

Original Article

Two-week Toxicity of Multi-walled Carbon Nanotubes by Whole-body Inhalation Exposure in Rats

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Abstract: To evaluate pulmonary toxicity of multi-walled carbon nanotubes (MWCNTs), F344 rats of both sexes were exposed by inhalation to 0.2, 1 or 5 mg/m³ MWCNT aerosol for 6 h/day, 5 days/week for 2 weeks using a whole-body exposure system. At the end of the 2-week exposure period, one-half of the rats were necropsied, and at the end of an additional 4-week postexposure period, the remaining rats were necropsied. MWCNTs were deposited in the lungs of all MWCNT-exposed groups and mostly remained in the lungs throughout the 4-week postexposure period. Granulomatous changes in the lung were found in the rats exposed to 5 mg/m³ MWCNTs, and these changes were slightly aggravated at the end of the 4-week postexposure period. In the bronchoalveolar lavage fluid (BALF), the numbers of neutrophils, percentages of bi- and multinucleated alveolar macrophages, levels of ALP activity and concentrations of total protein and albumin were elevated in the rats exposed to 1 and 5 mg/m³ MWCNTs. At the end of the 4-week postexposure period, the values of the BALF parameters tended to remain elevated. In addition, goblet cell hyperplasias in the nasal cavity and nasopharynx were observed in the rats exposed to 1 and 5 mg/m³ MWCNTs, but these lesions had largely regressed by the end of the postexposure period. Based on the histopathological and inflammatory changes, the no-observed-adverse-effect level (NOAEL) for inhalation of MWCNTs for 2 weeks was 0.2 mg/m³. (DOI: 10.1293/tox.26.131; J Toxicol Pathol 2013; 26: 131–140)

Key words: multi-walled carbon nanotube, pulmonary toxicity, inhalation, whole-body exposure, rat

Introduction

Nanotechnology provides our society with materials with exceptional electrical, mechanical and thermal properties. Carbon nanotubes, which were discovered by Iijima in 1991¹, are nanomaterials with numerous applications in industry. The total volume of production and import of multi-walled carbon nanotubes (MWCNTs) was approximately 500 tons for the fiscal year 2008 in Japan². With the rapid growth of the MWCNT industry, however, concern has been raised about the health of workers who are exposed to MWCNTs in their occupational settings.

Neither epidemiological nor medical case studies have been reported on the health consequences of MWCNT exposure; therefore, MWCNT hazard is primarily assessed by toxicity studies using rodents. Since humans are exposed primarily by inhalation to MWCNT aerosol during its manufacture, handling and cleanup, toxicity data obtained from inhalation exposure of rodents to aerosolized MWCNTs is

the best method of determining risk assessment and the influences of MWCNTs on the health of MWCNT-exposed humans: Inhalation studies use either MWCNTs dissolved in a solvent and then aerosolized or dry MWCNTs that are directly aerosolized.

There are several reports of acute and subchronic studies of inhalation exposure of rodents to aerosolized MWCNTs. Nose-only exposure of rats to aerosolized dry MWCNTs resulted in pulmonary toxicity after a single exposure³ and in 13-week studies^{4,5}. In contrast to these studies using rats, a study using mice reported that whole-body exposure of mice to aerosolized dry MWCNT for 7 or 14 days did not result in pulmonary toxicity⁶. However, a more recent study using mice reported pulmonary inflammation and damage in mice after a single whole-body exposure to aerosolized dry MWCNTs⁷. In another study, Morimoto *et al.*⁸ reported whole-body exposure of rats to aerosolized MWCNTs using a nebulizer and mist dryer for 4 weeks resulted in a transient pulmonary inflammatory response. However, inhalation toxicity studies using whole-body exposure of rats to aerosolized dry MWCNTs have not yet been reported.

In addition to the acute and subchronic studies noted above, carcinogenicity studies have reported that intraperitoneal or intrascrotal injection of MWCNTs resulted in the development of mesotheliomas in p53-heterozygous mice⁹ and in F344 rats¹⁰. These studies demonstrated that the type

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of fibers formed by MWCNTs have the potential to present a risk similar to that of asbestos and induce inflammation and mesothelioma in the pleura¹¹. Furthermore, Mercer *et al.*¹² showed that exposure of mice to MWCNTs by pharyngeal aspiration resulted in MWCNT fiber penetration of the visceral pleural surface and suggested the need to investigate the chronic toxicity of MWCNTs for risk of mesothelioma development in humans. All these data indicate that a carcinogenicity study of MWCNTs by inhalation exposure using experimental animals is needed. For a long-term inhalation study, OECD guidance documents^{13,14} recommend using whole-body chambers.

Our ultimate goal is to carry out a two-year carcinogenicity study in rats exposed to aerosolized dry MWCNTs by whole-body inhalation exposure. To this end, we have developed a whole-body MWCNT exposure system¹⁵. In the study reported here, we conducted a 2-week MWCNT inhalation study of rats using this new system as a preliminary study for a two-year carcinogenicity study. The current study presents the pulmonary toxicity data after 2 weeks of whole-body exposure to aerosolized dry MWCNTs.

