The Interactions Between Asbestos Fibers and Metaphase Chromosomes of Rat Pleural Mesothelial Cells in Culture

A Scanning and Transmission Electron Microscopic Study

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Rat pleural mesothelial cells (PMCs) in culture at the exponential growing phase were exposed to 5 μg/ml of chrysotile (CH) or crocidolite (CR) asbestos fibers: the cells and their chromosomes were studied 48 hours thereafter by light, scanning, and transmission electron microscopy (LM, SEM, TEM). PMCs phagocytized both CH and CR. Mild vacuolar cytoplasmic changes by LM and a few small surface blebbings by SEM were present, mainly in cells treated with CH. Metaphase chromosomes were well separated and retained surface details by SEM in the control group. Chromosomes were frequently entangled with, adherent to, and severed or pierced by long and thin curvilinear CH with occasional chromatin fibers threading over the partly severed asbestos. Similar chromosomal changes were much less frequently found in CR-treated cells; TEM confirmed the same findings. CH and CR have different physicochemical properties and also appear to have direct, intricate, but different interactions with chromosomes, as well as the cytoplasm, of PMCs. (Am J Pathol 1987, 126:343-349)

EPIDEMIOLOGIC and experimental evidence indicate that asbestos fibers induce mesotheliomas.[1,2] The detailed mechanisms of their carcinogenesis are not completely clear: both physical and chemical properties of the fibers have been implicated.[3,4] The two major classes of asbestos, the serpentines and the amphiboles, have different physicochemical properties and also appear to cause different incidences of mesotheliomas in humans[1,5,6]; their tumorigenic effects in experimental animals are, however, similar.[2,3]

Rat pleural mesothelial cells (PMCs) in culture phagocytize both chrysotile (serpentine, CH) and crocidolite (amphibole, CR) asbestos fibers.[7] Vacuolar changes of the cytoplasm, prolonged population doubling times, and polyploidy, however, have been found mainly in CH and less commonly in CR-treated cells, especially at a low dosage (5 μg/ml culture medium).[7] By light microscopy (LM), CH also induces chromosomal aberrations, including fragmentation and breaks in PMCs.[8] CR also induces chromosomal changes in PMCs and other types of cells in culture but with a lower incidence than CH.[9,10] These findings indicate that these two types of asbestos cause different degrees or patterns of cytoplasmic and nuclear changes in cultured cells and also suggest the possibility of a direct interaction between asbestos fibers and chromosomes.[8] The present study investigated the metaphase chromosomes of PMCs in culture by light, scanning, and transmission electron microscopy (LM, SEM, TEM) for their possible interactions with CH and CR.

Materials and Methods

Rat pleural mesothelial cells (PMCs) were cultured in Falcon flasks with or without glass coverslips as detailed previously.[7] Briefly, PMCs were obtained and cultured in Ham's F-10 medium (Biopro, France) supplemented with 10% fetal bovine serum, 10 mM

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Hepes, 100 U/ml penicillin, and 50 μg/ml streptomycin.

The culture medium of PMCs, between 12 and 20 passages and 24 hours after plating, was replaced by a complete culture medium containing 5 μg/ml of UICC chrysotile A or crocidolite. The asbestos fibers were dispersed by sonication in the complete culture medium for 5 minutes (50 kHz, 20 W) immediately before the medium change. In the control group the complete culture medium, without fibers, was replaced at the same time.

After 48 hours of incubation, some of PMCs grown on coverslips were fixed with 3% glutaraldehyde in 0.1 M Na-cacodylate buffered solution for LM, SEM, and TEM. The coverslips were examined directly by an inverted phase-contrast microscope or stained routinely with the Giemsa stain for LM. For SEM, the cells were postfixed in 1% osmium tetroxide, dehydrated in graded acetone, critical-point-dried with CO_2, and coated with a layer of gold.[11] For TEM, the cells were processed as above and then detached from the coverslip in Epon.[7] Sixty to 100-nm-thick sections were stained with lead citrate and uranium acetate.

