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# Morphological Transformation *in Vitro* of Normal Human Fibroblasts by Chrysotile

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**Pathologic response of tissue to asbestos *in vivo* gives rise to fibromatoma, granuloma and mesothelioma. We are attempting to develop a model system *in vitro* using human cells in order to investigate the possible mechanisms responsible for these pathologies.**

**Within the first 12 hr of exposure to chrysotile, the fibroblasts showed distinctive morphological changes. Cells appeared elongated with occasional vacuolated nuclei and granular cytoplasm. Cells showed no other obvious morphological changes by light microscopy and were serially passaged at confluence. The cells with vacuolated nuclei were successfully serially passaged. Binucleated cells were first observed 48 hr after passaging. As time in culture increased (3 days to 2 weeks) many cells lost their distinctive bipolar properties and developed "stress striations" and multiple vacuoles in the cytoplasm. Multinucleated giant cells (2-11 nuclei/cell) with lobate nucleoli became more numerous. With increasing passages, the confluent cell density decreased and cell size increased. Cells usually had condensed nucleoli and had lost all control of directional growth. Preliminary indications suggest that these *in vitro* morphological transformations are due—at least in part—to a lack of control over cytokinesis.**

## Introduction

Asbestos has been known for its flame retardant and insulating properties since ancient times. The lamps of the Vestal Virgins contained asbestos wicks. Legend states that Charlemagne would throw the tablecloth into the fireplace for cleaning to the amazement of his guests. As we headed into the industrial revolution our usage of asbestos became more sophisticated. In the mid-1800s, physicians noted an increased incidence of pulmonary distress in individuals working with asbestos. It has been determined that an excess of $1 \times 10^4$ individuals will die each year until the turn of the century due to past exposure (1940 to 1980) (*1*). The period from initial exposure to the onset of asbestosis is frequently in excess of 20 years.

Research has mainly been concerned with acute effects of asbestos exposure (*2-5*). Since we are interested in why there is a latency period of 20 years, we decided to look at the effect of chronic exposure to asbestos. We have an ongoing study on the morphological changes associated with chronic asbestos exposure due to a single inoculation *in vitro*.

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## Material and Methods

### Morphology

CIs (normal human dermal fibroblasts) at passage 16, 80% confluence, were exposed to NIEHS short chrysotile or NIEHS amosite in PBS (phosphate buffered saline) at a concentration of 63 $\mu g/cm^2$. Control cells were exposed to the same amount of PBS. Cells were observed hourly, by inverted phase microscopy, for the first 6 hr and then every 12 hr until confluence, which occurred approximately 2 days following exposure. Medium (EMEM supplemented with 10% FBS) was changed 24 hr after exposure due to a change in the pH. At confluence the cells were washed twice with PBS and split in the same manner as described in Stephens et al. (*2*). Cells were observed 6 hr after plating and then observed daily until confluence, at which time they were serially passaged. Every third day, cells were fed with supplemented EMEM plus 10% FBS. CIs were checked monthly for viral, bacterial, *Mycoplasma* sp. contamination by the Microbiology Laboratories, Department of Pathology, The Ohio State University, Columbus, OH.

## Transmission Electron Microscopy

Confluent flasks (control and experimental) of CI $P_{23}$ (seven passages P after exposure) were washed twice with PBS and trypsinized with 0.01% trypsin. Trypsin was inactivated with ten times the volume of cold PBS and centrifuged at 1500 rpm for 10 min. Supernatant was aspirated, and cold 2% gluteraldehyde was added for 15 min. Gluteraldehyde was decanted and the pellet was cut into pieces. Fresh gluteraldehyde was added for 2 hr, aspirated and 0.122 *M* phosphate buffer was added. At 15 hr

