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Cell Toxicity, Hemolytic Action and Clastogenic Activity of Asbestos and Its Substitutes

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Abstracts: The cell toxicity, hemolytic and clastogenic activity were examined in various kinds of asbestos and some asbestos substitutes with reference to the their mineralogical and physicochemical characteristics. There were thirty-five fibrous and non-fibrous samples including UICC chrysotile, size-selected samples of UICC chrysotile, chrysotile altered by heating and grinding, Yamabe (Japan) chrysotile with long and short fibers, Coalinga (U.S. A.) chrysotile with short fibers, UICC crocidolite, amosite, and 19 non-asbestos samples such as, glass fibers, calcium silicates, sepiolites and some clay minerals. The cell toxicity and the hemolytic and clastogenic activity of asbestos were the strongest for chrysotile among all of the asbestos samples tested, and their strengths varied with fiber length and with the conditions of grinding and heating. These cellular effects of Yamabe chrysotile with long fibers and size-selected UICC chrysotile with long fibers were stronger than those of chrysotile of the same origin but with short fibers. These effects were weaker in chrysotile altered by heating and grinding. Among the asbestos substitutes, the cell toxicity, hemolytic and clastogenic activities of thin glass fibers were more marked than those of thick glass fibers. The four types of sepiolite were strongly hemolytic, but their cell toxicity and clastogenicity varied according to their grade of crystallinity and/or fiber size. These effects of calcium silicates and some clay minerals were generally low but varied with mineral species. In general, the cell toxicity, hemolytic and clastogenic activities of the asbestos substitutes tested here were mild compared with those of asbestos.

Key words: Asbestos—Long fiber—Short fiber—Altered asbestos—Asbestos substitutes—Cell toxicity—Hemolysis—Chromosome aberrations

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INTRODUCTION

Asbestos inhalation is strongly associated with an increased risk of asbestosis, carcinoma of the lung and pleural and peritoneal mesothelioma.^{1,2)} These adverse health effects have been confirmed by many epidemiologic studies and by animal experiments. Pleural mesothelioma can be induced in rats by intrapleural injection of asbestos fibers, and pulmonary carcinoma by inhalation exposure.³⁻⁵⁾

General recognition of the hazardous effects of asbestos for human health has led several (inter)national agencies to recommend the prohibition or restriction of asbestos uses. These policies have promoted worldwide development and production of various asbestos substitutes. However, their toxic effects are not well known. Stanton *et al.*⁶⁾ and Pott⁷⁾ reported that long, thin fibers were more carcinogenic than short, thick fibers, and that fiber carcinogenicity depends more on the fibers' durability than their physicochemical properties. If so, the dimensions of the asbestos substitutes become an important factor in their carcinogenicity. However, whether the other factors, such as chemical composition and the surface properties, affect the carcinogenicity or not is still obscure.

In 1988, the Japanese Environmental Agency reported⁸⁾ that in the air of the areas along motorways and some arterial roads, asbestos fibers shorter than $2\mu\text{m}$ or asbestos fibers with altered structures were several times more abundant than in other areas such as residential zones. Braking by automobiles was found to be responsible for the increased air levels of both the short asbestos fibers and the altered non-crystalline dusts whose original asbestos morphology and crystal structure had been destroyed by the heating and grinding during braking.

These studies show the importance of further study to reveal the biological effects of various kinds and sizes of fibers, such as short fibers less than $2-3\mu\text{m}$ in length, altered asbestos, and asbestos substitutes. In this investigation, we prepared samples of asbestos with long fibers and short fibers, and of altered-asbestos and asbestos substitutes such as glass fibers, calcium silicates and sepiolites, and assessed their *in vitro* biological effects, such as the cell toxicity and chromosome aberration in Chinese hamster lung cells, and the hemolysis of human erythrocytes.

MATERIALS AND METHODS

1. *Mineral samples of asbestos and asbestos substitutes and their method of preparation.*

Thirty-five kinds of fibrous and non-fibrous mineral samples including asbestos, glass fiber, calcium silicate fiber, sepiolite and some clay minerals, were prepared for *in vitro* experiments as shown in Table 1. The asbestos samples prepared were chrysotile UICC-B (from Canada); its size-selected samples and altered samples which were heated and/or ground; Coalinga chrysotile from California,

Table 1 Samples and the origins prepared for in vitro experiment.