To study chromosomes in metaphase, mitosis was arrested by adding 0.4 μg/ml of Colcemid to the culture medium for 2–3 hours. PMCs in mitosis on the Falcon flask were detached with 0.25% trypsin for 1 minute, incubated in 75 mMKCl solution at 37 C for 15 minutes, fixed with two changes of 3:1 methanol/acetic acid, spread on glass slides or coverslips, and air-dried (detached preparation.[8] PMCs in mitosis grown on coverslips were prepared similarly, except that the cells remained *in situ* throughout the processing (*in situ* preparation). A portion of PMCs in metaphase was also detached and fixed in 3% glutaraldehyde as above for routine TEM.

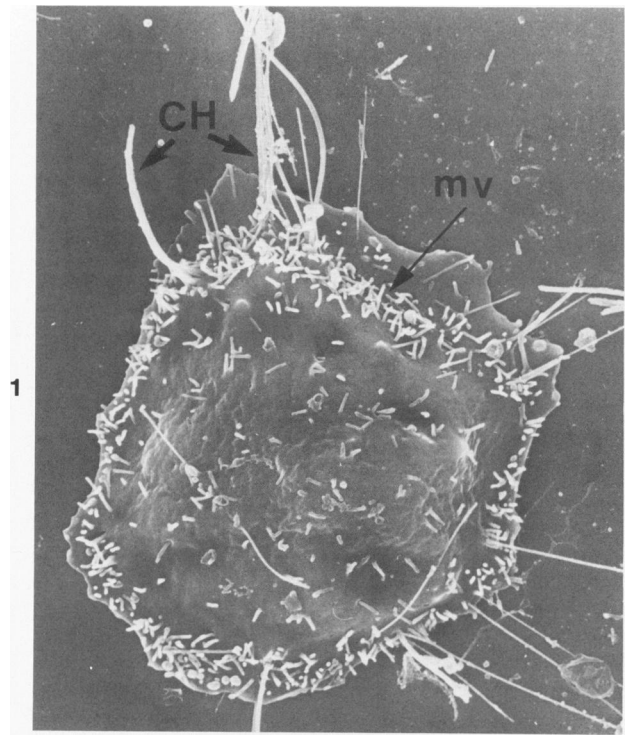
The coverslips with air-dried metaphase chromosomes were processed further, 2–7 days later, by washing with 1% Triton X-100 for 1 minute, incubated with 0.025% trypsin for 30 seconds at room temperature, washed with three changes of phosphate-buffered normal saline solution, refixed with the glutaraldehyde solution, and processed for LM, SEM, and TEM as above.

Results

By LM all preparations showed near confluent cell growth with many cells in mitotic process. As detailed in the previous study, there was no evidence of cytolysis, and asbestos fibers were readily found in the cytoplasm of most cells in both experimental groups.[7] Mild to moderate vacuolar changes of the cytoplasm were found in some CH-treated but seldom in CR-treated or control cells.

By SEM surface microvilli and pinocytic vesicles were moderate in number and unevenly distributed but appeared similar in all three groups. A few small surface blebbings of 0.5–1 μ in size were, however, noted in some CH-treated but rarely in CR-treated or control cells. The CH fibers were mostly in groups, curvilinear, with fragmented or frayed ends, and partly enveloped by or inserted into the PMCs (Figures 1 and 2). The CR fibers, on the other hand, were mostly isolated and straight, showed less fragmentation, and were more likely to lie adjacent to or glued on, instead of inserted into, the PMCs (Figures 3 and 4). Both types of fibers were frequently long and thin and quite varied in size, ranging from more than 10 μ to less than 1 μ in length and more than 1 μ to less than 0.1 μ in diameter. The variations in the size were more striking in CH than in CR. By TEM most intracellular fibers were membrane-bound, and more in number than suspected by SEM. Focal vacuolar changes and surface blebbings of the cytoplasm were mainly seen in the CH-treated cells. Intranuclear fibers were short and thin and were found only very rarely in CH-treated cells.

