

Magnetometric Evaluation of the Effects of Man-Made Mineral Fibers on the Function of Macrophages Using the Macrophage Cell Line RAW 264.7

Kaori SHIBATA^{1*}, Yuichiro KUDO¹, Masashi TSUNODA¹, Mayuko HOSOKAWA¹,
Yasuhiro SAKAI², Makoto KOTANI³ and Yoshiharu AIZAWA¹

¹Department of Preventive Medicine and Public Health, Kitasato University School of Medicine, 1–15–1 Kitasato, Sagami-hara-shi, Kanagawa 228-8555, Japan

²Department of Anatomy, Kitasato University School of Medicine, Kanagawa, Japan

³Department of Electronic Engineering, Tokyo Denki University, Tokyo, Japan

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Abstract: The toxic effects of man-made mineral fibers (MMMFs) have been evaluated by cell magnetometry using alveolar macrophages (AMs). Recently, on the other hand, the murine macrophage cell line, RAW 264.7, became available and has been used as an *in vitro* model of AMs. The objective of this study was to determine whether or not cell magnetometry using RAW 264.7 cells can be used to evaluate the toxic effects of MMMFs. RAW 264.7 cells were exposed to one of the MMMFs, potassium octatitanate (PT) or silicon carbide whisker (SiC) at 0, 20, 40 and 60 $\mu\text{g}/\text{ml}$, or chrysotile as a positive control at 0, 15, 20 and 25 $\mu\text{g}/\text{ml}$. The toxic effects of fibers were evaluated by cell magnetometry and LDH assay. For this comparison, AMs were also exposed to chrysotile fibers (CF). In the RAW 264.7 cells exposed to PT 20, 40, 60 or SiC 20, 40, 60, CF 15, 20 and 25 $\mu\text{g}/\text{ml}$, significant delayed relaxation were observed compared with the respective control. In the LDH assay, significant increases in LDH in the supernatant of the cells exposed to PT 20, 40, 60, SiC 20, 40, 60 and CF 15, 20, 25 $\mu\text{g}/\text{ml}$ were observed. In AMs exposed to CF 20, 25 $\mu\text{g}/\text{ml}$ significant delayed relaxation and significant increases in LDH compared with the control were observed. The levels of MMMFs that induced significant differences were similar for cell magnetometry and LDH. The levels of CF that induced significant differences in cell magnetometry and LDH were identical for RAW 264.7 cells and AMs. Our results suggest that cell magnetometry using RAW 264.7 cells is adequate to evaluate the cytotoxicity of exposure to MMMFs.

Key words: Magnetometry, Cytoskeleton, Cell line, Man-Made Mineral Fibers, Cytotoxicity

Introduction

Man-made mineral fibers (MMMFs) have been developed as substitutes for asbestos, since the associations between exposure to asbestos and diseases such as pulmonary cancer and malignant mesothelioma have been reported^{1,2}. MMMFs are considered safer than asbestos, based on their physical and chemical characteristics^{3,4}. However, Stanton *et al.*⁵

have suggested that not only asbestos but also any long and thin fibers that are retained in the lung for a long time, could cause lung injury, fibrosis and lung cancer. The current evaluations of toxic effects of MMMFs are insufficient. Therefore, safety evaluations of MMMFs are urgently needed.

MMMFs are taken into the body by inhalation and phagocytosed by alveolar macrophages (AMs)⁶. In previous studies, AMs obtained from rats have been used for assessments of MMMFs^{7,8}. A unique method called cell magnetometry for the evaluation of toxic effects of fibrous

*To whom correspondence should be addressed.

materials on the functions of AMs was developed in 1996⁹⁾. It provided useful information for the toxic effects of MMMFs, such as silicon carbide whiskers¹⁰⁾ (SiC), potassium octatitanate whiskers¹¹⁾ (PT), rock wool¹²⁾ and micro glass fibers¹³⁾, as described in previous studies.

Rats must be sacrificed to obtain AMs for the cell magnetometry. The number of AMs obtained from each rat is limited to several millions, therefore, many animals are needed to evaluate each substance. Recently the murine macrophage cell line RAW 264.7 became available and has been used as an *in vitro* model of AMs^{14–16)}. Cell magnetometry using RAW 264.7 cells may be useful.

The purpose of this study is whether or not RAW 264.7 cells can be used in the evaluation of toxic effects of MMMFs by cell magnetometry. PT and SiC, which have a high toxicity on AMs among other MMMFs^{10, 11)}, were chosen as representative MMMFs. RAW 264.7 cells were exposed to PT, SiC or chrysotile fiber (CF), which is one of the toxic asbestos fibers, as a positive control. The toxic effects of fibers were evaluated by cell magnetometry and LDH in the supernatant of the cells. LDH level is the index of cell membrane damage and cell death. Electron microscopic observations were used to confirm that RAW 264.7 cells engulfed fibers. The observation by the fluorescent antibody method¹³⁾ was used to check the change in cytoskeleton. For the comparison, AMs were also exposed to CF and the toxic effects were evaluated by cell magnetometry and LDH assay.