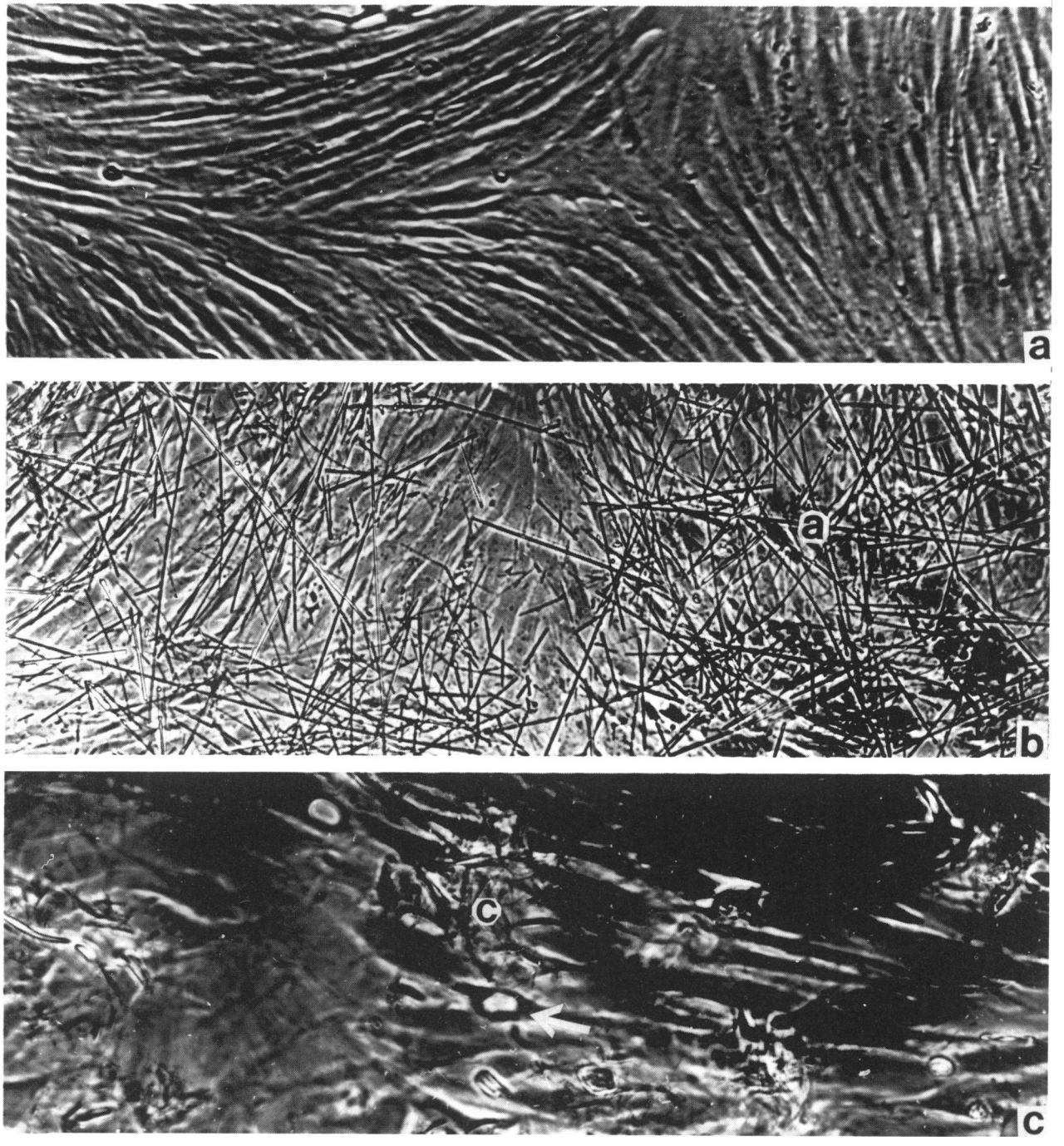


FIGURE 1. CI $P_{16}$, 12 hr post treatment: (*a*) control, nontreated, 100×; (*b*) amosite-treated, a = amosite, 100×; (*c*) chrysotile-treated, c = chrysotile, arrow indicates vacuolated nucleus, 200×.

