fiber, confirmed to be
relationship with thin cytoskeletal

lasm and are not removed
ght on an intimate connec-
ersive X-ray microanalysis
t the electron-dense bodies
ationship with cytoskeletal
enabled us to better visual-
ect interaction between as-
igh magnification (Fig. 9c)
ber and some cytoskeletal
uld probably belong to the

material added to epithelial

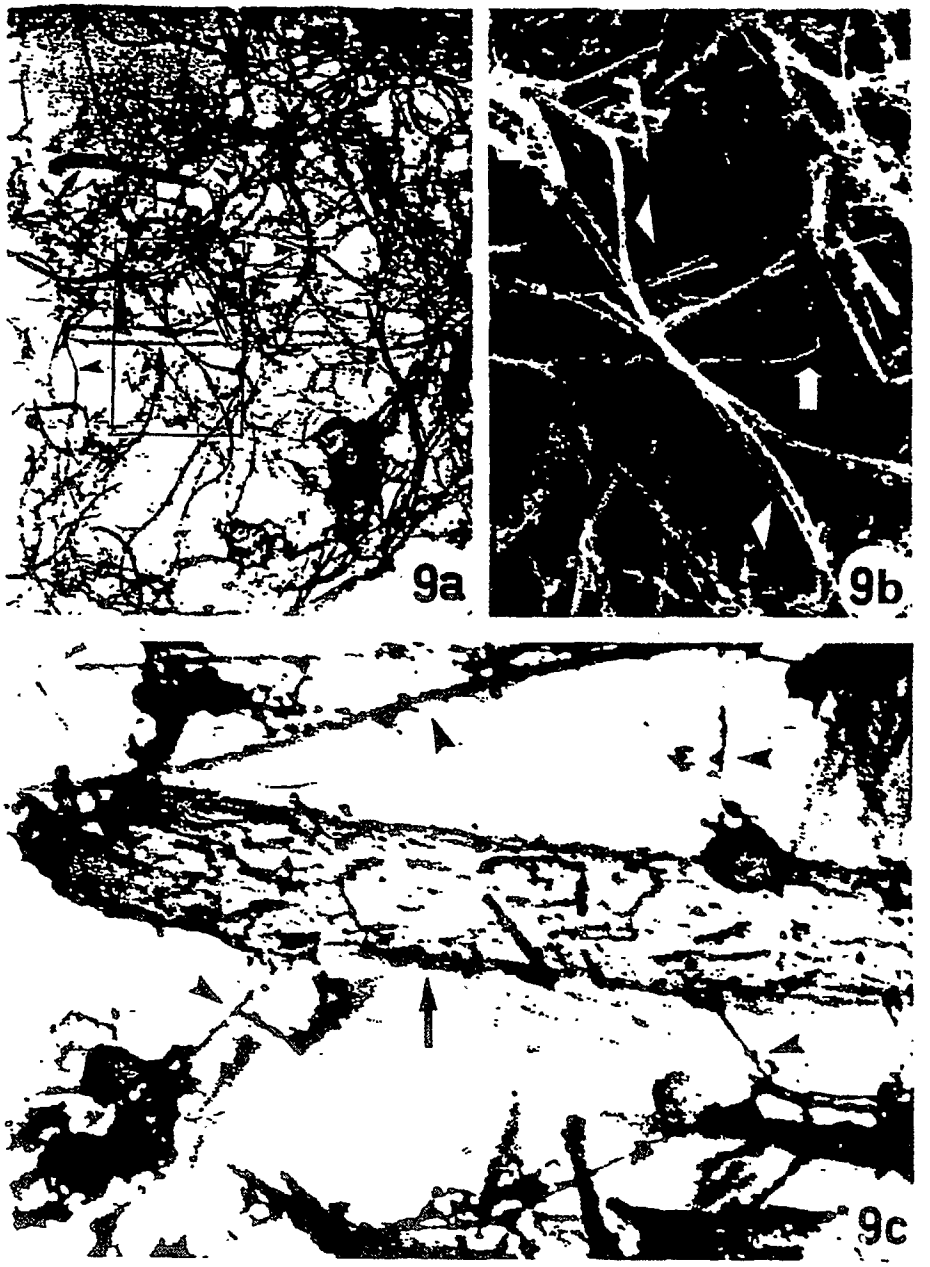


FIG. 9. Scanning electron microscopy. Triton-permeated CG5 cells treated with chrysotile fibers for 6 hr. Cytoskeletal organization appears well preserved. Relationships between asbestos fibers and cytoskeletal elements can be observed (a, b). At higher magnification (c) interaction seems to exist between some cytoskeletal elements and the internalized fiber. (a and c) Reverse contrast. (b) Enlargement of the outlined area shown in (a). Arrows: chrysotile fiber; arrowhead: cytoskeletal elements. (a, ×800; b, ×3500; c, ×14,000).

cell culture media appears to be associated with cytoskeletal elements. Internalized and transported fibers were detected in the nuclear envelope of interphase cells.