Materials and Methods

Materials

PT and SiC were supplied by the Japan Fibrous Materials Research Association (JFM) (Tokyo). Chrysotile fibers (CF) were supplied by the Japan Association for the Working Environment Measurement (Tokyo). In Fig. 1a–c, the SEM image of CF, PT and SiC fibers is shown, respectively. The geometric mean of the length (geometric SD) and that of the width (geometric SD) of CF was 2.60 (2.30) μm and 0.15 (1.80) μm , respectively. The geometric mean of the length (geometric SD) and that of the width (geometric SD) of PT was 6.00 (2.04) μm and 0.35 (1.51) μm , respectively. The geometric mean of the length (geometric SD) and that of the width (geometric SD) of SiC was 6.40 (2.45) μm and 0.30 (1.58) μm , respectively, according to the study of Kohyama *et al.*¹⁷⁾. The composition of PT was Ti 84.73%, Nb 0.23%, Zr 0.03%, and K 15.01%; that of SiC was SiC 99.6% and Al_2O_3 0.16%; and that of CF was Mg 56.88%, Si 39.98% and Fe 3.14%. These materials were suspended in phosphate buffered saline (PBS), pH7.4, sterilized by

autoclaving at 121°C for 20 min.

Cell line

A murine peritoneal macrophage cell line RAW 264.7 was obtained from Dainippon Sumitomo Pharma Co., Tokyo. The cells were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, ICN Biomedicals, Inc., Aurora, OH).

Preparation of alveolar macrophage

Male F344 rats were obtained from CLEA Japan Inc. (Tokyo). Each rat was anesthetized and bled to death by incising the abdominal aorta. Bronchoalveolar lavage was performed by instilling 4 ml cold PBS, pH 7.4, containing 0.1% EDTA (ethylenediaminetetraacetic acid) through a tracheal catheter, followed by gentle aspiration. This procedure was repeated 9 times. Six rats were used for each experiment. Fluid from each lavage contained AMs and was pooled and centrifuged at 650 \times g for 10 min. The AMs pellet was suspended in serum free medium (Macrophage-SFM liquid, Life Technologies, Inc., Rockville, MD, USA).

Cell culture

The viabilities of RAW264.7 or AMs used were 90% or more determined by the trypan-blue exclusion test. Cells were counted with hemacytometer and RAW 264.7 cells were adjusted to 25×10^4 cells/ml and AMs were adjusted to 10^6 cells/ml. The cell number set from preliminary experiment. Each cell population was seeded into glass tube (Shibata Scientific Technology, Ltd., Tokyo). The base of the glass tube was 2.5 cm^2 .

Cell magnetometry

Cell magnetometric measurement was performed by a method previously reported by Keira *et al.*⁹⁾. The diameter of Fe_3O_4 ranged from 0.08 to 0.57 μm , and the geometrical mean diameter of Fe_3O_4 was 0.26 μm . The cells were exposed to particles of Fe_3O_4 (Toda Kogyo Co., Hiroshima) suspended in PBS at the concentration of 50 $\mu\text{g}/\text{ml}$ added to each glass tube containing cells. One hour after exposure to Fe_3O_4 , the cells were exposed to each tested material.

RAW 264.7 cells were exposed to CF 0, 15, 20 or 25 $\mu\text{g}/\text{ml}$, PT or SiC 20, 40 or 60 $\mu\text{g}/\text{ml}$ and incubated in a humidified incubator with 5% CO_2 at 37°C for 48 h. AMs exposed to CF 0, 15, 20, 25 $\mu\text{g}/\text{ml}$ and after the exposure incubated in a humidified incubator with 5% CO_2 at 37°C for 18 h. The incubation times were set from our preliminary experiments. There were 6 tubes for each concentration of tested glass tubes.

After the incubation, the cells in the glass tubes were

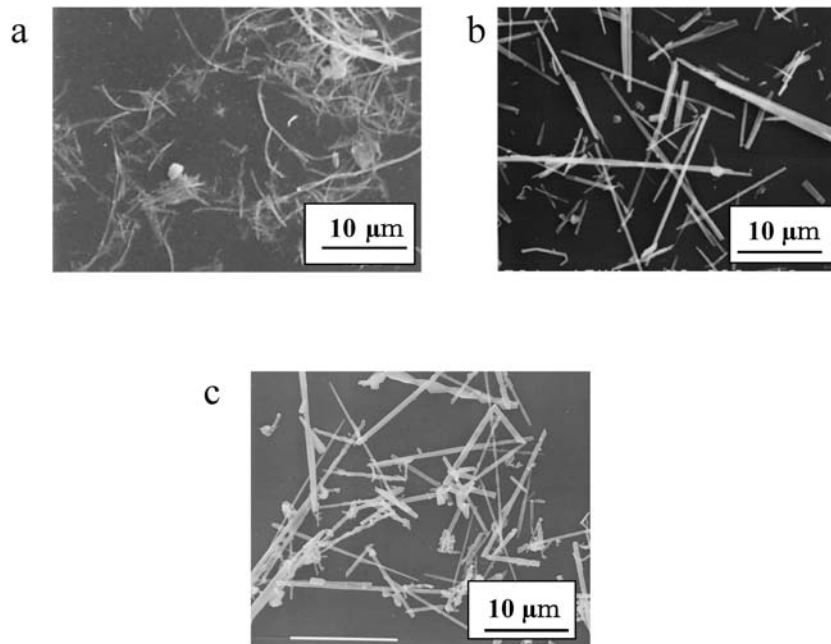


Fig. 1. The SEM image of asbestos fiber (CF) and MMMFs (PT, SiC).

a. Chrysotile fibers (CF), b. potassium octatitanate whisker (PT), and c. silicon carbide whisker (SiC), respectively. PT image from Watanabe *et al.*¹¹⁾.

magnetized by a magnetizer at 70 mT for 10 ms using the magnetizer of a cell magnetometry apparatus. Immediately after magnetization, the remnant magnetic field (RMF) was measured for 20 min with a fluxgate magnetometer and recorded with a pen recorder. The glass tubes were set on the sample plate. The strength of magnetic field was measured every 6 s. The temperature was maintained at 37°C by an air fan with a thermostat under a magnetic shield.

