Mitotic Disturbances and Micronucleus Induction in Syrian Hamster Embryo Fibroblast Cells Caused by Asbestos Fibers

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Asbestos and other mineral fibers have long been known to induce lung cancer and mesothelioma. However, the primary mechanisms of fiber-induced carcinogenesis still remain unclear. We investigated the occurrence of mitotic disturbances induced by asbestos (amosite, crocidolite, chrysotile) in an *in vitro* approach using Syrian hamster embryo (SHE) fibroblast cells. The following endpoints were investigated: micronucleus formation as a result of mitotic disturbances and characterization of the induced micronucleus population by kinetochore staining and visualization of the spindle apparatus. Supravital UV–microscopy was used to analyze changes in interphase chromatin structure, impaired chromatid separation, and blocked cytokinesis. All three asbestos fiber types induced a high frequency of micronucleus formation in SHE cells (>200/2000 cells) in a dose–dependent manner (0.1–5.0 μg/cm^2), with a maximum between 48 hr and 66 hr exposure time. At higher concentrations (more than 5.0 μg/cm^2) the micronucleus formation decreased again as a result of increased toxicity. Kinetochore staining of micronuclei revealed that 48 ± 2% of asbestos–induced micronuclei reacted positively with CREST (antikinetochore) serum. Furthermore, spindle apparatus deformations occurred in cells with disturbed metaphases and anaphases, while the spindle fiber morphology appeared unchanged. Our results show that asbestos fibers may cause both loss and breakage of chromosomes in the absence of direct interaction with spindle fibers. Key words: asbestos fibers, kinetochore, micronuclei, mitotic disturbances, SHE cells, supravital UV–microscopy. *Environ Health Perspect* 103:268–271 (1995)

Based on sufficient evidence from epidemiological studies and animal experiments, asbestos is now regarded as an established carcinogen (*1,2*); however, despite numerous investigations, the mechanism of its carcinogenic action remains unclear (*3*). Asbestos fibers do not cause gene mutations, but they may act as tumor promoters (*4–6*).

Alternatively, because asbestos treatment alone induces tumors and fiber dimensions appear to be important in this process (*2,7*), asbestos may affect cells by more direct mechanisms. In this respect, aneuploidy is a common characteristic of asbestos–induced tumors, and it has been hypothesized that such a shift in chromosome complement plays a major role in the early stages of neoplastic development (*6,8*). This condition can also be caused by chemicals whose targets include components of the cytoskeleton and chromosome condensation or the spindle apparatus. Once inside an interphase cell after phagocytosis, asbestos is accumulated preferentially in the perinuclear region (*3,9*). As a result, asbestos fibers are frequently identified within the mitotic apparatus. These observations have led to the hypothesis that asbestos causes aneuploidy primarily by interfering with the normal course of mitosis (*3,10*).

Due to these disturbances during mitosis, micronucleus formation is observed (*11*). Micronuclei originate either from acentric chromosome fragments or from whole chromosomes or chromatids that are not incorporated into daughter nuclei when cell division is completed (*12*). The results of our previous investigations show that the Syrian hamster embryo (SHE) micronucleus assay is a short-term test of high predictive value (*13*). In combination with the immunofluorescent staining of kinetochores in micronuclei using antikinetochore (CREST) serum, the SHE assay allows the detection of clastogenic events, as well as those that affect the regular distribution of chromosomes in mitosis. Furthermore, supravital-UV microscopy (*11*) allows direct observation of movement of chromosomes and chromatids during mitosis.

In our experiments, we used three different types of asbestos fibers: amosite, crocidolite, and Rhodesian chrysotile (UICC standard). Average dimensions for chrysotile, crocidolite, and amosite, respectively, were 0.10 μm, 0.25 μm, and 0.24 μm in diameter; and 2.24 μm, 1.71 μm, and 2.50 μm in length. The percentage of fibers with length ≥5 μm was approximately 5% for all three types of fibers. Number of fibers, expressed as millions per microgram, were 11.2 (chrysotile), 1.4 (crocidolite), and 2.0 (amosite). These data basically agree with those reported by Coffin et al. (*14*).