SAMPLE	ORIGN	PREPARATION
<u>ASBESTOS</u>		
1. Chrysotile	UICC-B, CAN	0
2. "	UICC-A, Rhodesia	0
3. "	Coalinga 3778, Ca, USA	0
4. "	Y-A, Yamabe, JPN	1
5. "	Y-H(3), Yamabe, JPN	2
6. "	Y-C, Yamabe, JPN	1
7. "	UICC-B- # 200	3
8. "	UICC-B- # 635	3
9. "	UICC-B-R	3
10. "	UICC-B-600°C	4
11. "	UICC-B-800°C	5
12. "	UICC-B-G2	6
13. "	UICC-B-G3	7
14. "	UICC-B-800G	8
15. Crocidolite	UICC	0
16. Amosite	UICC	0
<u>CLAY MINERALS</u>		
17. Antigolite	Ant-Ko-C, Kōmori, JPN	9
18. "	Ant-Ko-F, Kōmori, JPN	1
19. Halloysite	Argeria	1
20. Kaolinite	KGa-1, Georgia, USA	1
21. Mg-Chlorite	Wanibuchi, Tottori, JPN	1
22. Hemalite	Brucite, Quebec, CAN	10
<u>CLASS FIBER</u>		
23. MN # 100	Manville, USA	11
24. MN # 104	Manville, USA	11
25. MN # 108A	Manville, USA	11
26. MN # 108B	Manville, USA	11
<u>CALCIUM SILICATE</u>		
27. Gyrolite	Synthesized by M	12
28. 11Å Tobermolite	Synthesized by M	12
29. α -dicalchum silicate hydrate	Synthesized by M	12
30. Xonotlite	Synthesized by M	12
31. Wollastonite	Quebec, CAN	9
<u>SEPIOLITE</u>		
32. Sepiolite	Sepio-C, China	13
33. "	Sepio-K, Kuzuu, JPN	1
34. "	Sepio-S, Spain	13
35. "	Sepio-T, Turkey	13

U.S.A.; Yamabe chrysotile from Hokkaido, Japan; UICC-amoi; and UICC-crocidolite. The glass fibers, calcium silicate fibers and sepiolite studied in this experiment have been widely used as asbestos substitutes in modern industrial countries. The clay minerals in Table 1 are of non-fibrous forms except for halloysite and nemalite.

The sample preparation methods, keyed to the numbers for each mineral in Table 1, were as follows :

- 0: The samples, i.e. UICC standard asbestos (chrysotile-A and B, amosite and crocidolite) and chrysotile from the Coalinga mine, California, were used as obtained. Details of the sample preparation method have been published.^{9,10)}
- 1: Fiber-sizes were separated by an elutriation method using a suspension of crushed original mineral to produce particles with $<10\mu\text{m}$ equivalent spherical diameter ($<10\mu\text{m}$ e.s.d.).
- 2: After the above elutriation procedure, the fiber sample was stirred with a magnetic stirrer to remove a small amount of coexisting magnetite.
- 3: Fiber-sizes were separated using a dry size-selective procedure with a 2-component fluidized bed, wire screens (200 and 635 mesh) and virtual impactor.
- 4: Heating for 1 h at 600°C .
- 5: Heating for 1h at 800°C .
- 6: Grinding for 1 h with ball mill.
- 7: Grinding for 2 h with a ball mill.
- 8: Heating for 1 h at 800°C and then grinding for 1 h with a ball mill.
- 9: Grinding by hand in an agate mortar.
- 10: Grinding for 30 min in an ethanol solution using a ball mill.
- 11: Crushing at an impact pressure of 25 ton/inch² using an oil-pressure apparatus.
- 12: Synthesis from a mixture of CaO and silicic acid by a hydrothermal formation procedure.
- 13: Pulverizing with an automatic milling machine and collection of small fractions by an air-stream separation method.

These samples were analyzed by X-ray diffraction, electron microscopy, thermal, chemical, and surface charge analysis. Some characteristics of each sample were as follows (Further details will appear elsewhere.):

[1] *Chrysotile UICC-B*: Chrysotile UICC-B consisted mostly of long fibers, as shown in the transmission electron micrograph in Fig. 1a, and did not contain any other fibrous minerals. However, there were small amounts of platy minerals, such as brucite, pyroaurite, lizardite and antigolite.

[2] *Size-selected chrysotile (UICC-B-# 200, UICC-B-# 635, and UICC-B-R)*: Chrysotile UICC-B was separated into various size fractions by a dry procedure using a 2-component fluidized bed, wire screens (200 and 635 mesh) and a virtual impactor with a cut-off point of $2\mu\text{m}$.¹¹⁾ The size distribution of the fraction sizes is shown in Fig. 2. Eighty-one percent of the short-fiber fraction (UICC-B-# 635) consisted of fibers less than $5\mu\text{m}$ long and 96% less than $10\mu\text{m}$ long. Eighty percent of the middle fiber fraction (UICC-B-# 200) consisted of fibers less than $10\mu\text{m}$ long. The residue fraction consisted mostly of long fibers and was designated UICC-B-R. X-ray diffraction analysis revealed that the crystalline state of these fractions was not markedly changed.

[3] *Chrysotile from Yamabe (Y-A, Y-H(3), and Y-C)*: Chrysotile from

Fig. TEM photographs of the asbestos and asbestos substitutes used in this study.