The RMF over 20 min after the magnetization was plotted. The rapid reduction of the RMF after the termination of magnetization is called relaxation. The relaxation at 20 min after magnetization, B_{20} (%), was obtained from the formula $B_{20} (\%) = 100/B_0$, where B_{20} is RMF 20 min after the termination of magnetization. The logarithm of RMF for the first 2 min after magnetization was calculated to obtain the intercept with the y-axis. The decay constant (λ) was obtained from the formula $B = B_0 e^{-\lambda t}$, where B_0 is the intercept with the y-axis, and B is RMF at t seconds after the termination of external magnetization.

Lactate dehydrogenase assay

For the lactate dehydrogenase assay (LDH) measurement, the RAW 264.7 cells and AMs were exposed to tested material as described above. After the exposure, the supernatant was sampled and centrifuged at 1,400 rpm for 5 min. After centrifugation, the concentration of LDH in the supernatant

was determined by CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega Corp., Madison, WI). The absorbance at 492 nm was measured by using a microplate-reader (MPR-A4, Tosho Corp., Tokyo). LDH level in each sample was expressed as a percentage of the PBS-added group.

Morphological observation

The RAW 264.7 cells, adhered to a poly-L-lysine-coated glass disc in a glass tube, were exposed to PBS 50 μ l, CF 20, PT or SiC 60 μ g/ml and followed by incubation with 5% CO₂ at 37°C for 48 h as described above.

For the FITC method¹³⁾, the cultured cells were fixed with acetone for 10 min. After washing and blocking, cells were stained for 90 min with α -tubulin FITC conjugate (Sigma-Aldrich, Tokyo) by the direct antibody method. The each samples was dehydrated with 50% ethanol, and mounted in glycerol. The cells were examined by immunofluorescence micrography (Axioplan 2, Zeiss Co., Oberkochen, Germany).

For electron microscopic observation, cells were treated as follows¹³⁾. The samples were exposed to PBS 50 μ l, CF 20 μ g/ml, PT or SiC 60 μ g/ml, and incubated with 5% CO₂ at 37°C for 48 h. After the incubation, the cells were allowed to adhere to polycationic-treated glass slides by pipetting. The adherent cells were washed with 0.1 M cacodylate buffer (pH7.4) and prefixed with 1% glutaraldehyde at 4°C for 3 h. They were washed again with 0.1 M cacodylate buffer

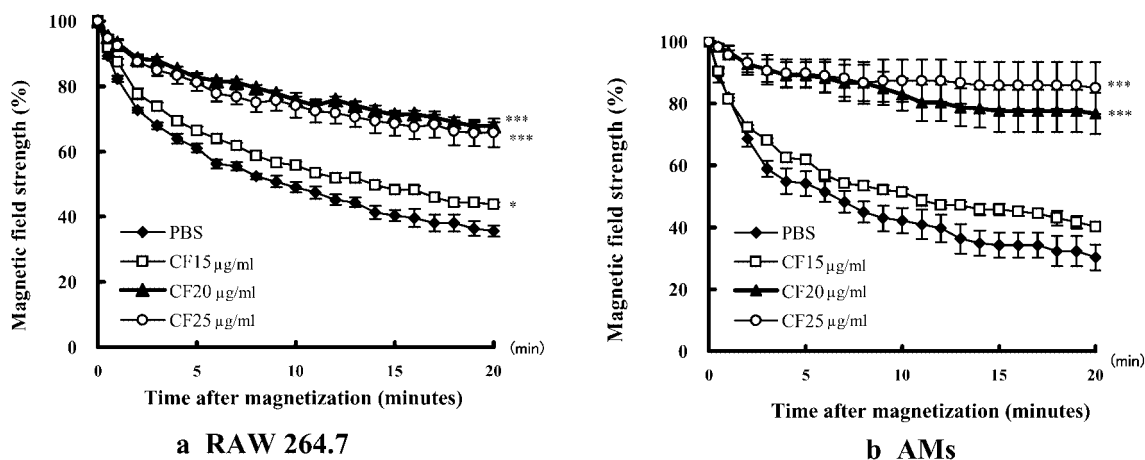


Fig. 2. The relaxation curve of RAW 264.7 cells and alveolar macrophage (AM) exposed to CF after magnetization.
 a. RAW 264.7 cells. b. AMs. The remnant magnetic field strength (RMF) was expressed as a percentage of the initial RMF value. The mean RMF over 20 min after magnetization was plotted. Bars represent SE. Mean values of the B_{20} (%) were compared among the groups (* $p < 0.05$, *** $p < 0.001$; compared with the PBS groups).

(pH7.4) fixed with 1% osmium tetroxide at 4°C for 3 h. Each sample was subsequently re-washed with 0.1 M cacodylate buffer (pH7.4) for observation with a transmission electron microscope (TEM). The fixed cells were subjected to a process of dehydration, resin embedding, ultrathin sectioning with an ultramicrotome and electron staining with uranyl acetate and lead citrate. TEM observation of the cells was performed using a Hitachi H-600 (Hitachi Ltd., Tokyo). For observation with a scanning electron microscope (SEM), the cells were subjected to a process of conductive staining, dehydration, drying and conductive treatment. SEM observation of the cells was performed using a Hitachi S-4500FE (Hitachi Ltd., Tokyo).