Metaphase chromosomes, prepared by the detached and *in situ* methods for SEM, appeared similar within the same groups and will be described together. The chromosomes in the control groups were mostly well separated, rod or oval in shape, with the detailed surface appearance of chromatin (Figures 5 and 6). Fibers connecting chromatids and adjacent chromosomes were frequently seen. Variations in the separation and surface details of chromosomes were, however, present between slides and within the same slide. Polyploidy was noted in all, but more in the CH-treated groups. CH fibers were found in 21 of 23 groups of metaphase chromosomes: CH fibers appeared to sever, puncture, or adhere to and mingle with chromosomes, causing deformities (Figures 7 and 8). Occasionally chromatin fibers appeared to thread over the asbestos fiber in the partly severed chromosome (Figure 7). Usually chromosomal alterations were multiple within the same cell: chromosomes that appeared to be uninvolved at low magnification might have small, thin, and long fibers inserted in them (Figures 9 and 10). Contrary to CH, most CR fibers were located adjacent to or between metaphase chromosomes; obvious fiber-chromosome interactions, similar to those observed in the CH group, were found only in 2 of 21 groups of metaphase chromosomes (Figure 11). By TEM asbestos fibers were present mostly adjacent to the chromosomes but occasionally were also found within a chromosome (Fig-



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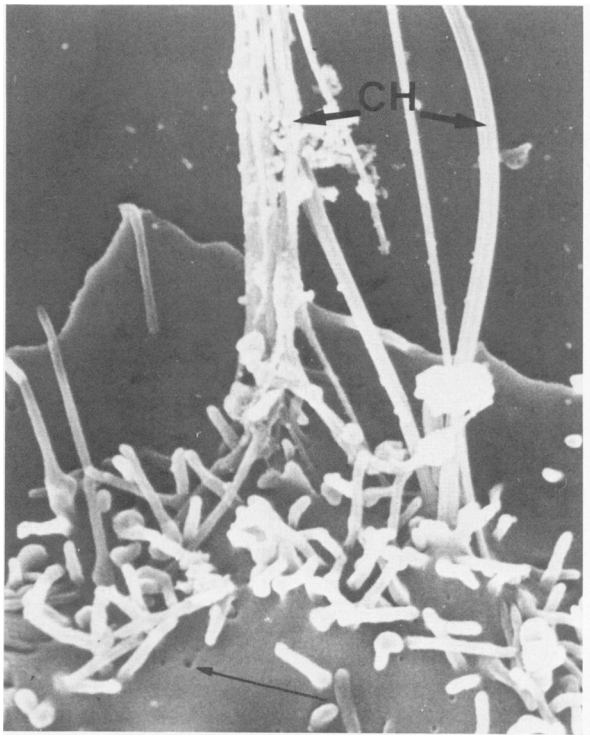

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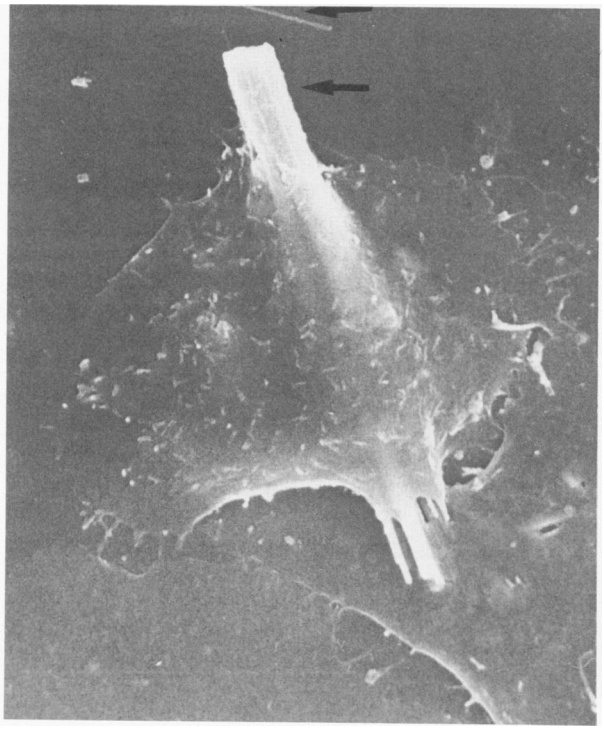