1a: Chrysotile UICC-B

1b: Chrysotile Y-A

1c: Chrysotile Y-H(3)

1d: Glass fiber MN # 100

1e: Xonotolite synthesized

1f: Sepiolite Sepi-C

Scale indicates 5μ

Fig. 2. Distribution of fiber length in size-selected chrysotile B

Yamabe, Hokkaido, Japan, was categorized into 3 different types, the long-fiber type (Y-A), the short-fiber type [Y-H(3)], the short fiber type with low-crystallinity (Y-C) containing 4 different polytypes.¹²⁾ TEM photographs show that Y-A consisted of very long fibers and Y-H(3) very short fibers mostly less than 2 μm long (Figs. 1b and 1c). A small amount of magnetite contamination in Y-H(3) was mostly removed with a magnetic stirrer. Chrysotile (Y-C) was an unusual type showing a greenish-white color and containing one-layer clino-, 2-layer clino-, 2-layer ortho-, and 6-layer ortho-chrysotils. The fiber length was mostly under 2 μm , similar to Y-H(3), but the crystallinity was low according to X-ray diffraction analysis.

[4] *Chrysotile samples altered by heating and grinding:* Chrysotile UICC-B was heated at 600°C and 800°C for 1 h (UICC-B-600 and UICC-B-800, respectively) and was ground for 1 h and 2 h (UICC-B-G2 and UICC-B-G3, respec-

tively). The typical X-ray diffraction pattern of chrysotile still remained in UICC-B-600, but the peak intensity was slightly weakened. The fibrous form of chrysotile was still evident by TEM observation. The chrysotile was transformed into forsterite in UICC-B-800. The fibrous form, however, still remained in this sample, but was very brittle and changed easily into small particles when ground in the agate mortar. Some fibrous form of chrysotile still remained in UICC-B-G2, but in UICC-B-G3, it had completely disappeared and changed into the fine particles of less than $0.1\text{ }\mu\text{m}$.

[5] *Glass fiber (MN # 100, MN # 104, MN # 108A and MN # 108B)*: The glass fibers used were made commercially by Manville Co., U.S.A., code # 100, having a nominal diameter of $0.29\text{--}0.32\text{ }\mu\text{m}$; code # 104, $0.39\text{--}0.53\text{ }\mu\text{m}$; code # 108A, $0.69\text{--}1.1\text{ }\mu\text{m}$; and code # 108B, $1.2\text{--}2.4\text{ }\mu\text{m}$. The fiber samples were crushed by the impact pressure method. In the crushed sample of MN # 100, 90% were less than $5\text{ }\mu\text{m}$ long and 95% were less than $10\text{ }\mu\text{m}$ long (Fig. 1d), the other crushed samples (MN # 104, MN108 # A and MN # 108B) were of similar fiber length. X-ray diffraction analysis showed that these samples were in an amorphous state and did not contain any mixed or crystalline materials.

[6] *Calcium silicate fibers*: In this experiment, some synthetic calcium silicates such as xonotolite, alpha-dicalcium silicate hydrate, $11\text{\AA}$ -tobernolite and gyrolite were examined, as well as a natural wollastonite. According to TEM analysis, the $11\text{\AA}$ -tobernolite had a thin sheet-like or elongated hexagonal platy form a few μm in diameter. The xonotolite had a fibrous form about $5\text{ }\mu\text{m}$ in length and less than $0.1\text{ }\mu\text{m}$ in diameter (Fig. 1e). The gyrolite had a thin platy form and the alpha-dicalcium silicate hydrate had various forms ranging from gel-like to fibrous. The natural calcium silicate, wollastonite, from Quebec, Canada, had a one-layer triclinic structure and a wide range of fiber sizes, mostly long and thick fibers.

[7] *Sepiolite (Sepio-C, Sepio-K, Sepio-S and Sepio-T)*: Sepiolite samples from four different natural sources were used. They were obtained from China (Sepio-C), Kuzuu, Japan (Sepio-K), Spain (Sepio-S) and Turkey (Sepio-T). X-ray diffraction analysis revealed that the crystallinity of these sepiolites descended in the order Sepio-C, Sepio-K, Sepio-S and Sepio-T. Small amounts of talc and calcite were detected as contaminants in sample Sepio-C, but never in the other samples. The sample of Sepio-C consisted mostly of fibers $1\text{--}10\text{ }\mu\text{m}$ long and $0.05\text{--}0.1\text{ }\mu\text{m}$ in diameter, but long and thick fibers over $20\text{ }\mu\text{m}$ long and over $1\text{ }\mu\text{m}$ in diameter were also present (Fig. 1f). The fibers of Sepio-K were mostly $3\text{--}7\text{ }\mu\text{m}$ long and $0.01\text{--}0.07\text{ }\mu\text{m}$ in diameter. The fibers of Sepio-S and Sepio-T were mostly $3\text{--}5\text{ }\mu\text{m}$ long and $0.01\text{ }\mu\text{m}$ in diameter. The order of crystallinity of these sepiolite samples were well coordinated with the fiber size (length and diameter).

[8] *Clay minerals*: To compare the in vitro experiments with non-fibrous minerals with those of the fibrous minerals above, some clay minerals with a platy form were selected. These were antigolite from the Kōmori mine, Kyoto,

Japan (with a lath shape), kaolinite from Georgia, U.S.A. (with a hexagonal platy form), and magnesium chlorite from Wanibuchi mine, Tottori, Japan (with an irregular platy form). In addition, halloysite, a kind of kaolin mineral with a short fibrous form, and nemalite, a kind of brucite with a fibrous form, were also prepared.