Statistical analysis

Data are shown as mean \pm SE of the results obtained from six experiments conducted for both the control and experimental groups. The statistical significance of differences among the groups was examined by one-way ANOVA (analysis of variance) using StatView 5.0 (SAS, Cary, CA), and Fisher's PLSD (protected least significant difference) test was used as a post hoc test (significance level: $p < 0.05$).

Results

The relaxation curves are shown for RAW 264.7 cells and AMs exposed to CF in Fig. 2a, 2b respectively. In the RAW 264.7 cells, the mean values of B_{20} (%) in the CF 15, 20 and 25 $\mu\text{g/ml}$ groups were significantly higher compared

to that in the PBS groups. In the AMs, the mean values of B_{20} (%) in the 20, 25 $\mu\text{g/ml}$ groups were significantly higher compared to those in the PBS groups. The mean values of decay constant are given for RAW 264.7 cells and AMs exposed to CF in Fig. 3a, 3b respectively. The mean decay constants of RAW 264.7 cells exposed to 15, 20 and 25 $\mu\text{g/ml}$ of CF were significantly lower compared to those of the respective PBS groups. The mean value decay constants of AMs exposed to 20 and 25 $\mu\text{g/ml}$ of CF were significantly lower compared to those of the PBS groups.

The relaxation curves are shown for RAW 264.7 cells exposed to PT and SiC in Fig. 4a, 4b, respectively. In the PT groups, RAW 264.7 cells exposed to 20, 40 and 60 $\mu\text{g/ml}$ the mean values of B_{20} (%) were significantly higher compared to those of the respective PBS groups. In the SiC groups, RAW 264.7 cells exposed to 20, 40 and 60 $\mu\text{g/ml}$ groups, the mean values of B_{20} (%) were significantly higher compared to those in the respective PBS groups. The extent of the delay in relaxation occurs dose-dependently for PT and SiC. The mean values of decay constants are given for RAW 264.7 cells exposed to PT and SiC in Fig. 5a, b, respectively. In the PT groups, the mean decay constants of the groups exposed to 20, 40 and 60 $\mu\text{g/ml}$ were significantly lower compared to those of the respective PBS groups. In the SiC groups, the mean decay constants of the groups exposed to 20, 40 and 60 $\mu\text{g/ml}$ were significantly lower compared to those of the respective PBS groups. For both groups exposed to SiC and PT, the decay constants of the groups became smaller as the concentrations increased.

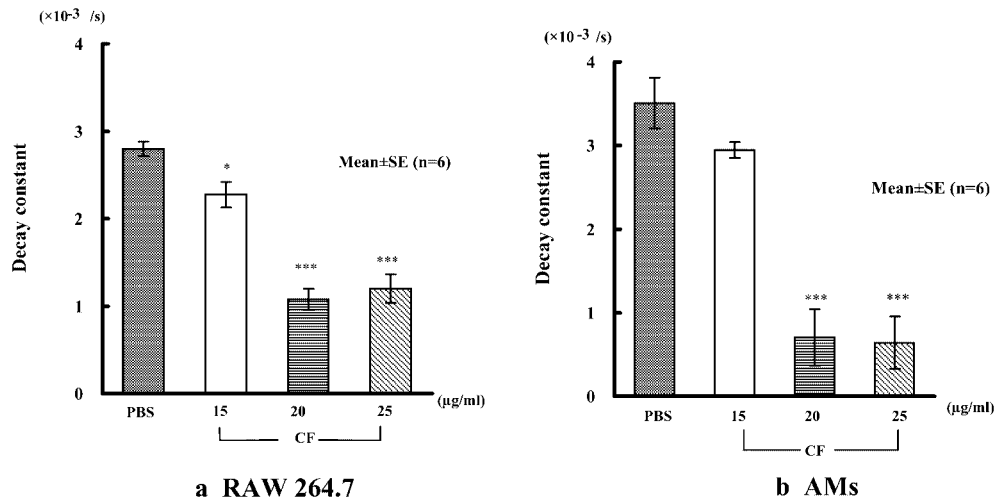


Fig. 3. The decay constant of RAW 264.7 cells and alveolar macrophage (AM) exposed to CF after magnetization.

a. RAW 264.7 cells. b. AMs. Bars represent mean values, and error bars represent SE (n=6). The mean values of decay constants (λ) were compared among the groups (* p <0.05, *** p <0.001; compared with the PBS groups).

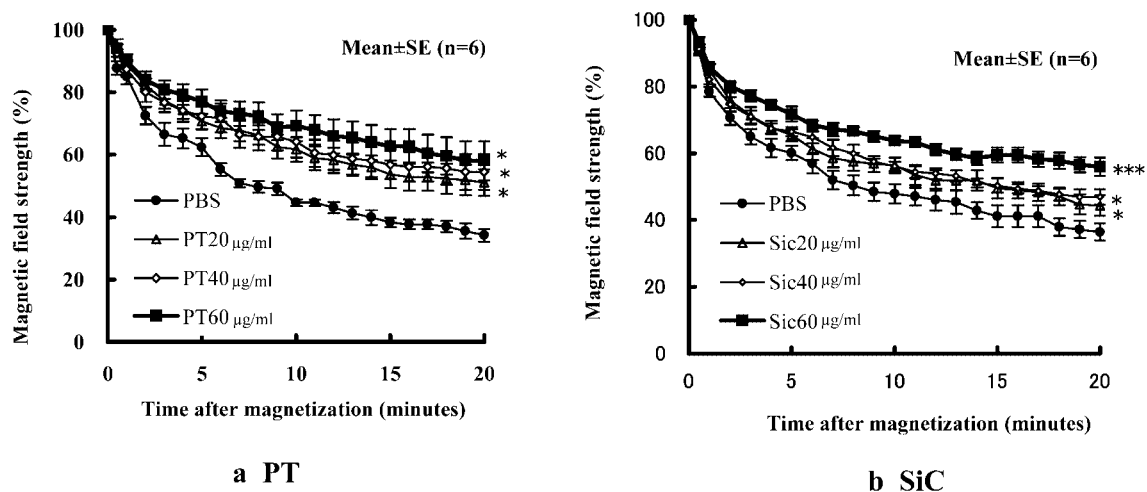


Fig. 4. The relaxation curve of RAW 264.7 cells exposed to PT or SiC after magnetization.