SHE cell cultures were established as described by Pienta et al. (*15*). For detection of micronuclei (*16*) SHE cells were grown on coverslips in a humidified atmosphere (12% CO_2 in air at 37°C), fixed in cold methanol (-20°C), and stained with bisbenzimide (Hoechst 33258, 1 μg/ml). Only structures smaller than one-third of the nucleus by area were counted to avoid confusion with nuclei of dividing cells. In addition, only micronuclei clearly separated from the cell nucleus were taken into consideration (Fig. 1). For each dose of asbestos fibers, the number of micronuclei (MN) was determined per 2000 cells in at least 3 experiments. Ten to 15% of treated cells showed more than one MN/cell. More than three MN/cell occurred in 2–8% of cases.

The results of the micronucleus assay showed that chrysotile, crocidolite, and amosite induce micronuclei in SHE cells in a dose–dependent manner. The MN frequency depends on exposure time, reaching a rather late maximum between 48 and 66 hr (frequency up to 15%). The time course is similar for the different types of fibers. This is shown in Tables 1 and 2, and in histogram form for better visibility in Figure 2. In contrast, the concentration dependence appears different (Tables 1 and 2; Fig. 2). Amosite is the most potent fiber, with the highest MN frequency at the lowest level of fiber concentration, compared with crocidolite and chrysotile (Fig. 2). This result correlates with the known carcinogenic potency of amosite (*17*). Chrysotile, crocidolite, and amosite, respectively, reached a maximum in MN formation at 5.0 μg/cm^2 (fiber concentration) and 66 hr (exposure time), 5.0 μg/cm^2 and 66 hr, 0.25 μg/cm^2 and 66 hr (Tables 1 and 2; Fig. 2). At higher concentrations and longer exposure times, the occurrence of MN decreased again as a result of an increased cytotoxicity. We tested fiber concentrations up to 184 μg/cm^2 (chrysotile), 200 μg/cm^2 (crocidolite), and 120 μg/cm^2 (amosite). At these concentrations we observed a very high toxicity and only 24.5 ± 3.7 MN/2000 cells. Lower amosite concentrations than 1.0 μg/cm^2 were tested because the maximum of MN formation was found at 0.25 μg/cm^2. As a negative control, we treated SHE cells with calcium sulfate ($CaSO_4$; Table 3).

For further analysis of the induced micronuclei, kinetochores were stained. This was carried out by incubating the fixed cell preparations with CREST serum (60 min) in a humidified chamber at 37°C. After rinsing with phosphate-buffered saline, the cells were incubated with fluorescein isothiocyanate (FITC) conjugated goat anti–human antibodies before apply-

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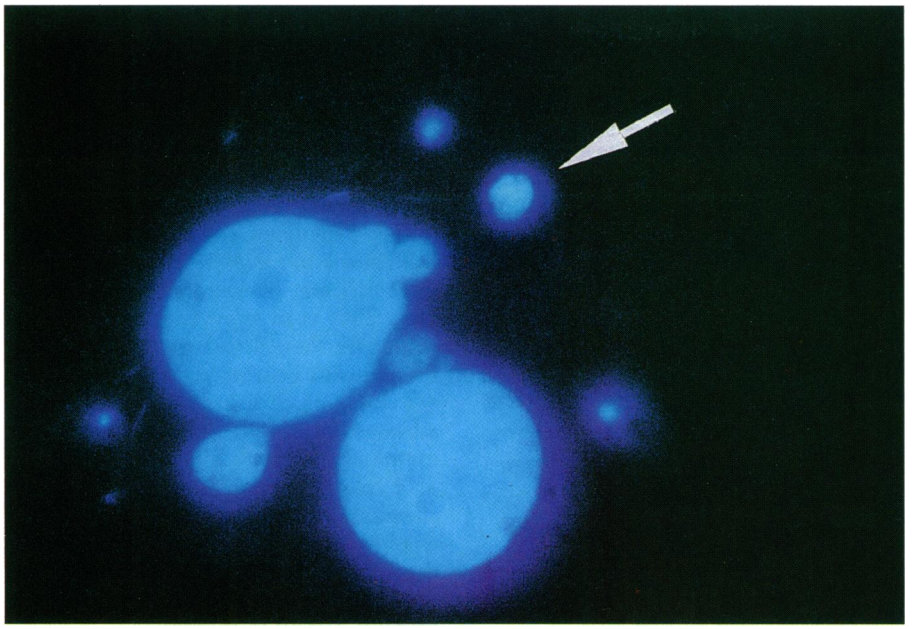

Figure 1. Typical micronucleus formation (arrow) and chromatin budding in fixed Syrian hamster embryo cells after treatment with asbestos fibers.