2. Cell toxicity

Chinese hamster lung (CHL) cells were obtained from Dainippon Pharmaceutical Co. Ltd.

CHL cells were grown in minimal essential medium (MEM) (GIBCO) supplemented with 10% fetal calf serum (GIBCO) and with penicillin (50 units/ml) and streptomycin (50 μ g/ml) (GIBCO).

Survival of CHL cells in the absence or presence of the mineral dusts was determined by colony-forming efficiency from a single-cell suspension. Approximately 200 cells were seeded into 60-mm-diameter Petri dishes (Falcon) along with the test dust. The surviving cells were allowed to grow and form colonies for 7 days. They were then fixed with 95% ethanol and stained with Giemsa solution.

3. Hemolysis

Human blood was taken from the cubital vein, and blood cells were washed three times with veronal-buffered saline. An erythrocyte suspension (approximately 2%) was prepared by making up 2 ml of packed erythrocytes to 100 ml of veronal-buffered saline. To 1 ml of this suspension, 1 ml of veronal-buffered saline containing various amounts of mineral dusts was added, and the mixture was incubated for 1 h at 37°C with shaking at 40 strokes for 1 min. Then, 0.2 ml of a 2.5% solution of glutaraldehyde was added to each sample to stop hemolysis. The samples were then centrifuged at 2000 r.p.m. for 10 min and the optical density of the clear supernatant was read at 541 nm using a spectrophotometer. Complete lysis was obtained by preparing a 2% erythrocyte suspension in distilled water with two drops of Triton X-100 (Sigma Chemical Co. U.S.A.) and 0.2 ml of 2.5% glutaraldehyde solution. The hemolytic activity of the test dusts was calculated from the average optical density of three replicates and was expressed as a percentage of the optical density of the totally lysed control (100%).

4. Cultures and chromosome preparation

A pseudo-diploid CHL cell line was used. The cells were grown in MEM supplemented with 10% fetal calf serum. The cells (4×10^5 cells) were seeded in a 25-cm² Falcon plastic flask, and various amounts of the test dusts were added to the cultures. All culturing was at 37°C in a 5% CO₂ atmosphere for 46 h and 0.05 μ g of colcemid/ml was added for the final 2 h. After incubation,

the cells were collected by scraping with a spatula and treated with 0.075 M calcium chloride for 15 min, followed by fixation with a mixture of ethanol and acetic acid (3:1) at 0°C. The fixed cells were spread on a glass slide then stained with Giemsa solution. The Giemsa-stained slides were examined for chromosome and chromatid aberrations, each type of aberration in one cell being scored separately.

RESULTS

1. Cytotoxicity

The toxic doses (TD_{50} : the amounts of dust necessary to inhibit the colony-forming efficiency of CHL cells by 50%) of mineral samples are shown in Table 2. Among the UICC reference asbestos samples, chrysotile was the most toxic, followed by amosite and crocidolite. The toxic doses of chrysotile A, chrysotile B, amosite and crocidolite were respectively, 1.6, 1.2, 4.5 and 7 $\mu\text{g}/\text{ml}$. As to the relationship between cell toxicity and fiber length, Yamabe chrysotile with long fibers (Y-A) was more toxic than the same chrysotile with short fibers [Y-H (3)]. The toxic dose of Yamabe chrysotile with short fibers was 3.7 $\mu\text{g}/\text{ml}$ and that of Yamabe chrysotile with long fibers was 0.2 $\mu\text{g}/\text{ml}$. Among the size-selected chrysotile UICC-B, the short-fiber fraction (UICC-B-# 635) had the weakest toxicity for cells and the toxicity increased with increasing fiber length. The toxic dose of the long-residue fiber fraction (UICC-B-R) was 0.3 $\mu\text{g}/\text{ml}$, those of the middle-fiber (UICC-B-# 200) and short-fiber fraction (UICC-B-# 635) were 0.9 $\mu\text{g}/\text{ml}$ and 1.1 $\mu\text{g}/\text{ml}$, respectively. The toxic dose of Coalinga chrysotile with short fibers was 0.9 $\mu\text{g}/\text{ml}$. The toxic dose of chrysotile altered by grinding for 2 h was 25 $\mu\text{g}/\text{ml}$ and that of the chrysotile heated at 800°C for 1 h and ground for 1 h was 67 $\mu\text{g}/\text{ml}$.

The toxic doses of glass fiber codes # 100, 104, 108A and 108B (Manville Co.) were 10, 11, 18 and 27 $\mu\text{g}/\text{ml}$, respectively.