Cell culture studies have been extensively used to investigate toxic and long-term effects of asbestiform minerals (Hext and Richards, 1976; Hext *et al.*, 1977; Chamberlain *et al.*, 1979; Wang *et al.*, 1987). Results present considerable differences which depend upon fibrous material, cell type, fiber processing, and the extent and duration of exposure. However, some general rules can be traced. Chrysotile stimulates collagen production, proteoglycans, and an aggregation of pericellular fibronectin (Vartio *et al.*, 1986), which is the prerequisite of fibrosis *in vivo* (Rahman *et al.*, 1975; Richards and Morris, 1979). Chrysotile asbestos also seems able to bind sialic acid groups to membranes (Brody *et al.*, 1983), and the interaction between the positively charged fibers and cell membranes could be related to cell damage (Allison *et al.*, 1975; Mossman *et al.*, 1982, 1983a). In accordance with these observations, chrysotile fibers seem to enter the epithelial cell cytoplasm, damaging both the plasma membrane and the underlying cytoskeletal network. Nevertheless, our data seem to indicate that both plasma membrane and cytoskeletal elements can undergo rapid rearrangement and recovery.

The involvement of cytoskeletal elements in intracellular transport of asbestos fibers has recently been hypothesized (Rüttner *et al.*, 1987). In fact, as has been previously described (Richards *et al.*, 1977; Joseph *et al.*, 1983), ingested fibers are not free in the cytoplasm, but seem to interact with some cytoskeletal elements. The results reported here not only confirm previous observations (Rüttner *et al.*, 1987), but also suggest that the cytoskeletal apparatus plays a structural role in the interaction of chrysotile fibers with cell cytoplasm. However, a functional role of cytoskeletal elements cannot be ruled out.

Phagocytosed asbestos fibers that are not removed from the cell by detergent extraction are revealed by scanning and transmission electron microscopic observations; they are shown to be in close contact with some cytoskeletal components. This could therefore shed some light on the close relationship between fibers and the cytoskeleton. Previous studies have shown that the uptake of fibers causes an increase in the filamentous (polymerized) to nonfilamentous actin ratio in epithelial cells (Brody *et al.*, 1983), and that individual actin filaments are probably attached to the fiber surfaces (Brody *et al.*, 1986). In fact, after treatment with cytochalasin B, a specific actin-depolimerizing agent, chrysotile fibers in cytoskeletal extracts were not detectable. This could suggest an important role of the relationships between actin filaments and chrysotile asbestos fibers next to a passive "aspecific" internalization.

Several *in vitro* studies on cell growth kinetics were also carried out in epithelioid cell lines after treatment with asbestos fibers (Neugut *et al.*, 1978; Reiss *et al.*, 1980; Kaw *et al.*, 1982; Tilkes and Beck, 1983; Kenne *et al.*, 1986). Growth inhibition and a more prolonged mitosis, as well as a high sensitivity to chrysotile fibers of cells undergoing mitosis, were suggested (Neugut *et al.*, 1978). An increase in bi- or polinucleated cells was also observed by using another component of asbestos fibers, i.e., crocidolite, in treated Chinese hamster ovary cells (CHO) (Kenne *et al.*, 1986). In fact, it is possible that many cells which try to undergo

mitosis fail to complete cytoplasmic division; as a consequence, they end up forming giant multinucleated cells with several micronuclei which could derive from a chromosome fragment (Grote and Revell, 1972). These giant cells undergo DNA replication with no cell division, remaining in the culture for long periods of time (up to 1 month) and growing in size before they die. Moreover, nuclear division may also occur as a result of the so-called amitotic process, previously described by other authors (Clegg, 1963; De Martino *et al.*, 1985). In fact, the higher-order structure of the interphase eukariotic cell nucleus is still a matter of conjecture. The increased nuclear foldings, particularly along the axis of the nucleus, together with the increased eccentricity and reduced size of the nucleolus, point to amitotic division (Clegg, 1963; Hervàs *et al.*, 1985). Previous studies also hypothesized that changes in the contractile microfilaments and microtubules may result in a constriction of the nucleus which can lead to nuclear segmentation and fragmentation (Ghadially, 1982; Neftel *et al.*, 1983).