Table 1. Induction of micronucleus formation in Syrian hamster embryo cells by crocidolite asbestos[a]

Asbestos dose (μg/cm^2)	Treatment duration (hr)				
	18	24	48	66	72
1.0	86.0 ± 0.5	99.3 ± 11.7	145.3 ± 5.0	119.7 ± 50.9	106.0 ± 46.6
5.0	117.5 ± 0.7	153.0 ± 9.9	164.0 ± 25.5	218.5 ± 80.3	128.0 ± 52.3
10.0	119.0 ± 15.5	126.0 ± 25.5	145.3 ± 18.9	192.0 ± 28.3	107.5 ± 39.2
Control	31.3 ± 4.0	32.0 ± 5.6	30.8 ± 7.6	32.3 ± 4.2	29.3 ± 6.4

[a]Data represent the mean of 3 counts of 2000 cells (± SD) of different experiments. The difference in micronucleus formation between asbestos-treated and untreated cells is in all cases significant (p<0.001).

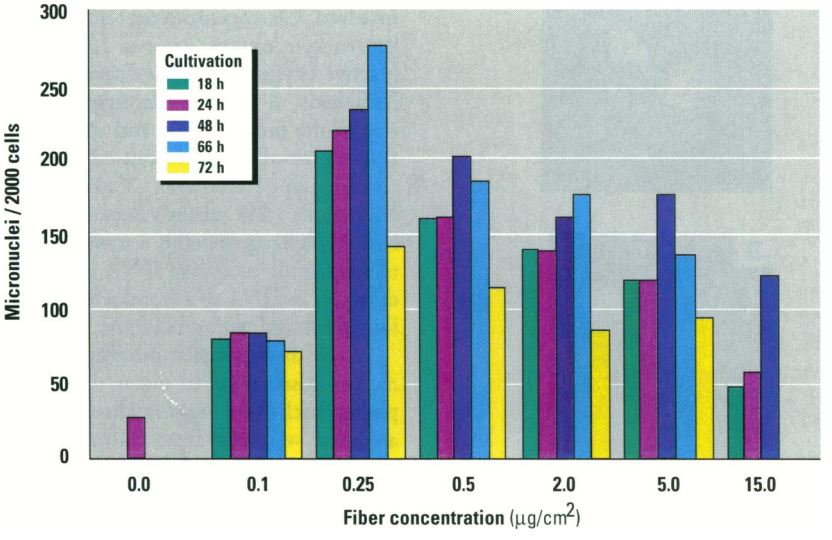

Figure 2. Micronucleus formation in Syrian hamster embryo cells after treatment with various concentrations of amosite asbestos (0.0–15.0 μg/cm^2) and different treatment durations (18–72 hr). The experiments were repeated several times with consistent results (SD ≤12%).

ing bisbenzimide. The numbers of kinetochore–positive (K^+) and kinetochore–negative (K^-) micronuclei were nearly equal (Table 4) in cells treated whith asbestos fibers. In this case the different fiber concentrations were used only for induction of a high number of micronuclei.

In addition, we stained the spindle apparatus to investigate a possible interaction between asbestos fibers and tubulin structure. This was achieved by incubating the fixed cells with tubulin antibodies (human anti-α-tubulin and FITC–anti-mouse antibodies, 60 min at 37°C) before applying bisbenzimide.

Disturbances during mitosis caused by asbestos fibers were directly monitored by supravital-UV microscopy. This method allows the observation of the spatial arrangement and movement of chromatin elements throughout mitosis in living cells. The intensity of the UV light is attenuated by neutral density filters. The faint cellular DNA signal is picked up by a silicon intensifier target camera and further improved by digital computer enhancement (*11*). For fluorescence microscopy analysis, SHE cells were plated in special chambers (*11*) and treated with amosite (0.25 μg/cm^2), crocidolite (1.0–5.0 μg/cm^2), or chrysotile (1.0–5.0 μg/cm^2). Exposure time was 24 hr–48 hr. Live cells were stained with 0.1 μg/ml bisbenzimide (here Hoechst 33342 was used) and the course of mitosis was observed. Figure 3 shows micronucleus formation following a disturbed mitosis. We also observed typical changes in interphase chromatin structure [chromatin budding (Fig. 1), compaction, and nuclear holes] and impaired chromatid separation in mitosis. This frequently resulted in the formation of chromatin bridges and chromosome displacement in metaphase and anaphase. In numerous cases, cytokinesis was blocked. We observed an increase in mitotic disturbances during and after the second cell cycle (delayed, blocked, or uncompleted mitoses and sticky and lagging chromosomes). Abnormal events of this type were not observed in untreated cells (more than 50 mitoses were analyzed).