The calcium silicate samples used were synthetic materials such as fibrous xonotolite, α -dicalcium silicate hydrate, 11Å tobermolite, gyrolite and natural wollastonite. The toxic doses of fibrous xonotolite, α -dicalcium silicate hydrate and natural wollastonite were more than 50 μg , respectively, and that of 11 Å tobermolite was 45 $\mu\text{g}/\text{ml}$. The toxic dose for gyrolite was 8 $\mu\text{g}/\text{ml}$.

The toxic dose of sepiolite from China was 0.9 $\mu\text{g}/\text{ml}$, which was the same level as that of UICC chrysotile. The toxic dose of sepiolite from Kuzuu was 2.5 $\mu\text{g}/\text{ml}$. Those of sepiolites from Turkey and Spain were 30 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$, respectively.

2. Hemolysis studies

The hemolytic doses (HD_{50} : dust dose necessary to cause 50% hemolysis of human erythrocytes) of various fibrous samples are shown in Table 3.

Table 2. Cell Toxicity

TD ₅₀ (μ g/ml)	Asbestos				Asbestos substitutes, etc.			
	UICC (raw)	Long fiber	Short fiber	Heating, grinding	Glass fiber	Sepiolite	Ca silicate	Others
<1		Y-A (Yamabe) (chrysotile) UICC-B- # 200 (chrysotile) UICC-B-R (chrysotile)	Coalinga 3778 (chrysotile)			China		
<3	Chrysotile A Chrysotile B		UICC-B- # 635 (chrysotile)	UICC-B-600° C (chrysotile) UICC-B-800° C (chrysotile) UICC-B-G2 (chrysotile)		Kuzuu		
<5	Amosite		Y-H(3), Yamabe (chrysotile)					
<10	Crocidolite				Code # 100		Gyrolite	Antigolite F (No 18) Mg-chlorite Nemalite
<30			Y-C, Yamabe (chrysotile)	UICC-B-G3 (chrysotile)	Code # 104 # 108A # 108B	Turkey		Antigolite C Kaolinite
<50						Spain	11Å Tobermolite	Halloysite
>50				UICC-B-800G (chrysotile)			Xonotolite α -dicalcium silicate hydrate wollastonite	

TD₅₀: The amounts of dust necessary to inhibit the colony-forming efficiency of CHL cells by 50%.

Table 3. Hemolysis

HD ₅₀ (μg/ml)	Asbestos				Asbestos suastitutes, etc.			
	UICC (raw)	Long fiber	Short fiber	Heating, grinding	Glass fiber	Sepiolite	Ca silicate	Others
< 100								
< 200	Chrysotile A Chrysotile B	Y-A (Yamabe) (chrysotile) UICC-B-R (chrysotile)	Coalinga 3778 (chrysotile) Y-H(3) Yamabe (chrysotile)			Kuzuu Spain		
< 400		UICC-B- # 200 (chrysotile)	UICC-B- # 635 (chrysotile) Y-C, Yamabe (chrysotile)	UICC-B-G2 (chrysotile)		Turkey China	Gyrolite α-dicalcium silicate hydrate Xonotolite	Halloysite Mg-chlorite
< 600				UICC-B-G3 (chrysotile)				
< 1000					Code # 100			
> 1000	Amosite Crocidolite			UICC-B-800G (chrysotile)	Code # 104 # 108A # 108B		11Å Tobermolite Wollastonite	Antigolite C Antigolite F Kaolinite Nemalite

HD₅₀: Dust dose necessary to cause 50% hemolysis of human erythrocytes.

To achieve 50% hemolysis, less than 200 $\mu\text{g/ml}$ of UICC chrysotile A and B, Yamabe chrysotile with long fibers (Y-A) and Coalinga chrysotile was needed. UICC amosite and crocidolite rarely produced 50% hemolysis at any concentration tested.

Yamabe chrysotile with long fibers (Y-A) was slightly more hemolytic than chrysotile with short fibers [Y-H(3)]. The residue fraction (UICC-B-R) of the size-selected UICC chrysotile was more hemolytic than the middle-fiber (UICC-B-#200) or short-fiber fraction (UICC-B-#635). The hemolytic activity of chrysotile altered by grinding was reduced with increased grinding time. Also, the UICC chrysotile altered by grinding for 1 h and heating for 1 h at 800°C (UICC-B-800G) was less hemolytic than chrysotile altered by grinding only.

Among the asbestos substitutes, the hemolytic potential of sepiolite was the stronger than glass fiber, calcium silicates and other clay minerals. In the sepiolite group, the sample from Kuzuu was the most hemolytic, followed by sepiolite from Spain and Turkey. Their hemolytic doses were 110, 195 and 210 $\mu\text{g/ml}$, respectively. The sepiolite from China was the least hemolytic. The sepiolite from China, showing the highest cytotoxic activity, had the weakest hemolytic activity, 350 $\mu\text{g/ml}$. Among the glass fibers, the hemolytic dose of the thinnest fiber (Code 100) was 800 $\mu\text{g/ml}$, and those of thicker glass fibers (Code #104, 108A and B) were above 1000 $\mu\text{g/ml}$. To achieve 50% hemolysis, 210 $\mu\text{g/ml}$ of gyrolite was required, whereas 260 $\mu\text{g/ml}$ of α -dicalcium silicate hydrate, 380 $\mu\text{g/ml}$ of xonotolite and more than 1000 $\mu\text{g/ml}$ of 11Å tobermorite were needed. Among clay mineral fibers, the hemolytic doses of halloysite and Mg-chlorite were 225 and 350 $\mu\text{g/ml}$, respectively. Those of antigolite C, antigolite F (prepared under 5 μ), kaolinite, and fibrous nemalite were more than 1000 $\mu\text{g/ml}$.