Materials and Methods

Test substance

MWCNTs, surface area 24–28 m²/g and purity 99.8% (wt/wt), were purchased from Hodogaya Chemical, Co., Ltd. (MWNT-7, Lot No. 080126, Tokyo) and used in the present study without further purification or sieving. The MWCNTs had a mean \pm SD width of 88 \pm 5 nm and length of 5.0 \pm 4.5 μ m with 38.9% $>$ 5 μ m length¹⁶.

Animals

Five-week-old F344/DuCrjCrj rats of both sexes were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The animals were cared for in accordance with the Guideline for Animal Experimentation¹⁷. The present study was approved by the ethics committee of the Japan Bioassay Research Center (JBRC). The animals were quarantined and acclimated for a week before the start of the experiment. The animals were housed individually in stainless steel wire hanging cages (150W \times 216 D \times 176 H mm) that were placed in stainless steel inhalation exposure chamber with a volume of 1.24 m³. The environment in the chamber was maintained at 22.1–22.6°C and a relative humidity of 54.6–57.0% with 12 air changes per hour (248 liters/min). Fluorescent lighting was controlled automatically to give a 12-hr light/dark cycle. Except during MWCNT exposure, animals had free access to sterilized water and γ -irradiation-sterilized commercial pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan).

Experimental design

Groups of 10 rats of both sexes were exposed to either clean air (control) or MWCNT aerosol at a target concentration of 0.2, 1 or 5 mg/m³ for 6 hrs/day, 5 days/week for 2 weeks. Five mg/m³ was selected as the highest concentra-

tion of MWCNTs because it is the Occupational Safety and Health Administration (OSHA) permissible exposure limit (PEL) value for synthetic graphite¹⁸, and this value is often cited on material safety data sheets by manufacturers. At the end of the 2-week exposure period, 5 rats from each group were necropsied. The remaining rats were necropsied at the end of a 4-week postexposure period without any treatment.

Aerosol generation and inhalation exposure to MWCNTs

The system and method for the generation of MWCNT aerosols and inhalation exposure of rats to the dry aerosol in the inhalation chamber has been described previously¹⁵. In the present study, MWCNT aerosols of 0.2, 1 or 5 mg/m³ were generated using the cyclone sieve method (sieve of 200 mm in diameter and 53 μ m in pore size), and 10 unrestrained, individually housed rats of both sexes were exposed to one of the aerosols in the inhalation chamber.

Monitoring of MWCNT aerosol in the exposure chamber

The methods for determination of concentrations and size distribution of MWCNT aerosol in the inhalation exposure chamber were also described in our previous report¹⁵. Briefly, the concentration of the MWCNT aerosol in the exposure chamber was continuously monitored with an optical particle controller (OPC) (OPC-AP-600, Sibata Scientific Technology Ltd., Tokyo, Japan). The OPC electric signal was fed into the dust feeder with a feedback control system so that chamber aerosol concentrations were maintained at a constant level. The mass concentrations of MWCNTs were determined gravimetrically by collecting the aerosol on a Teflon-binder filter three times (1, 3 and 5 hours) every exposure day. Chamber atmosphere samples were taken adjacent to the animals' breathing zone. The size distribution of MWCNT particles was determined for mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) using a micro-orifice uniform deposit cascade impactor (MOUDI) (Model 125B NanoMoudi-II, MSP, Shoreview, MN, USA).

Clinical observations and pathological examinations

The animals were observed daily for clinical signs and mortality. Body weight and food consumption were measured weekly throughout the study period. At the end of the 2-week exposure period, one-half of the rats were necropsied, and at the end of an additional 4-week postexposure period the remaining rats were necropsied. Blood was collected for hematology and blood biochemistry from the abdominal aorta of rats under pentobarbital anesthesia after overnight fasting. Organs including the thymus, adrenal, testis, ovary, heart, left lung, kidney, spleen, liver and brain were weighed, and all organs and tissues were examined for macroscopic lesions. The organs including the lung, trachea, nasal cavity, bronchus-associated lymphoid tissue (BALT), peritracheal lymph node, liver and kidney were fixed in 10% neutral buffered formalin and embedded in paraffin.

The nasal cavity was decalcified in a formic acid-formalin solution prior to trimming and was transversely trimmed at three levels as described previously¹⁹. Tissue sections of 5 μm in thickness were prepared and stained with hematoxylin and eosin (H & E). To detect MWCNTs deposited in the nasal cavity, lung and peritracheal lymph node, sections were stained with Kernechtrot stain (Merck, Darmstadt, Germany) for 1 min and washed with distilled water for 5 min. To identify collagen fibers, Sirius red-stained sections were stained with F3B/picric acid for 1-2 hour, washed with 0.01 N HCl for 1 min and counterstained with Mayer's hematoxylin for 2 min.