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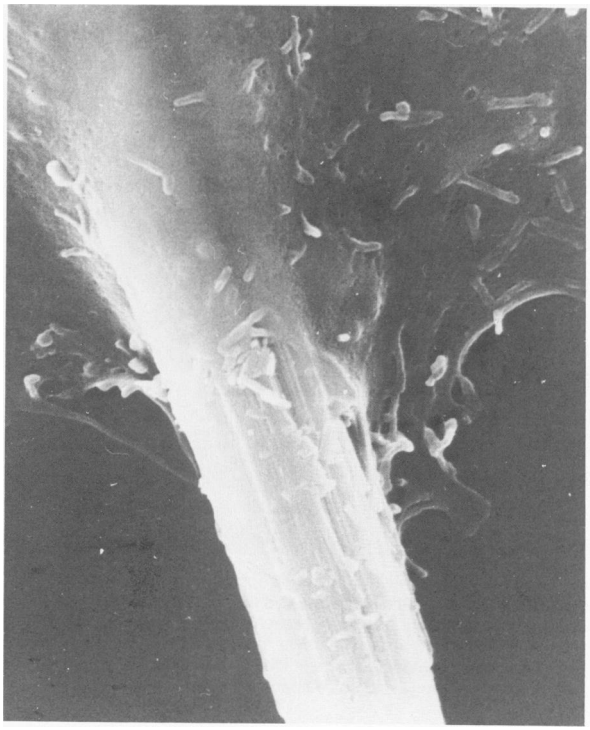