Visualization of the spindle apparatus of amosite-treated SHE cells revealed that about 10% showed a slightly deformed spindle apparatus, although the spindle fibers appeared to have normal morphology. However, a deformed spindle apparatus occurs only in connection with disturbed metaphases and anaphases. This effect may be due to impaired connection or disconnection at the spindle fibers.

In conclusion, we found that all three types of asbestos fibers are taken up by phagocytosis by the cells within 24 hr after administration. This is in agreement with Hesterberg et al. (*19*). Intracellular fibers accumulate at the perinuclear region of the cells 24–48 hr after exposure (*18*). When cells undergo mitosis, the physical presence of the fibers results in interference with chromosome segregation. This explains the late maximum of MN formation of 66 hr. The frequency of micronucleated cells reflects chromosomal damage (*19*). In the present study, chrysotile and crocidolite increased the level of MN sevenfold after

Table 2. Number of micronuclei (per 2000 cells) induced by various concentrations of chrysotile fibers[a]

Asbestos dose (μg/cm²)	Treatment duration (hr)				
	18	24	48	66	72
1.0	73.0 ± 11.8	97.5 ± 5.1	134.7 ± 18.0	121.0 ± 37.6	99.5 ± 6.4
5.0	106.0 ± 2.8	119.0 ± 18.4	137.0 ± 26.6	210.0 ± 56.5	151.5 ± 13.2
10.0	94.0 ± 5.3	101.3 ± 17.9	126.7 ± 17.5	125.0 ± 1.7	107.0 ± 7.2
Control	28.0 ± 1.4	29.0 ± 9.5	33.0 ± 8.0	28.0 ± 4.1	30.8 ± 7.2

[a]Data represent the mean of 3 counts of 2000 cells (± SD) of different experiments. The difference in micronucleus formation between asbestos-treated and untreated cells is in all cases significant ($p<0.001$).

Table 3. Occurrence of micronuclei in Syrian hamster embryo cells after treatment with calcium sulfate ($CaSO_4$, negative control)[a]

$CaSO_4$ dose (μg/cm²)	Treatment duration (hr)			
	18	24	48	72
0.5	47.0 ± 4.2	45.5 ± 1.7	59.1 ± 5.0	48.0 ± 2.0
1.0	56.6 ± 9.2	46.0 ± 9.9	58.2 ± 8.5	51.0 ± 15.5
5.0	46.5 ± 5.6	40.3 ± 5.5	60.5 ± 18.9	57.5 ± 10.6
Control	41.3 ± 4.0	49.0 ± 5.6	50.8 ± 7.6	45.3 ± 6.4

[a]Data represent the mean of 3 counts of 2000 cells (± SD) of different experiments. In all cases, the differences in micronucleus formation between CaSO4-treated and untreated cells are not significant.

Table 4. Presence of kinetochores in micronuclei in asbestos treated Syrian hamster embryo cells[a]

Asbestos fiber	Fiber concentration (μg/cm²)	No. of cells	No. of micronuclei	%CRMN⁺
Chrysotile	10	3520	220	48.2 ± 3.7
Amosite	5	1662	133	47.4 ± 5.9
Crocidolite	1	1869	100	49.0 ± 1.0
Control	0	36000	300	27.0 ± 4.0[b]

[a]No. of cells, total number of cells scored for micronuclei; no. of micronuclei, number of micronuclei scored for presence of kinetochores; %CRMN⁺: percentage of the detected micronuclei that react positively to anti-kinetochore serum (± SD).
[b]Data taken from Schiffmann and De Boni (*11*) for comparison.