The remnant magnetic field strength (RMF) was expressed as a percentage of the initial RMF value. The mean RMF over 20 min after magnetization was plotted. Bars represent SE. Mean values of the B_{20} (%) were compared among the groups (* p <0.05, *** p <0.001; compared with the PBS groups).

LDH assay

The mean concentration of LDH in the supernatant of RAW 264.7 cells exposed to CF is shown in Fig. 6a, while that of AMs is shown in Fig. 6b. RAW 264.7 cells exposed to CF 15, 20 and 25 μ g/ml had significantly higher mean values compared to the control. AMs exposed to CF 20 and 25 μ g/ml had significantly higher mean values compared to the respective control. The concentration of LDH in the supernatant of RAW 264.7 cells exposed to PT or SiC are

shown in Fig. 7. Significantly higher mean values were observed in the PT 20, 40 and 60 μ g/ml groups, and the SiC 20, 40 and 60 μ g/ml groups compared to the respective control.

Morphological observation

Immunofluorescent micrographs of the cells stained with FITC anti- α tubulin are shown in Fig. 8a–d for the PBS, CF, PT and SiC groups, respectively. The fine network of

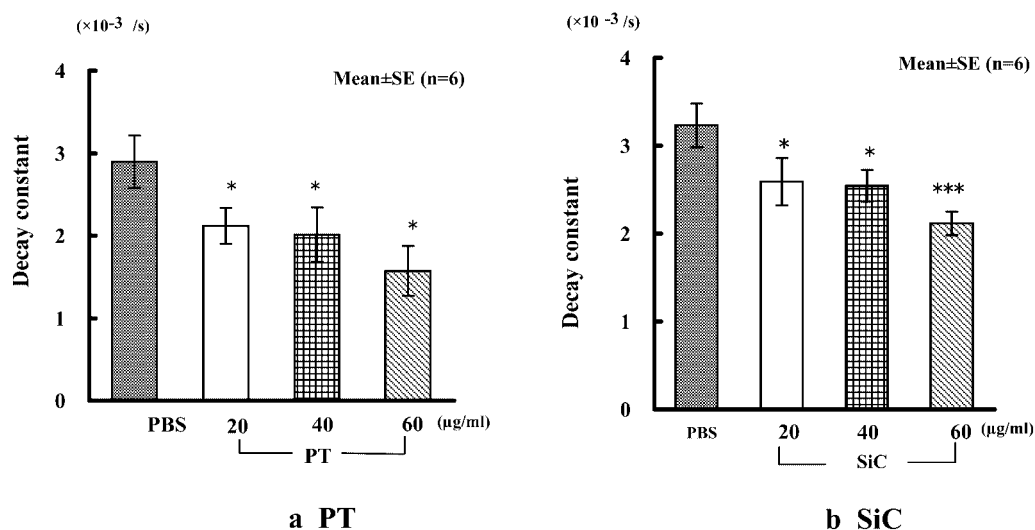


Fig. 5. The decay constant of RAW 264.7 cells exposed to PT or SiC after magnetization.

Each bar represents mean value, and error bars represent SE (n=6). The mean values of decay constants (λ) were compared among the groups (* p <0.05, ** p <0.001; compared with the PBS groups).

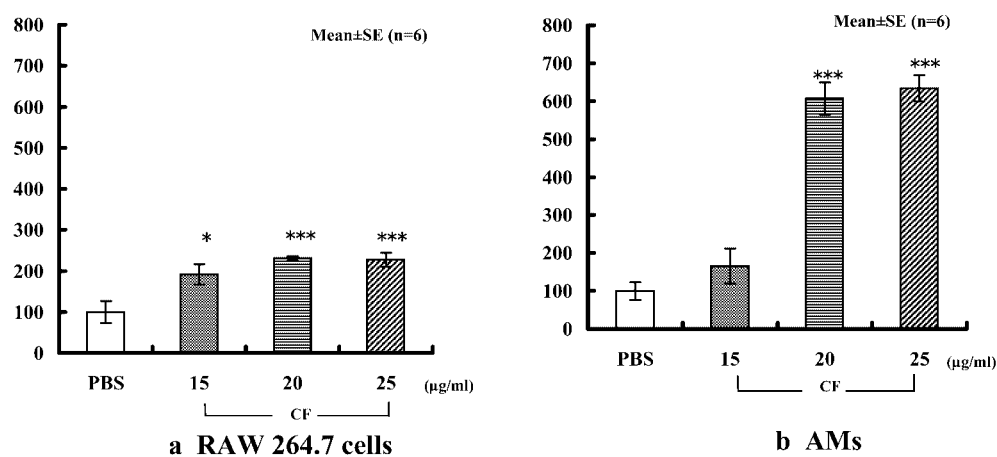


Fig. 6. The mean value of LDH in the supernatant of RAW 264.7 cells and alveolar macrophage (AM) exposed to CF.