In addition, several studies were performed on the direct interactions between chrysotile fibers and chromosomes. In particular, metaphase and anaphase abnormalities were detected in different cultured cells (Hesterberg and Barret, 1985). Bridges, abnormalities, and misaggregations of chromosomes resulting in aneuploidy could also be induced by abnormalities in nuclear division (Hervàs *et al.*, 1985). On the other hand, a direct interaction between chrysotile fibers and the cytoskeletal apparatus, mainly microtubules and microfilaments, could also contribute to chromosomal misaggregation via an impairment of the mitotic spindle. In fact, the sensitivity of epithelial cells *in vitro* has been related to the plasticity of its cytoskeleton resulting in chromosomal damage and aneuploidy (Haugen and Harris, 1982).

In conclusion, our results support the hypothesis of a direct, active involvement of cytoskeletal elements in chrysotile fiber translocation in the cytoplasm of cultured epithelial cells. These findings, together with those on the "stored" fibers detected in interphase nuclei, might suggest an active contribution of epithelial cells to their own pathological or carcinogenic potential.

REFERENCE

Allison, A. C., Harington, J. S., and Badami, D. V. (1975). Mineral fibres: Chemical physiochemical and biological properties. *Adv. Pharmacol. Chemother.* 12, 291–402.

Beck, E. G., and Bignon, J. (1980). *In vitro* effects of mineral dusts. *NATO ASI Ser.*.

Brody, A. R., Gerwyn, G., and Hill, L. H. (1983). Interactions of chrysotile and crocidolite asbestos with red blood cell membranes: Chrysotile binds to sialic acid. *Lab. Invest.* 49, 468–475.

Brody, A. R., Hill, L. H., Barrett, J. C., and Adler, K. B. (1986). Intracellular translocation of inorganic particles. *In* "The Cytoskeleton, a Target for Toxic Agents" (T. W. Clarkson, P. R. Sager, and T. L. M. Syversen, Eds.), pp. 221–227. Plenum, New York.

Chamberlain, M., Brown, R. C., Davies, R., and Griffits, D. M. (1979). *In vitro* prediction of the pathogenicity of mineral dusts. *Brit. J. Exp. Pathol.* 60, 320–327.

Clegg, E. J. (1963). Studies on artificial cryptorchidism: Morphological and quantitative changes in the Sertoli cells of the rat testis. *J. Endocrinol.* 26, 567–574.

De Martino, C., Marcante, M. L., Malorni, W., Citro, G., Floridi, A., Scorza-Barcellona, P., and Capanna, E. (1985). Morphological and karyological studies on *in vitro* Sertoli cells of adult Crab-Eating Macaques (*Macaca fascicularis*). *Amer. J. Primatol.* 9, 173–187.

Environmental Health Criteria 53 (1986). "Asbestos and Other Natural Mineral Fibres." World Health Organization, Geneva.

Ghadially, F. N. (1982). Radial segmentation of the nucleus. *In* "Ultrastructural Pathology of the Cell and Matrix" (F. N. Ghadially, Ed.), 2nd ed., p. 8. Butterworths, London.

Grote, S. J., and Revell, S. H. (1972). Correlation of chromosome damage and colony-forming ability in Syrian Hamster cells in culture irradiated in G_1. *Curr. Top. Radiat. Res. Q.* 7, 303–309.

Harington, J. S., Miller, K., and Macnab, G. (1971). Hemolysis by asbestos. *Environ. Res.* 4, 95–98.

Haugen, A., and Harris, C. (1982). Asbestos carcinogenesis: Asbestos interactions and epithelial lesions in cultured human trecheobronchial tissues and cells. *Recent Results Cancer Res.* 82, 32–41.

Hervàs, J. P., Lòpez-Sàez, J. F., and Giménez-Martìn, G. (1985). Proliferation of multinucleate cells with unbalanced nuclei. *Cell Biol. Int. Rep.* 9, 91–101.

Hesterberg, T. W., and Barret, J. C. (1985). Induction by asbestos fibers of anaphase abnormalities: Mechanism for aneuploidy induction and possibly carcinogenesis. *Carcinogenesis* 6, 473–475.

Hext, P. M., and Richards, R. J. (1976). Biochemical effects of asbestiform minerals on lung fibroblast cultures. *Brit. J. Exp. Pathol.* 57, 281–286.

Hext, P. M., Hunt, J., Dogdson, K. S., and Richards, R. J. (1977). The effects of long-term exposure of lung fibroblast strains to chrysotile asbestos. *Brit. J. Exp. Pathol.* 58, 160–167.

Hunt, J., Pooley, F. D., and Richards, R. J. (1981). Biological reactivity of calcium silicate composites: *In vitro* studies. *Environ. Res.* 26, 51–55.