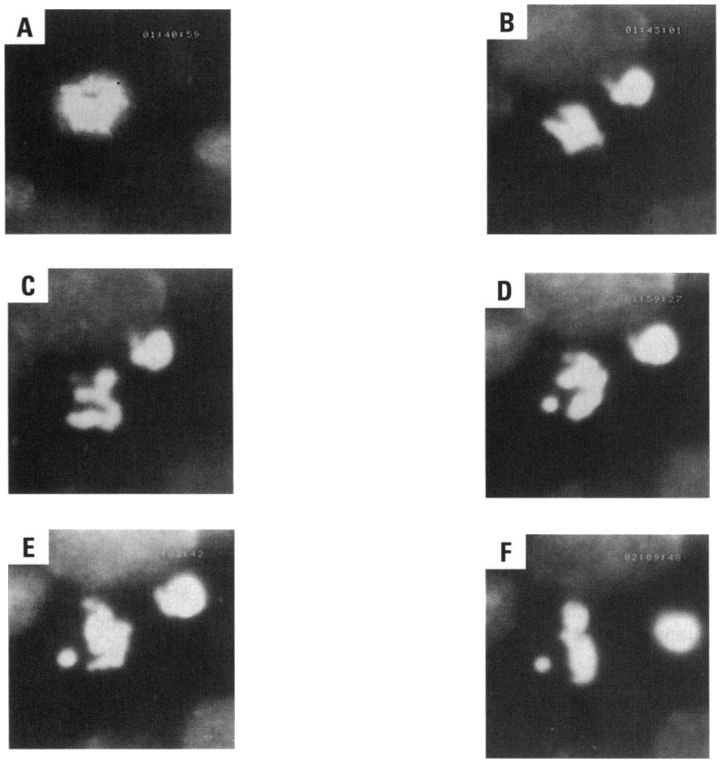

Figure 3. Supravital UV–microscopy analysis showing micronucleus formation during disturbed mitosis (anaphase) after treatment (24 hr) of Syrian hamster embryo live cells with chrysotile (1 μg/cm²).

66 hr treatment with 5 μg/cm². Amosite increased the MN formation ninefold after 66 hr treatment with 0.25 μg/cm². The maximum micronucleus frequency triggered by amosite (>250 MN/2000 cells) is the highest of all compounds tested in the SHE cell-system so far.

In our investigations, amosite was the most potent fiber, but the differences in MN formation after treatment with the three different fiber types are not significant. This is in agreement with Barrett (*20*), who showed that no significant difference exists between the effects of different doses of chrysotile and crocidolite asbestos on the relative survival and the morphologic transformation frequency of SHE cells in culture. A comparison of the chemical composition of chrysotile and the amphiboles crocidolite and amosite shows that the iron content of amphiboles is substantially increased compared with the amounts present in chrysotile (*1*). We suggest that chrysotile decreases its biological activation over time. Jaurand et al. (*21*) reported that chrysotile undergoes dissolution in the lung and in pleural mesothelial cells and may not persist in human lungs over the extended period necessary for the development of tumors. The presence of iron seems to be a critical factor in asbestos–induced cytotoxicity and DNA breakage (*22*). Iron–containing particles can produce oxygen–activated species by oxidizing their iron.

Our results of kinetochore staining provide evidence that loss of whole chromosomes as well as clastogenic events are involved. Clastogenic events may be caused by reactive oxygen species (*23*). Among reactive oxygen species–induced base modifications, 8–hydroxydeoxyguanosine is one of the most specific and the most representative of damages caused by reactive oxygen species (*24*). Takeuchi and Morimoto (*25*) recently reported that 8-hydroxydeoxyguanosine, a molecule typical of mutagenic oxidative DNA damage, was detected in DNA of a human leukemia cell line incubated with crocidolite.

As with chromatin budding, MN formation also seems to occur during interphase. Sticky and lagging chromosomes in anaphase were also reported by Hesterberg and Barrett (*26*). In contrast to these studies, we worked with living cells and also observed abnormalities in metaphase. In connection with these disturbances, a deformed spindle apparatus occurred. Furthermore, typical changes in chromatin structure (compaction) may be related to apoptotic events.

Finally, our results show that asbestos fibers may cause both loss and breakage of chromosomes in the absence of direct interaction with spindle fibers. Further

studies should be carried out to analyze the direct interaction between asbestos fibers and chromatin or chromosomes.

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