Bars represent mean values and error bars represent SE (n=6). LDH mean values were compared among the groups (* p <0.05, ** p <0.001; compared with the PBS groups).

microtubules and the spherical shape of the cells were clearly seen in the PBS groups. In the CF group, the englobed fibers were found in the cytoplasm. The aggregation of microtubules was seen, and the spherical shape of the cells became unclear. In the PT groups, the aggregation of microtubules was seen while the spherical shape of the cell was also unclear. The englobed fibers were also found in the cytoplasm. In the SiC groups, the aggregation of microtubules was partly observed in the cells that englobed fibers. While the spherical shape of the cells was relatively clear compared with the CF or PT- exposed cells. SEM

images of the RAW 264.7 cells are shown in Fig. 9a–d for the PBS, CF, PT and SiC groups, respectively. In the PBS group, the cell was spherical, and many microvilli were observed on the surface. In the CF groups, the spherical shape of the cell was lost, the microvilli on the surface disappeared, and many fibers twined around and englobed the cells. Likewise, in the PT and SiC groups, fibers tangled around and englobed the cells. The depletion of microvilli from the surface of the cells was also observed. TEM images of the RAW 264.7 cell are shown in Fig. 10a–d of the PBS, CF, PT and SiC groups, respectively.

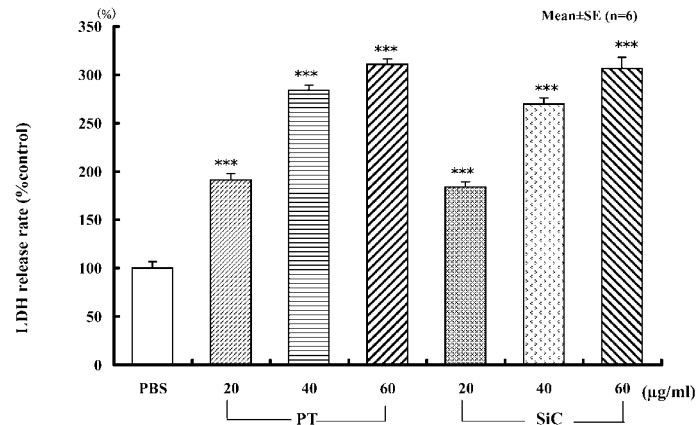


Fig. 7. The mean value of LDH in the supernatant of RAW 264.7 cells exposed to PT or SiC.

Bars represent mean values and error bars represent SE (n=6). LDH mean values were compared among the groups (***) $p < 0.001$; compared with the PBS group).

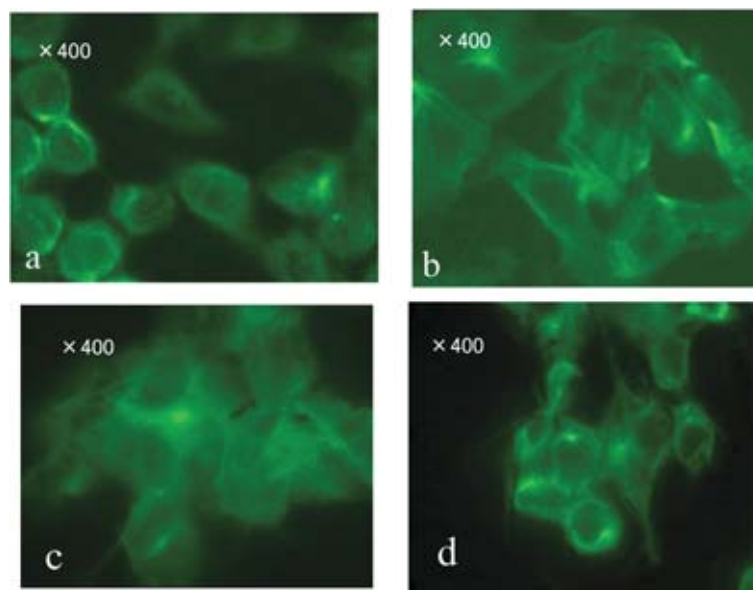


Fig. 8. Immunofluorescent micrographs of RAW 264.7 cells exposed to CF, PT or SiC.

Cells stained with α -tubulin FITC conjugate (Sigma-Aldrich, Tokyo) by the antibody method $\times 400$. a. A fine network of microtubules was observed in the PBS group. b. An aggregation of microtubules was observed in the CF 20 $\mu\text{g/ml}$ group. c. An aggregation of microtubules were observed while the spherical shape of the cells were unclear in the PT 60 $\mu\text{g/ml}$ group. d. An aggregation of microtubules was partly observed in the cells which englobed fibers in the SiC 60 $\mu\text{g/ml}$ group.

In the PBS group, a horseshoe-shaped nucleus, phagocytic vacuoles and lysosomes were observed. In the CF group, many vacuoles were observed, and a few fibers were englobed in the cytoplasm. In the PT group, many vacuoles also were observed, and many fibers were englobed in the cytoplasm.

SiC, being an extremely hard material, made it difficult to slice samples. The micrograph was relatively unclear, however, a few fibers and many vacuoles in the cytoplasm could be observed.