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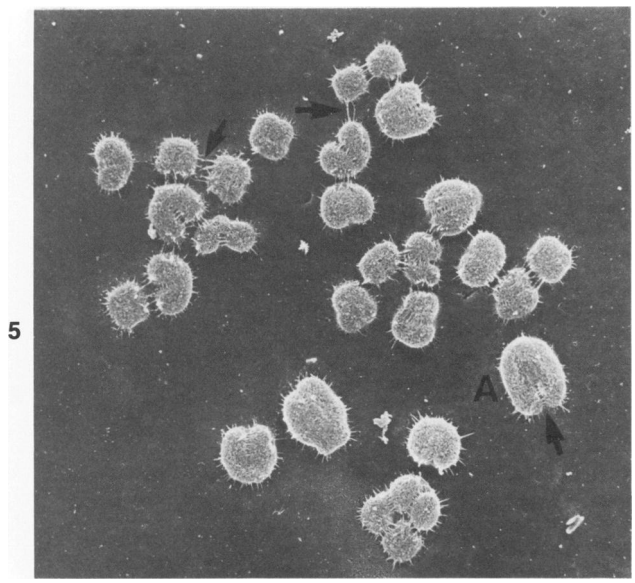
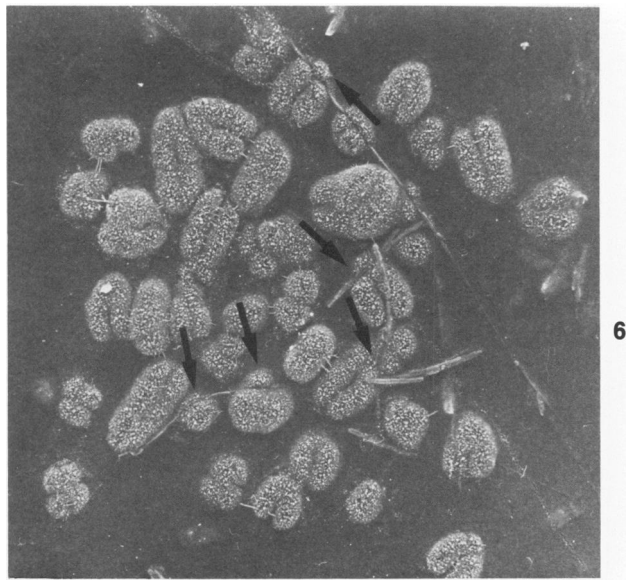
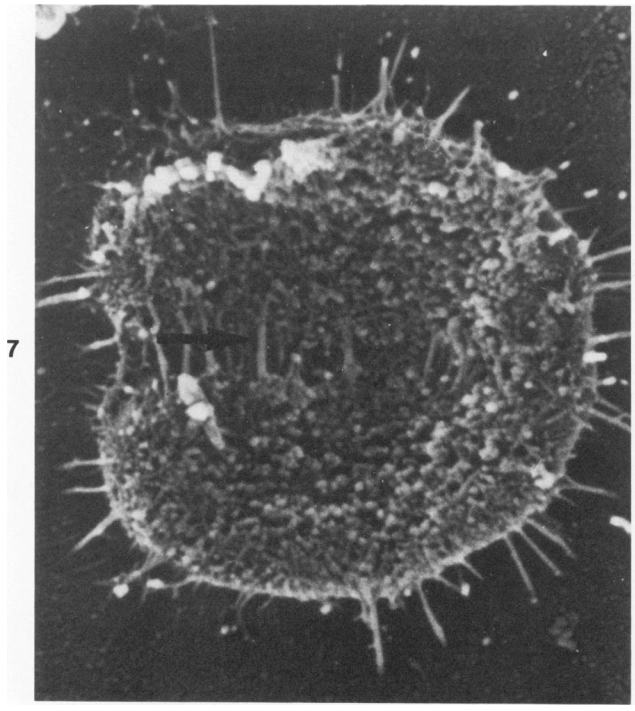
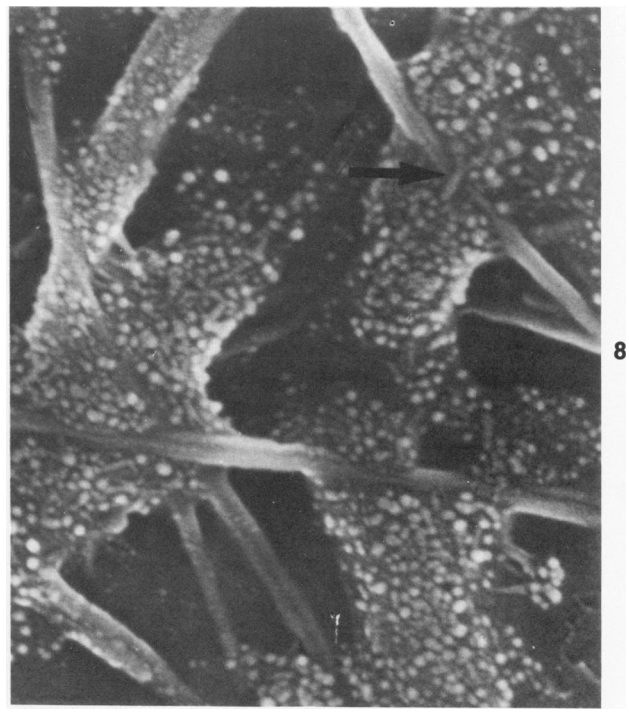
Figures 1 and 2—Curvilinear chrysotile fibers (CH) are frequently inserted into the mesothelial cells. Chrysotile fibers appear to fray easily *(arrow)*. Microvilli *(mv)* and pinocytic vesicles *(small arrow)* are more commonly found in the peripheral portion of the cytoplasm. (**Figure 1**, SEM, ×6340; **Figure 2**, SEM ×24,400) **Figures 3 and 4**—Crocidolite fibers are mostly straight and isolated individually *(arrows)*. They usually do not fray and more frequently appear adherent to, rather than inserted into or phagocytized by, the mesothelial cell in culture. (**Figure 3**, SEM, ×5500; **Figure 4**, SEM, ×19,000)