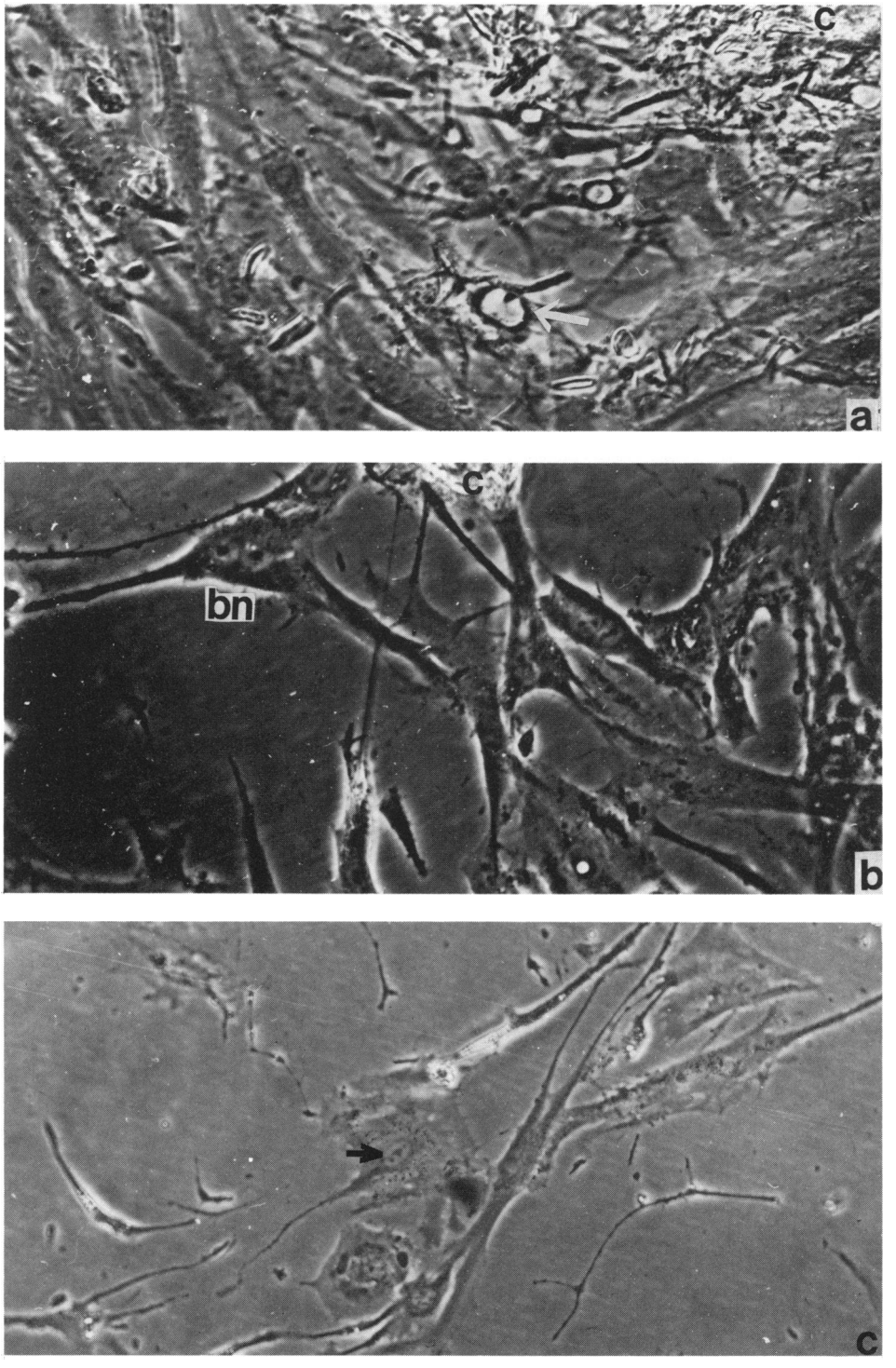


FIGURE 2. CI, various times post treatment: (*a*) CI $P_{16}$, 48 hr post treatment; note elongation of cells and development of intercellular spaces; arrow indicates vacuolated nucleus, c = chrysotile, 176 ×. (*b*) CI $P_{17}$, 48 hr post passage; note elongated cellular processes, binucleated cells (bn) and chrysotile (c), 188 ×. (*c*) CI $P_{23}$ approximately 2 months post treatment; arrow indicates nucleus; note multiple granular bodies surrounding nuclei, 94 ×.