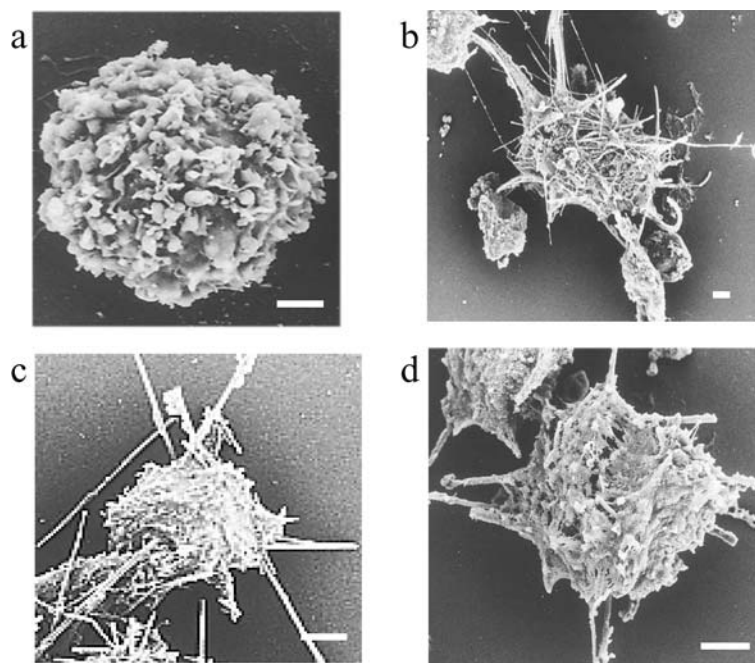


Fig. 9. Scanning electron micrograph of RAW 264.7 cells exposed to CF, PT or SiC.

RAW 264.7 cells exposed to PBS, CF 20 $\mu\text{g/ml}$, PT 60 $\mu\text{g/ml}$, SiC 60 $\mu\text{g/ml}$ were incubated at 37°C in 5% CO_2 for 48 h. Bar=1 μm . a. RAW 264.7 cells exposed to PBS, a large number of microvilli on the surface of ball-shaped cells were observed. b. Exposed to CF 20 $\mu\text{g/ml}$, many fibers that had twined around the cells were visible. c. Exposed to PT 60 $\mu\text{g/ml}$, show that many fibers pierce the cells. d. In cells exposed to SiC 60 $\mu\text{g/ml}$, many fibers were seen piercing the cells from various directions.

Discussion

Cell magnetometry using AMs is a useful method for screening the toxic effects of fibrous materials⁹⁻¹³. Magnetic particles of Fe_3O_4 are engulfed by macrophages obtained from bronchoalveolar lavage, and the cells are magnetized externally. The RMF, generated from magnetized Fe_3O_4 in cells, is measured right after external magnetization. The rapid reduction of RMF is called relaxation. Unidirectional particles are unified by external magnetization. However, after the cessation of magnetization, these particles rotate randomly due to phagocytic movement of the cytoskeleton¹⁸, the orderly arranged alignment of magnetic particles is disturbed according to time, and as a result, RMF is attenuated. The cytoskeleton is mainly composed of microtubules, microfilaments and intermediate filaments, and plays an important role in the maintenance of the structure of the cytoplasm, intracellular transport^{19, 20}. Delayed relaxation observed in association with exposure to toxic substances would be attributable to physical and chemical properties of toxic substances^{18, 21}.

The murine cell line RAW 264.7 is lately being used as an *in vitro* model of alveolar macrophage. If RAW 264.7 cells can be used in cell magnetometry, it would be possible to evaluate the toxicities of fibrous materials without the limitations of animal experiments. To examine the usefulness of RAW 264.7 cells in cell magnetometry, we compared RAW 264.7 cells and AMs exposed to CF with cell magnetometry.

In cell magnetometry in RAW 264.7 cells and AMs, rapid relaxation was observed in the PBS groups on the experimental protocol in this study. In RAW 264.7 cells, the groups exposed to CF 15, 20 and 25 $\mu\text{g/ml}$ demonstrated significantly delayed relaxation compared to that in the respective PBS groups. In AMs, the groups exposed to CF 20 and 25 $\mu\text{g/ml}$ demonstrated significantly delayed relaxation compared to that in the respective PBS groups. The mean decay constants of RAW 264.7 cells exposed to CF 15, 20 and 25 $\mu\text{g/ml}$ were significantly lower compared to those in the PBS groups. In AMs, the groups exposed to CF 20 and 25 $\mu\text{g/ml}$ were significantly lower means compared to those in the respective PBS groups. RAW 264.7

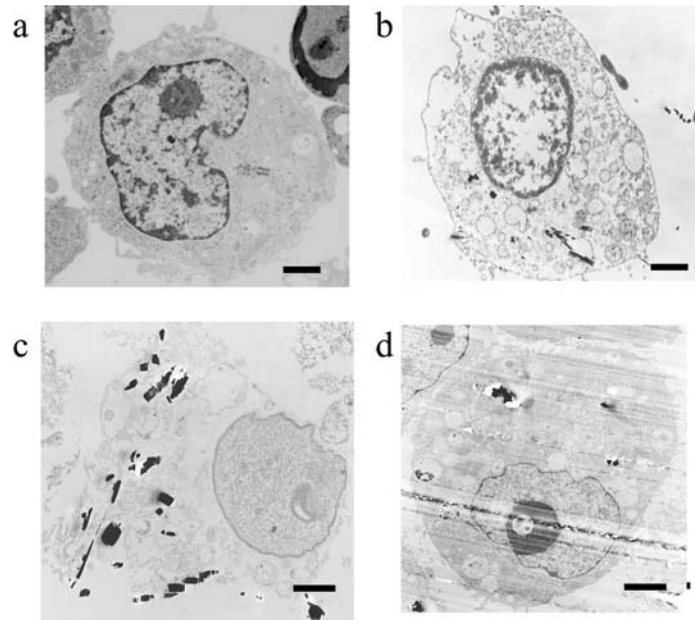


Fig. 10. Transmission electron micrographs of RAW 264.7 cells exposed to CF, PT or SiC.