3. Chromosomal aberrations and induction of polyploidy

UICC chrysotile, crocidolite and amosite induced structural chromosomal aberration and polyploidy. As shown in Table 4, chrysotile was the most clastogenic, followed by crocidolite and amosite. The polyploidy occurred frequently in the amosite sample, whereas the structural chromosomal aberrations were mainly chromatid gaps. The aberrations in the cultures treated with chrysotile-UICC-B included gaps, chromosome and chromatid breaks, chromatid exchanges, dicentric and polyploidy.

Upon treatment of the cultures with size-selected chrysotile-UICC-B, the long-residue fractions (UICC-B-R) had the highest clastogenicity followed by the middle-fiber fraction (UICC-B-#200) and the short-fractions (UICC-B-#635) (Table 5). Table 5 shows that long-residue fraction induced high levels of polyploidy and structural chromosome aberrations. The aberrations in the cultures treated with the long-residue fraction included gaps, chromatid breaks, dicentric and chromatid exchanges. Those in the cultures treated with the

Table 4. Chromosomal aberrations in CHL cells treated with UICC asbestos.

Treatment	Dose ($\mu\text{g/ml}$)	Abnormality (%)					Polyploids (%)
		Gaps	Breaks	Dicentrics	Exchanges	Others	
Control	0	2	0	0	0	0	0
Chrysotile	5	7	0	2	1	0	38
	10	12	3	2	2	0	34
	30	5	2	2	1	0	50
	100	not observed					
Crocidolite	10	4	0	2	0	0	24
	30	12	0	0	0	0	32
	100	not observed					
Amosite	10	2	0	0	0	0	18
	30	2	0	0	0	0	36
	100	6	0	0	0	0	42

Table 5. Chromosomal aberrations in CHL cells treated with size-selected chrysotile.

Treatment	Dose ($\mu\text{g/ml}$)	Abnormality (%)					Polyploids (%)
		Gaps	Breaks	Dicentrics	Exchanges	Others	
Control	0	2	0	0	0	0	0
Short-fraction (UICC-B- # 635)	10	4	0	0	0	0	34
	30	4	0	0	0	0	34
	100	4	0	0	0	0	38
Middle-fraction (UICC-B- # 200)	10	6	0	4	0	0	46
	30	4	2	4	0	0	38
Residue-fraction (UICC-B-R)	10	4	2	6	4	0	66
	30	12	0	6	6	0	65

short-fiber fraction included only gaps and low levels of polyploidy.

In the Yamabe chrysotile sample with long fibers (Y-A), the aberrations observed also included gaps, chromatid breaks, dicentrics, chromatid exchanges and rings, whereas the Yamabe chrysotile with short fibers [Y-H(3)] and Coalinga chrysotile did not induce any significant increase in the incidence of such chromosome abnormalities, but mainly increased the incidence of chromatid gaps, isochromatid gaps and polyploidy. Table 6 shows the frequency of chromosome abnormalities found in metaphase spreads of CHL cells exposed to Yamabe chrysotile with long and short fibers. Table 7 shows that chrysotile altered by grinding for 2 h (UICC-B-G3) did not induce structural chromosome abnormalities, but only low levels of polyploidy.

The results obtained from treatment with asbestos substitutes are shown in Table 8. Glass fibers, codes #100 and #104, did not significantly increase structural chromosome aberrations. They caused only relatively high levels of polyploidy. Code #108A induced neither structural nor numerical chromosome aberrations up to dosage of 30 $\mu\text{g/ml}$. Among calcium silicates, synthetic 11Å tobermolite, α -dicalcium silicate hydrate, gyrolite and natural wollastonite did not significantly increase polyploidy and structural chromosome aberration. But in the xonotolite induction of polyploidy and structural chromosome aberration, they

Table 6. Chromosomal aberrations in CHL cells treated with long fiber-chrysotile and short fiber-chrysotile.

Treatment	Dose ($\mu\text{g}/\text{ml}$)	Abnormality (%)					Polyploids (%)
		Gaps	Breaks	Dicentrics	Exchanges	Others	
Control	0	2	0	0	0	0	0
Yamabe (Y-A)	5	10	4	4	2	2 (ring)	48
(long)	10	10	0	4	2	0	54
	30	not observed					
Yamabe (Y-H(3))	10	2	0	0	0	0	18
(short)	30	4	0	0	0	0	30
	100	4	2	3	1	0	40
Coalinga	10	2	0	0	0	0	22
(short)	30	8	0	0	0	0	34

Table 7. Chromosomal aberrations in CHL cells treated with chrysotile altered by grinding and heating.