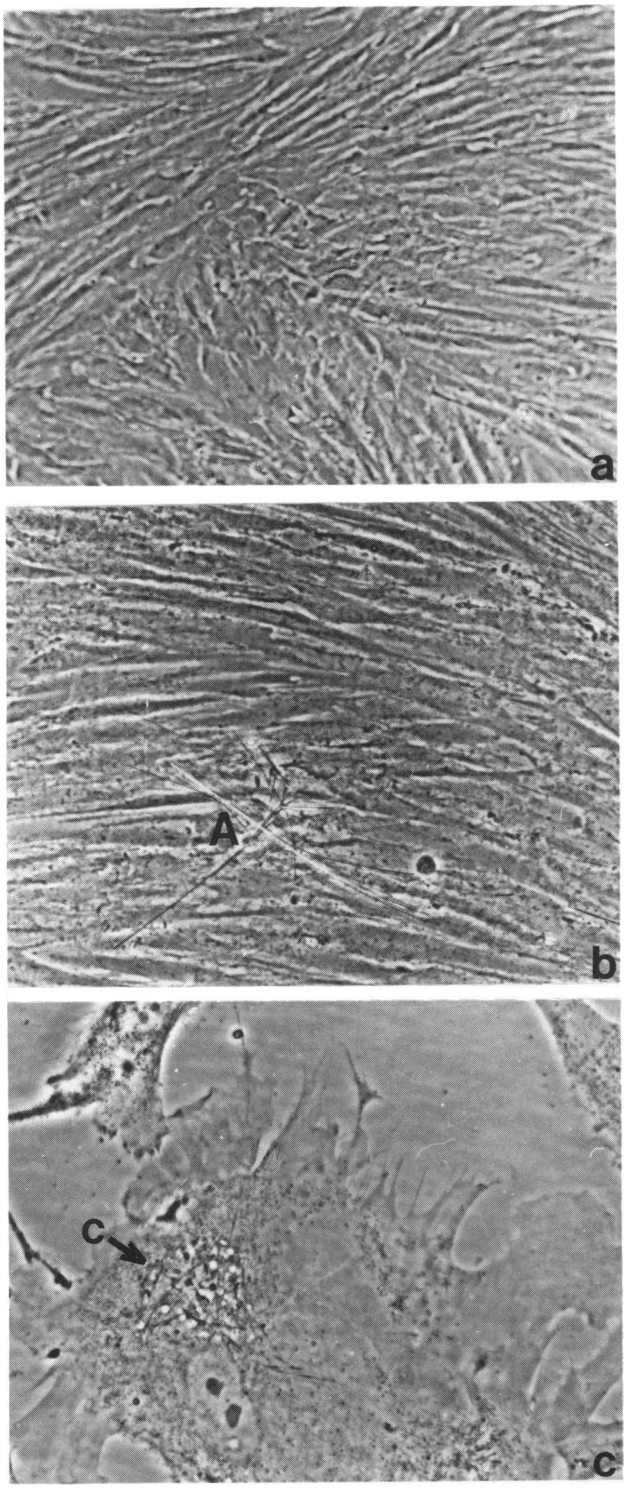


FIGURE 3. CI $P_{23}$, 2 months post treatment: (*a*) control, nontreated, 91 ×. (*b*) amosite-treated; note some granularity of cytoplasm and presence of amosite, 91 ×. (*c*) chrysotile-treated; arrow indicates chrysotile within the cytoplasm; note vacuolation and granulation of the cytoplasm plus condensation of nucleolar material, 182 ×.

later, cells were postfixed with 2% osmium tetraoxide for 1 hr. Cells were then dehydrated through EtOH and propylene oxide. Spurr (Ernest Fullam) plus propylene oxide (1:1) was added for 2 hr and cells were then put in 100% Spurr overnight at room temperature. Then cells were placed in 100% Spurr in molds for 24 hr at 65°C. Spurr plugs were removed from molds, mounted, cut and placed on grids. Thin sections were stained with uranyl acetate and lead citrate.

## Results

CI passage 16 is a normal human fibroblast which at confluence displays the typical swirl growth pattern (Fig. 1*a*). Morphological changes become evident (Figs. 1*b* and 1*c*) 12 hr after application of asbestos; NIESH amosite (Fig. 1*b*) applied to the cells did not cause the same morphological changes as the chrysotile. NIEHS chrysotile (Fig. 1*c*) caused definite elongation of the fibroblasts and what appears to be cleared nuclei.

At 48 hr after chrysotile exposure, fibroblasts became very elongated and pulled apart from each other (Fig. 2*a*). There was an increased number of cells exhibiting clearing of the nuclei and an increased granulation of the cytoplasm. At 72 hr after inoculation with asbestos, the cells were serially passaged (1:2). Two days later, binucleated cells began to appear in the chrysotile inoculated cultures (Fig. 2*b*). Also evident were condensation of the nucleoli, vacuolation of the cytoplasm and "stress striations" in the cytoplasm. Chrysotile was still evident in the cultures. The cell density at confluence of CI $P_{17}$ was as follows: control $1.41 \times 10^5$ cells/cm$^2$; amosite-treated $1.26 \times 10^5$ cells/cm$^2$; and chrysotile-treated $3.2 \times 10^4$ cells/cm$^2$. By passage 23 the cell density at confluence of the chrysotile-treated cells was $5.3 \times 10^3$ cells/cm$^2$ compared to the control density of $5.5 \times 10^4$ cells/cm$^2$ and the amosite-treated cells were $4.2 \times 10^4$ cells/cm$^2$. The chrysotile-treated cells at passage 23 (CI $P_{23}$) had lost their typical fibroblast morphology (Fig. 2*c*), and there was an increased granularity of the cytoplasm and no binucleated cells were evident.