Jaurand, M. C., Kheuang, L., Magne, L., and Bignon, J. (1986). Chromosomal changes induced by chrysotile fibres or benzo-3,4-pyrene in rat pleural mesothelial cells. *Mutat. Res.* 169, 141–148.

Joseph, L. B., Stephens, R. E., Ottolenghi, A. C., Lipetz, P. D., and Newman, H. A. I. (1983). Morphological transformation *in vitro* of normal human fibroblasts by chrysotile. *Environ. Health Perspect.* 51, 17–22.

Kaw, J. L., Tilkes, F., and Beck, E. G. (1982). Reaction of cells cultured *in vitro* to different asbestos dusts of equal surface area but different fibre length. *Brit. J. Exp. Pathol.* 63, 109–115.

Kenne, K., Ljungquist, S., and Ringertz, N. R. (1986). Effects of asbestos fibers on cell division, cell survival, and formation of thioguanine-resistant mutants in Chinese Hamster Ovary cells. *Environ. Res.* 39, 448–464.

Mossman, B. T., Adler, K. B., Jean, L. J., and Craighead, J. E. (1982). Mechanisms of hypersecretion in rodent tracheal explants after exposure to chrysotile asbestos. *Chest* 815, 235–255.

Mossman, B. T., Jean, L. J., and Landesman, J. M. (1983a). Studies using lectins to determine mineral interactions with cellular membranes. *Environ. Health Perspect.* 51, 23–25.

Mossman, B. T., Eastman, A., Landesman, J. M., and Bresnick, E. (1983b). Effects of crocidolite and chrysotile asbestos on cellular uptake, metabolism, and DNA after exposure of Hamster tracheal epithelial cells to benzo(*a*)pyrene. *Environ. Health Perspect.* 51, 331–336.

Neftel, K. A., Stahel, R., Müller, O. M., Morell, A., and Arrenbrecht, S. (1983). Radial segmentation of nuclei (Reider cells): A morphological marker of T cell neoplasms? *Acta Haematol.* 70, 213–223.

Neuberger, M., Kundi, M., and Friedl, H. P. (1984). Environmental asbestos exposure and cancer mortality. *Arch. Environ. Health* 39, 261–265.

Neugut, A. I., Eisenberg, D., Silverstein, M., Pulkrabek, P., and Weinstein, J. B. (1978). Effects of asbestos on epithelioid cell lines. *Environ. Res.* 17, 256–265.

Rahman, O., Beg, M. W., Viswanathan, P. N., and Zaidi, S. H. (1975). Biochemical changes in the lungs of rats caused by asbestos dust. *Scand. J. Work Environ. Health* 1, 50–53.

Reiss, B., Solomon, S., Weisburger, J. H., and Williams, G. M. (1980). Comparative toxicities of different forms of asbestos in a cell culture assay. *Environ. Res.* 22, 109–129.

Richards, R. J., Hext, P. M., Desai, R., Tetley, T., Hunt, J., Presley, R., and Dogdson, K. S. (1977). Chrysotile asbestos: Biological reaction potential. *In* "Inhaled Particulates" (J. Walton and M. C. McGovern, Eds.), pp. 477–493. Pergamon, New York.

Richards, R. J., and Morris, T. C. (1979). Collagen and mucopolysaccharide production in growing lung fibroblasts exposed to chrysotile asbestos. *Life Sci.* 12, 441–451.

Rüttner, J. R., Lang, A. B., Gut, D. R., and Wydler, M. U. (1987). Morphological aspects of interaction between asbestos fibers and human mesothelial cell cytoskeleton. *Exp. Cell Biol.* 55, 285–294.

Schliwa, M., and Van Blerkom, J. (1981). Structural interaction of cytoskeletal components. *J. Cell Biol.* 90, 222–235.

Sincock, A. M., and Seabright, M. (1975). Induction of chromosome changes in Chinese Hamster cells by exposure to asbestos fibers. *Nature (London)* 257, 56–58.

Tilkes, F., and Beck, E. G. (1983). Influence of well-defined mineral fibres on proliferating cells. *Environ. Health Perspect.* 51, 275–279.

Vartio, T., Virtanen, I., and Lehto, V. P. (1986). Aggregation of fibronectin by asbestos fibers in the pericellular matrix of cultured human fibroblasts. *Acta Pathol. Microbiol. Immunol. Scand. Sect. A* 94, 169–175.

Wang, M. D., Jaurand, M. C., Magne, L., Kheuang, L., Pinchon, M. C., and Bignon, M. D. (1987). The interactions between asbestos fibers and metaphase chromosomes of rat pleural mesothelial cells in culture. A scanning and transmission electron microscopic study. *Amer J Pathol.* 126, 343–349.