RAW 264.7 cells exposed to PBS or CF 20 $\mu\text{g}/\text{ml}$ or PT 60 $\mu\text{g}/\text{ml}$ or SiC 60 $\mu\text{g}/\text{ml}$ were incubated at 37°C in 5% CO_2 for 48 h. Bar=1 μm . a. RAW 264.7 cells exposed to PBS. The shape of the cell appears round. b. In the cell exposed to CF 20 $\mu\text{g}/\text{ml}$, many vacuoles were visible, and a few fibers were observed in the cytoplasm. c. Cells exposed to PT 60 $\mu\text{g}/\text{ml}$ show many vacuoles and fibers englobed in the cytoplasm. d. The cells exposed to SiC 60 $\mu\text{g}/\text{ml}$ show many vacuoles, and a few fibers were visible in the cytoplasm.

cells proved more sensitive than AMs to detect the difference in cell magnetometry. These results suggest that it is adequate to use RAW 264.7 cells in cell magnetometry. For the LDH level in the supernatant, the RAW 264.7 cells exposed to 15 $\mu\text{g}/\text{ml}$ of CF and the AMs exposed to 20 $\mu\text{g}/\text{ml}$ of CF had significantly higher mean values compared to their respective controls. These results were related with those of the cell magnetometry. Compared to the levels that induce significant difference in cell magnetometry and LDH in RAW 264.7 cells, the 15 $\mu\text{g}/\text{ml}$ of CF groups had significantly higher mean values compared to the control in both cell magnetometry and LDH. Therefore, it is suggested that cell magnetometry and LDH can be good indicators of cytotoxicity of fibers at the same level.

On the basis of these results, the toxic effects of PT or SiC were evaluated by cell magnetometry and LDH assay. RAW 264.7 cells exposed to PT 20, 40 and 60 $\mu\text{g}/\text{ml}$ and exposed to SiC 20, 40 and 60 $\mu\text{g}/\text{ml}$ demonstrated significantly delayed relaxations compared to those in the respective PBS groups. The mean values of B_{20} (%) were higher in the groups exposed to PT and SiC in a dose-

dependent manner. The mean values of decay constants of the groups exposed to SiC and PT were smaller as the concentrations increased. The decay constants of the PT 20, 40, 60, SiC 20, 40 and 60 $\mu\text{g}/\text{ml}$ groups were significantly lower compared with those of the respective PBS groups. These results from the mean value of B_{20} (%) and the decay constants suggested that the exposure to MMMFs as well as the exposed to CF impaired the function of RAW 264.7 cells. The level of LDH in the supernatant of RAW 264.7 cells exposed to PT or SiC increased in a dose-dependent manner. The significantly higher values were observed in the PT 20, 40 and 60 $\mu\text{g}/\text{ml}$ groups, and the SiC 20, 40 and 60 $\mu\text{g}/\text{ml}$ groups.

In exposed PT groups, the results of LDH levels were the same as those in AMs in a previous study¹¹. In exposed SiC groups, the results of LDH levels were related the cell magnetometry measurement. In previous studies, there were no significant increases in LDH levels in the supernatant of AMs exposed to SiC groups¹⁰. In present study, LDH measurement may be sensitive to detect the toxic effect of PT and SiC. PT and SiC fibers are relatively long and the

number of PT and SiC fibers per gram is larger than that of CF fibers²⁴). This may be a factor in the strong impairment of the cell membranes of RAW 264.7 cells.

In a previous study²⁵), the RAW 264.7 cells exposed to PT or SiC at 100 $\mu\text{g/ml}$ had a significantly higher level of LDH compared with the control. In addition, the increase in LDH was similar to cells exposed to crocidolite. Increases in LDH in the supernatants of RAW 264.7 cells exposed to high concentrations of PT or SiC were confirmed in this study at relatively lower levels. In inhalation studies of PT and SiC in rats, pulmonary fibrosis²⁶), and mesotheliomas and pulmonary fibrosis²⁷) were observed, respectively. This suggested the possible injurious nature of PT and SiC to the lungs. The impairment of macrophages by PT or SiC suggested in the present study could be a mechanism of lung injury induced by PT or SiC *in vivo*.

To examine actual phagocytosis and cytoskeletal changes, we conducted morphological observations using the electron micrograph and fluorescent antibody method (α -tubulin FITC conjugate). From the results of TEM, the cells actually phagocytosed CF, PT and SiC fibers. The increase in the number of vacuoles in the cytoplasm of the cells that engulfed CF, PT and SiC fibers were related the observations of AMs by TEM in previous studies^{10, 11, 22}). Phagocytosis of fibers was also confirmed in SEM observations. Decrease in the number of microvilli on the surface of the cells that engulfed CF, PT and SiC fibers also were related the observations of AMs by SEM in those studies. In morphological observations using immunofluorescent staining in the cells exposed to levels that showed significant increase in B_{20} (%), aggregation of microtubules was observed in the cell-englobed fibers. Microtubules, one of the components of the cytoskeleton, are related to rotational movement of phagosomes. From observations using immunofluorescent staining, impairment of the cytoskeleton was suggested in the cells that exhibited delayed relaxation.

In conclusion, these results of MMMFs suggest that cell magnetometry using RAW 264.7 cells is useful for the evaluation of toxic effects. The combined use of both LDH and cell magnetometry may be the most efficacious way to evaluate MMMFs.

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