Approximately 2 months after inoculation, the control ($P_{23}$) cells exhibited no change in growth morphology and minimal granulation of the cytoplasm (Fig. 3*a*). $P_{23}$ of the amosite-inoculated fibroblasts showed increased granularity of the cytoplasm but no change in the growth morphology or clumping of the nucleoli (Fig. 3*b*). Cells exposed to chrysotile (Fig. 3*c*) lacked typical fibroblast morphology and had vacuolation and granulation of the cytoplasm. Chrysotile can also be seen within the cell.

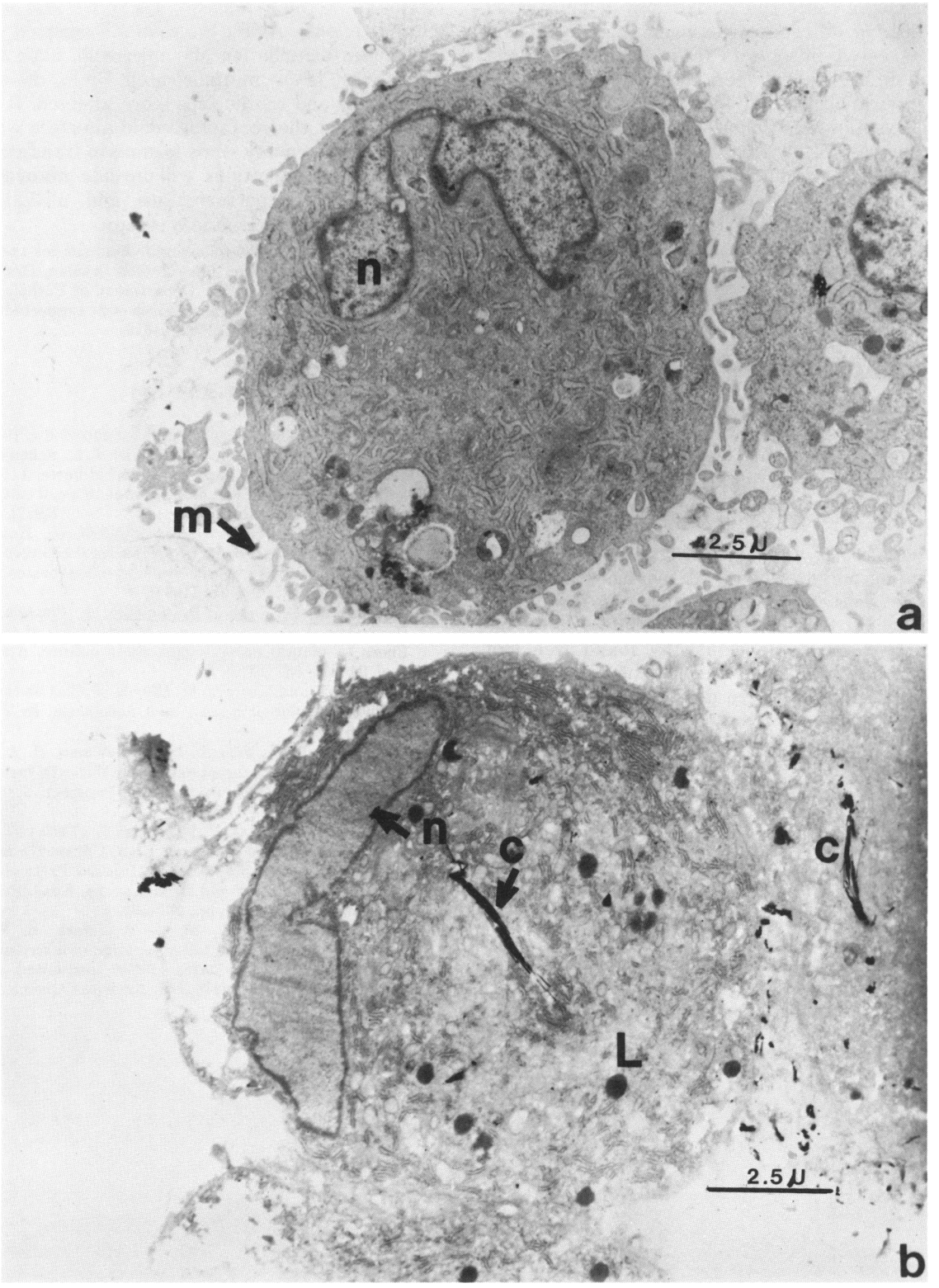


FIGURE 4. CI $P_{23}$ transmission electron microscopy (4032 × ). (*a*) control (nontreated cells), with normal chromatin pattern in the nucleus (n) and microvilli (m) at cell periphery; (*b*) chrysotile-treated cell, with immature nucleus (n), lysosomal bodies (L) and chrysotile (c) in the cytoplasm.