Treatment	Dose ($\mu\text{g}/\text{ml}$)	Abnormality (%)					Polyploids (%)
		Gaps	Breaks	Dicentrics	Exchanges	Others	
Control	0	2	0	0	0	0	0
Chrysotile	10	0	0	0	0	0	4
(grinding for 2 h)	30	0	0	0	0	0	4
	100	1	0	0	0	0	4
	300	3	0	0	0	0	4
Chrysotile	10	0	0	0	0	0	2
(heating at 800°C,	30	0	0	0	0	0	0
grinding for 1 h)	100	0	0	0	0	0	2
	300	0	0	0	0	0	2

were stronger than the other calcium silicates tested. The aberrations in the cultures treated with xonotolite included gaps and dicentrics. Among the sepiolite samples, the sepiolite from Kuzuu, Spain and Turkey did not significantly increase polyploidy and structural chromosome aberration up to a dosage of $100\mu\text{g}/\text{ml}$. But the sepiolite from China induced a relatively high level of polyploidy, approximately the same as glass fiber code #100. The aberrations in the cultures treated with the sepiolite from China included exchanges only by the addition of $10\mu\text{g}/\text{ml}$. In the additions of 30 and $100\mu\text{g}/\text{ml}$ they caused only chromatid gaps.

Figure 3 shows photographs of numerical and structural chromosome aberrations in the cells treated with fibers.

DISCUSSION

In an earlier study, we¹³⁾ demonstrated that among various asbestos and the related mineral samples, chrysotile had the highest hemolytic and cytotoxic effects on erythrocytes and macrophages. To estimate the cell toxicity of asbestos, several primary and cultured cell lines have been used as experimental materials since the 1970's. The results obtained from the experiments using macrophages

Table 8. Chromosomal aberrations in CHL cells treated with asbestos substitutes.

Treatment	Dose ($\mu\text{g/ml}$)	Abnormality (%)					Polyploids (%)
		Gaps	Breaks	Dicentrics	Exchanges	Others	
Control	0	2	0	0	0	0	0
Glass fiber							
Code # 100	10	2	0	0	0	0	16
	30	2	0	0	0	0	22
	100	2	0	0	0	0	26
	300	3	0	0	0	0	32
Code # 108A	10	2	0	0	0	0	4
	30	4	0	0	0	0	4
	100	2	0	0	0	0	14
	300	2	0	0	0	0	18
Ca. silicate							
Xonotolite	10	2	0	0	0	0	8
	30	0	0	2	0	0	10
	100	2	0	2	0	0	12
	300	2	0	0	0	0	20
11Å Tobermolite	10	0	0	0	0	0	4
	30	0	0	0	0	0	6
	100	0	0	0	0	0	4
	300	0	0	0	0	0	5
α -dicalcium silicate hydrate	10	0	0	0	0	0	4
	30	0	0	0	0	0	4
	100	0	0	0	0	0	4
	300	0	0	0	0	0	4
Gyrolite	10	2	0	0	0	0	6
	30	4	0	0	0	0	4
	100	2	0	0	0	0	4
	300	2	0	0	0	0	4
Wollastonite	10	2	0	0	0	0	2
	30	2	0	0	0	0	2
	100	2	0	0	0	0	4
	300	2	0	0	0	0	12
Sepiolite							
China	10	0	0	0	4	0	21
	30	4	0	0	0	0	26
	100	4	0	0	0	0	32
	300	not observed					
Kuzuu	10	0	0	0	0	0	6
	30	2	0	0	0	0	6
	100	0	0	0	0	0	8
	300	0	0	2	2	0	8
Spain	10	0	0	0	0	0	6
	30	0	0	0	0	0	8
	100	2	0	0	0	0	6
	300	4	0	0	1	0	16
Turkey	10	0	0	0	0	0	4
	30	2	0	0	0	0	6
	100	0	0	0	0	0	6
	300	0	0	0	0	0	8

Fig. 3. Photographs of chromosome of CHL cells
 1) Chromosome of CHL cells (no addition of fibers, control)
 2) Polyploid (addition of Yamabe chrysotile with short fibers)
 3) Polyploid including dicentric (addition of UICC chrysotile)
 4) Polyploid including chromatid exchange and break (addition of Yamabe chrysotile with long fibers)
 5) Polyploid including gap (addition of Yamabe chrysotile with short fibers)

and cultured cells showed almost the same tendencies. However, it seems difficult to infer the fibrogenicity and carcinogenicity of asbestos directly from their cellular effects. In 1978, Chamberlain and Brown¹⁴⁾ referring to data of Wagner³⁾ who conducted tumorigenicity experiment of the mineral dusts, found that there is a qualitative relationship between the tumorigenicity and in vitro cytotoxicity of fibrous minerals. That is, the activity of fibrous dust in reducing the colony-forming efficiency of V79-4 cells was related to the tumorigenic potential of the dusts. Therefore, using the same method, we tested the amounts of dust necessary for inhibiting the colony-forming efficiency of Chinese hamster lung cells (CHL cell line) by 50%.