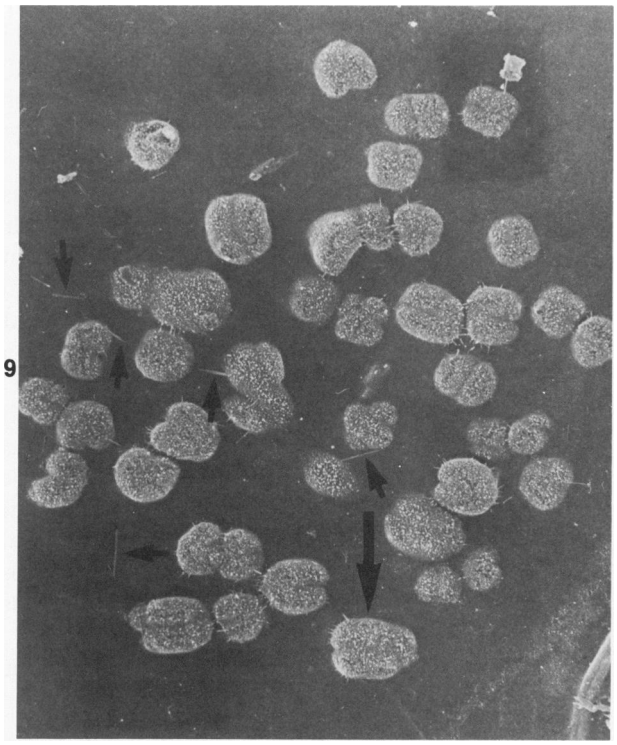


Figures 5 and 6—By SEM chromosomes appear ovoid or rod-shaped, usually well separated from each other, in the control rat mesothelial cells. Surface details of chromosomes are discernible, as shown in **Figure 6** from a chromosome (*A*) in **Figure 5**. Fine chromosome fibers (*arrows*) connecting chromatids or adjacent chromosomes are frequently present. (*In situ* preparation, SEM, **Figure 5**, ×4880; **Figure 6**, ×39,000) **Figure 7**—Long and thin chrysotile fibers intermingle with and also appear to sever, puncture, or adhere to chromosomes in multiple places (*arrows*). (*In situ* preparation, SEM, ×6100) **Figure 8**—Deformities of chromosomes, in addition to other alterations, shown in **Figure 7**, are prominent in areas with many chrysotile fibers. A repairlike change (*arrow*) can be seen over a chrysotile fiber that partly severs a chromosome. (SEM, detached preparation, ×48,800)

ure 12), especially in the CH-treated group, both in the detached and *in situ* preparations.

Discussion

Chromosomal aberrations can be induced by a variety of agents, and apparently also by a variety of mechanisms.[12] Our SEM findings suggest that asbestos fibers can induced chromosomal changes by direct physical or mechanical damage. The severing or puncturing of chromosomes could occur between prometaphase and metaphase when the nuclear membrane breaks down and the condensed chromosomes go through a stage of violent rotating and oscil-



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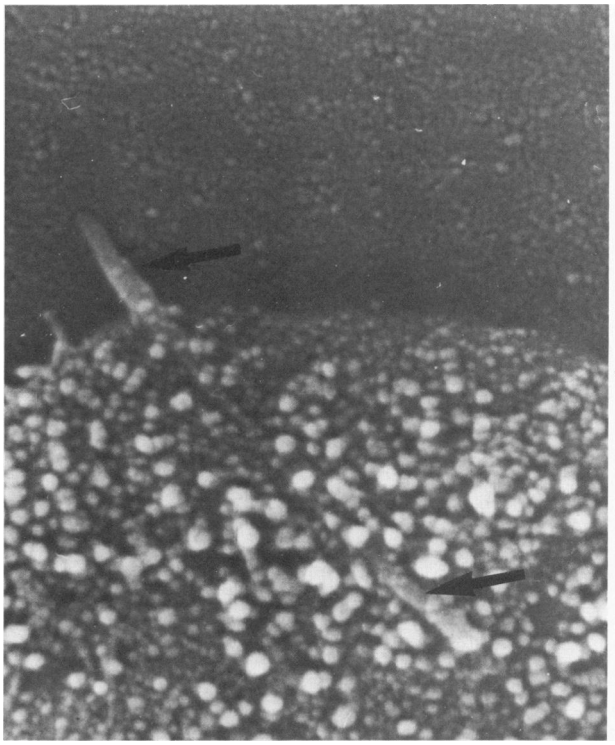