Transmission electron microscopy demonstrated no detectable differences between the control and amosite-treated fibroblasts (Fig. 4*a*); there was a marked difference between these cells and those treated with chrysotile. In the chrysotile-treated cells, chrysotile was found in the cytoplasm. No chrysotile was seen within the nucleus (Fig. 4*b*). The cell margin of the control cells showed distinct microvilli as compared to chrysotile-treated cells. Other perturbations seen in the chrysotile-treated fibroblasts (Fig. 4*b*) include stacking of the endoplasmic reticulum, immature nucleus which shows clearing of the chromatin and an increased amount of lysosomes.

## Discussion

Chronic effects of asbestos can be induced by a one-time exposure. Chrysotile is not easily removed from *in vitro* by normal cell handling techniques (i.e., feeding and passaging). Chrysotile may become adsorbed to the cell membrane (*6*) or ingested into the cell.

Ultrastructural work by Richards and others (*3*, *7*), including our own observations, has shown that chrysotile does not penetrate the nuclear membrane (Fig. 4*b*). Richards (*7*) stated (and we have confirmed) that chrysotile is usually found free (not membrane-bound) within the cytoplasm. At high resolution, however, there does seem to be a coating on the chrysotile; this is also consistent with Richards' observations. Work previously reported from our laboratory possibly suggests that the coating on the chrysotile could be a protein or protein-based molecule (*8*).

Multinucleated cells have been seen one passage post treatment ($P_{17}$) but, by $P_{20}$, the multinucleated cells were not evident. We agree with Richards (*3*) that this could suggest an abnormality in cell division.

If the primary destructive mode of chrysotile is physical penetration, then the nucleus is not the primary site of attack. The primary site of attack seems to be the cytoplasm, where we saw the first chronic effects. At $P_{18}$ the cells still showed normal chromatin distribution and microvilli, even though many were bi- or multinucleated. By $P_{23}$ the cellular membrane and the nucleus were affected. It seems possible that the chrysotile could interfere with one or more of the many steps leading to translation.

Future investigations will include measurement of translational perturbations and utilization of freeze fracture/electron microscopy.

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REFERENCES

1. Selikoff, I. J. *ABC News Night Line*, interview, 1981.
2. Stephens, R. E., Joseph, L. B., Daniel, F. B., Schenk, K. M., Newman, H. A. I., Lipetz, P. D., and Millette, J. R. Interactions of chrysotile and benzopyrene in a cell culture system. Environ. Health Perspect. 51: - (1982).
3. Richards, R. J., Hext, P. M., Blundell, G., Henderson, W. J., and Volcani, B. E. Ultrastructural changes in lung fibroblast cultures exposed to chrysotile asbestos. Brit. J. Exptl. Pathol. 55: 275-281 (1974).
4. Jaurand, M. C., Kaplan, H., Thiollet, J., Pinchon, M. C., Bernaudin, J. F., and Bignon, J. Phagocytosis of chrysotile fibers by pleural mesothelial cells in culture. Am. J. Pathol. 94: 529-532 (1979).
5. Absher, M., and Mortara, M. Effects of silica on the proliferative behavior of human lung fibroblasts. In Vitro 16: 371-376 (1980).
6. Ottolenghi, A. C., Joseph, L. B., Newman, H. A. I., and Stephens, R. E. Interactions of erythrocyte membranes with particulates. Environ. Health Perspect. 51: 253-256 (1982).
7. Richards, R. J., Hext, P. M., Desai, R., Tetley, T., Hunt, J., Presley, R., and Dodgson, K. S. Chrysotile asbestos: biological reaction potential. In: Inhaled Particulates, IV Pt. 2. (W. H. Walton and B. McGovern, Eds.), Pergamon Press, New York, 1977, pp. 477-493.
8. Joseph, L. B., Chang, M. J. W., Hart, R. W., and Stephens, R. E. Differential absorption of macromolecules to mineral fibers *in vitro*. Paper presented at 16th Midwest Meeting, Lincoln, NB, American Chemical Society, 1980.