Chrysotile and crocidolite asbestos have been reported to induce chromosome aberrations in Syrian hamster embryo cells^{15,16)} and Chinese hamster ovary cells,^{17,18)} V79-4 cells,¹⁹⁾ human mesothelial cells,²⁰⁾ and rat pleural mesothelial cells.²¹⁾ However, there has been no comparative study on the clastogenicity of asbestos with short fibers vs long fibers, originating from the same sample. In this study we obtained samples having different fiber size distributions originating from the same sources. To obtain a chrysotile sample with short fibers, we had not ground a long fiber sample with a ball-mill or an agate mortar. The short-chrysotile fibers were separated from the original (UICC) sample by aerodynamically²²⁾ or collected from the same geological environment in a mine (Yamabe, Hokkaido, Japan), where also producing long chrysotile fibers. Therefore, the crystal structure, especially the surface property of the short fibers was considered to be unchanged or to be equivalent with the long fiber sample.

It is significant that the cytotoxicity, clastogenic and hemolytic activities of the short-fiber chrysotiles, such as the short-fiber fraction of the size-selected UICC chrysotile or the short-fiber Yamabe chrysotile, were weaker than those of the middle-fiber and long-residue-fiber fractions of the size-selected UICC chrysotile and Yamabe chrysotile with long fibers. And in particular both the polyploidy and structural chromosomal aberrations induced by the long-fiber chrysotile were more marked than those induced by the short-fiber chrysotile. That is, dicentrics, chromatid exchanges and chromosome breaks were induced by the long-fiber chrysotile, whereas the short-fiber chrysotile induced mostly chromatid gaps.

There have been two reports of reduced cytotoxicity in the macrophages²³⁾ and V-79 cells¹⁴⁾ as a result of grinding asbestos. Also, it has been reported that the clastogenic activity of ground crocidolite in CHO-K1 cells^{17,18)} was reduced in comparison with the original crocidolite. In our study, the crystal structure of chrysotile disappeared with increasing grinding time, and the cytotoxicity and the clastogenic and hemolytic effects were reduced.

A ball milling was also not used in this study to obtain short fibers of asbestos substitutes. Glass fibers were crushed using an oil pressure apparatus and their fiber shapes were kept as shown in Fig. 1-d. The average diameter of the code # 100 glass fiber was approximately the same as that of the chrysotile fibers. The

average length of the code #100 was similar to short fraction of size-selected UICC chrysotile and was slightly longer than the Yamabe chrysotile with short fibers. The cytotoxicity, clastogenicity and hemolytic action of the Code #100 were slightly weaker than these chrysotile samples. Further, the cytotoxicity and the biological effects of the glass fibers were reduced with increasing diameter.

Among the sepiolite samples, the fiber size decreased in the order of their sources from China, Kuzuu, Spain and Turkey. The levels of hemolytic action of sepiolites from Kuzuu and Spain were similar to those of UICC chrysotile samples and were generally stronger than that of the other mineral fibers tested. The order of cell toxicity and clastogenic activities of the sepiolites seems to be well correlated with crystallinity and/or fiber size. The clastogenicity of the sepiolite from China was moderate, but that of the other sepiolite samples was weak. Since the clastogenicity varied with the sepiolite samples, the tumorigenic potential would vary according to the degree of crystallinity and fiber size, although Wagner²⁴⁾ has reported that a sepiolite from the U.S.A. did not induce mesothelioma after intrapleural injection into F344 rats. However, judging from these results, we supposed that a sepiolite fiber having good crystallinity and/or long and thin fiber-shape will be more carcinogenic and will induce mesothelioma in an animal experiment, and we²⁵⁾ produced mesothelioma in female SD rats' pleura by injection of sepiolite from China which is composed of long fibers, but no mesothelioma was produced by sepiolite from Spain with short fibers. Recently, Pott *et al.*²⁶⁾ reported similarly that sepiolite from Finland induced abdominal tumors (sarcomas or mesotheliomas excluding tumors of the uterus) after intraperitoneal injection in female Wistar rats, but sepiolite from Unalua under the same conditions did not.

Among the calcium silicate samples, such as xonotlite, 11Å tobermorite, gyrolite and α -dicalcium silicate hydrate, and natural wollastonite, the cytotoxicity, hemolytic action and clastogenicity were weaker than those of the chrysotile sample. Although some reports have shown that natural wollastonite is more toxic to macrophages²⁷⁾ or erythrocytes²⁸⁾ than the synthetic calcium silicates, the cellular effects of the wollastonite tested in our study were no worse than those of synthetic calcium silicates.

Stanton *et al.*²⁹⁾ have reported that the probability of developing pleural tumor was best correlated with the number of fibers with diameters less than $0.25\mu\text{m}$ and lengths greater than $8\mu\text{m}$. In our study, the frequency of polypoid induction was related to the length of fibers in various fibrous samples.

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