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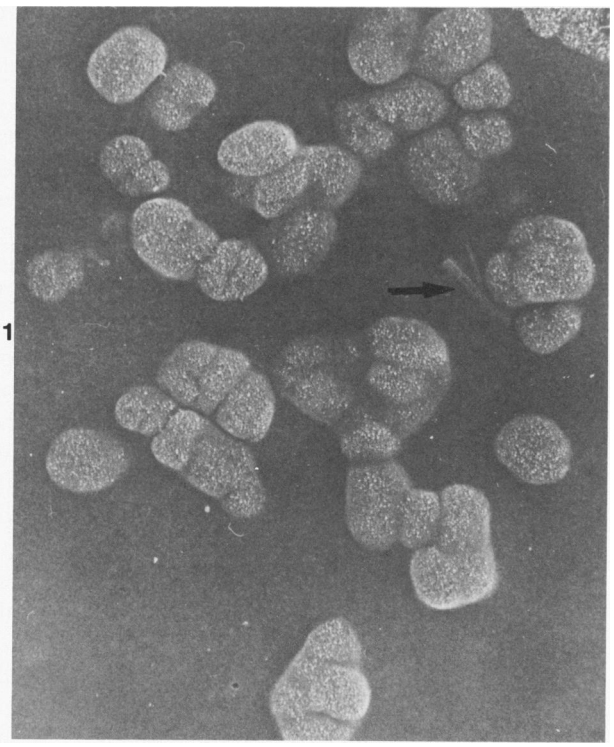

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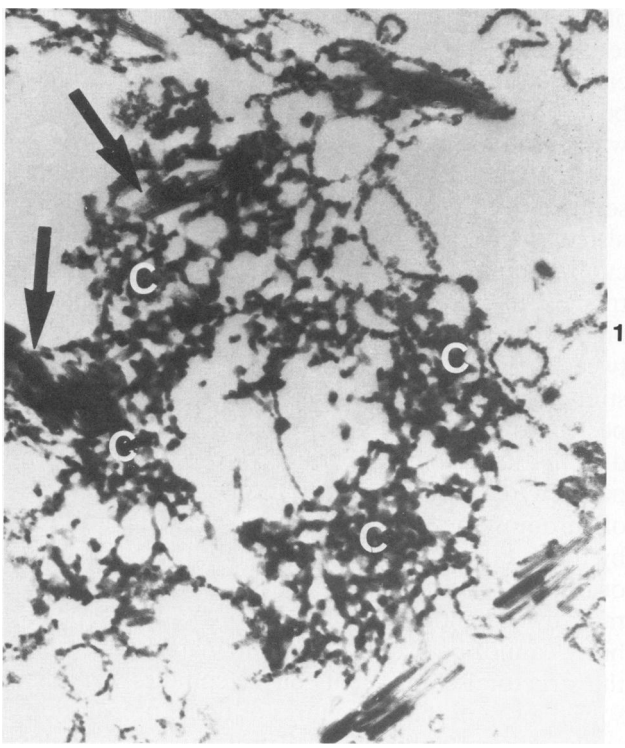

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Figures 9 and 10—Fine chrysotile fiber (*arrows*) appear mainly scattered between chromosomes at a low magnification. A higher magnification of a chromosome in **Figure 9** (*large arrow*), however, shows a thin fiber that is inserted into the chromosome (**Figure 10,** *arrows*). The surface details of chromosomes are discernible. (SEM, *in situ* preparation, **Figure 9**, ×5850; **Figure 10**, ×73,200) **Figure 11**—Although the shapes of chromosomes are somewhat altered, crocidolite fibers are mostly found adjacent to or between chromosomes. Direct interactions between the two, as seen in chrysotile (Figures 7 and 8), are rare. (SEM, *in situ* preparation, ×7800) **Figure 12**—Many small bundles of chrysotile fibers are seen around, in direct contact with (*arrows*), or within, the chromosomes. The chromosomes (*c*) are loosened up into networks after the trypsin treatment. (TEM, ×33,000)

lating movements, to and fro between the two poles, before aligning themselves at the equator.[13,14] The frequently long and thin curvilinear CH fibers with fragmented, serrated, or frayed surfaces and ends would appear to become entangled easily with and cause more damage to chromosomes than the usually single, unfragmented, and straight CR fibers. The small inserted CH fiber in apparently normal chromosomes (Figure 10) supported this assumption.

The violent movements of chromosomes between prometaphase and metaphase and physical properties of the asbestos fibers, however, were probably not the only factors in their interactions. Despite the fact that CR does not fray or disintegrate as readily as CH does, as many long and thin CR fibers were found among or abutted to, but not interacting with, chromosomes as CH fibers.

The surface properties of CH and CR are quite different. The surface of CH is rich in magnesium ions, positively charged, and high in electron transfer potential and catalytic activities to generate hydroxyl and superoxide radicals from H_2O_2, and shows high affinity to polar proteins, including chromosomes.[4,15-17] The negatively charged surface of CR has much less of these activities and also causes less cytotoxicity and hemolysis than CH. The different surface properties of CH and CR, therefore, may be responsible for the difference in their interactions with chromosomes.

Although asbestos fibers damage cells and chromosomes by direct contact, other mechanisms of toxicity also exist. Toxic cytoplasmic changes, including vacuolization and surface blebbing, as seen in our CH-treated cells, have been seen in the mesothelial cells of amosite-exposed guinea pigs.[18] Direct contact between mesothelial cells and fibers was not seen in that study. Cytoplasmic vacuolization, is, therefore, suspected to be due to chemically mediated stimuli either directly from the fibers or secondarily from parenchyma–asbestos interactions.[18] Abnormalities of chromosomes, in addition, could also be induced by the interference of microtubules, microfilaments, or other structural components of the spindle apparatus by CH and CR at cell division.[9] This last possibility cannot be evaluated in our materials because the structural components of the spindles were dissolved away in the procedures of tissue preparation. Both physical and chemical properties of asbestos fibers, therefore, might directly or indirectly affect their affinity and interactions with chromosomes, as they do with the cell membrane.

All our specimens went through the step of air-drying, which was necessary for the adherence, but more importantly, for the spreading of chromosomes on the coverslip.[19] Air-drying is carefully avoided in any fine structural studies of biologic material because it obliterates structural details. This rule apparently does not apply in chromosomal studies,[19] probably indicating the resilient properties of DNA after denaturation.

The interaction between chromosomes and asbestos fibers might occur during the processing of the tissue, including air-drying. This was, however, unlikely because of the same and consistent results obtained by the detached and in situ processing methods in our and other studies.[10] Threading of the chromatin fiber over the CH fiber (Figure 8) also argued for a prefixation event of probable chromosomal repair. Although the obvious interactions between asbestos fibers and chromosomes were readily discernible, precise interpretations of other chromosomal details were difficult, in part because of the inhomogeneity of the preparations. The application of SEM in diagnostic cytogenetic studies, however, appears feasible, with some improvements in the methodology.

The role asbestos may play in human carcinogenesis is not completely clarified. Neither CH or CR induces any detectable activities in common gene mutation assays at doses that are cytotoxic and cell-transforming.[20] Asbestos is, therefore, proposed to act primarily as a promoter or cocarcinogen.[21,22] Cancer may also occur secondarily to the induction of inflammation or fibrosis by asbestos.[23] Asbestos, however, induces mesothelioma *in vivo*[2,3] and chromosomal aberrations and cell transformation, in *in vitro* systems, with or without other carcinogenic contaminants.[8,20,24,25] Reduction of the fiber length of asbestos, however, reduces the incidence of the cell transformation,[24] as well as the induction of mesothelioma.[2] These observations suggest that asbestos could still be a complete carcinogen and that the length, or the length and diameter (aspect) ratio, of the fibers could play an important role in carcinogenesis.[3,4,20,24] Asbestos-mediated chromosomal changes have been suggested to play a role in asbestos carcinogenesis.[9,10,20] Our findings of the direct and intricate interactions between the long and thin fibers and chromosomes could explain and support further this concept.

Both CH and CR induced direct chromosomal alterations, but with a striking difference in the incidence. The significance of this difference in the incidence is unclear. The cytotoxicity of CH and that of CR are different, and the difference might be reflected in the incidence of chromosomal alterations. A higher dose of CR, therefore, might be necessary for the same incidence of chromosomal alterations induced by a lower dose of CH. The dosage of CH and CR used in

this study was, however, chosen with the consideration of maximal exposure with minimal cytotoxicity for this cell model[7]: the dosage was high for both CH and CR, compared with conceivable situations in human exposure.

The direct interaction of the asbestos fibers with chromosomes is, however, merely one of the many aspects of the inhalational carcinogenesis also governed by the physicochemical properties of the fibers. The same properties dictate the inhalability, deposition, migration, as well as durability of the fibers in the lung: all of them are sequential in the eventual development of cancer.

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