Biochemical and cytological analyses of the bronchoalveolar lavage fluid (BALF)

After euthanization under pentobarbital anesthesia, the left bronchus was tied in order to lavage only the right lung. The right lung was lavaged 2 times with 4 ml of physiological saline solution, and the lavage fluid was collected. For cytological analysis, total cells in the BALF were counted with an automatic cell analyzer (ADVIA120, Siemens Healthcare Diagnostics Inc. Tarrytown, NY, USA). The BALF was centrifuged at 700 rpm ($55 \times g$) for 5 min with a cytocentrifuge (Cytospin4, Thermo Fisher Scientific Inc. Waltham, MA, USA), and the cellular components were stained with May-Grünwald-Giemsa. The numbers of neutrophils, lymphocytes and alveolar macrophages were counted for a total of more than 500 cells under a light microscope, and then corrected for total cells/ μL BALF. For biochemical analysis, the BALF was centrifuged at 1960 rpm ($800 \times g$) and 4°C for 10 min, and aliquots of the acellular supernatant were used for biochemical analysis with an automatic analyzer (Hitachi 7080, Hitachi, Ltd., Ibaraki, Japan). Total protein (TP), albumin and alkaline phosphatase (ALP) activity were measured by conventional biochemical methods. TP and albumin were chosen as indicators of alveolo-capillary permeability, because they are believed to pass into the alveolar space by passive transudation from the serum. ALP activity was used as an indicator of the activity of type II epithelial cells.

Statistics

Body weight, organ weight and biochemical and cytological parameters in the BALF were analyzed by Dunnett's multiple comparison test. Differences between groups at $P < 0.05$ were considered significant.

Results

Concentration and particle size distribution

The MMAD (GSD) of the 0.2, 1 and 5 mg/m^3 MWCNT aerosols measured with a MOUDI were 1.3 (2.7), 1.2 (3.4) and 1.4 (2.4) μm , respectively, with 78 - 82% of the mass fraction below 3 μm , the inhalable fraction. The mass concentrations of the 0.2, 1 and 5 mg/m^3 MWCNT aerosols as determined gravimetrically with the Teflon-binder filter were 0.21 ± 0.02 , 1.07 ± 0.12 and 5.09 ± 0.35 mg/m^3 (mean

\pm SD).

Mortality and clinical signs

Neither death nor clinical signs were observed in any MWCNT or clean air control animals in the 2-week exposure or 4-week postexposure periods. There was no growth retardation of greater than 10% in any group exposed to MWCNTs for 2 weeks, although, body weights were lower than in the control groups (Table 1). There were no significant differences between the clean air controls and the MWCNT-exposed groups in the body weights at the end of the 4-week postexposure period (Table 1).

Pathological findings

The relative lung weights were slightly increased, by 1.15-fold, in the male and female rats exposed to 5 mg/m^3 at the end of the 2-week exposure period (Table 1). There were no significant differences between the clean air controls and the MWCNT-exposed groups in the weights of any of the organs at the end of the 4-week postexposure period (Table 1).

The deposition of MWCNTs in the upper and lower respiratory tracts is listed in Table 2. The MWCNTs were black, straight shapes and were deposited separately as single-like fibers. MWCNT fibers were deposited in the nasal cavity (respiratory epithelium) of the rats exposed to 1 and 5 mg/m^3 MWCNTs. Non-phagocytosed MWCNT deposition in the nasal cavity was primarily in the non-ciliated respiratory epithelium at levels 1 and 2. At the end of the 4-week postexposure period, MWCNT fibers were found in the nasal cavities of the rats exposed to 5 mg/m^3 MWCNTs, but not the rats exposed to 1 mg/m^3 MWCNTs.

MWCNT fibers were deposited in the lung (bronchi and alveolar space and alveolar wall) of all the exposed groups at the end of the exposure period, as shown in Table 2. MWCNTs were detected primarily within alveolar macrophages with, a few free MWCNT fibers found in the bronchi and alveolar space (Fig. 1). Although the incidence of MWCNT deposition in the bronchi and alveolar space were equal in the rats exposed to 1 and 5 mg/m^3 MWCNTs, the quantity of MWCNTs was higher in the rats exposed to 5 mg/m^3 MWCNTs. Somewhat longer MWCNT fibers tended to remain in the alveolar space at the end of the 4-week postexposure period. The incidence of MWCNT deposition in the alveolar wall in the rats exposed to 0.2 mg/m^3 MWCNTs increased slightly after the 4-week postexposure period. Persistent deposition of MWCNTs in the lung (bronchi and alveolar space and alveolar wall) was observed in all the exposed groups at the end of the 4-week postexposure period.

In the BALT and peritracheal lymph node, MWCNT deposition was found mainly in the rats exposed to 5 mg/m^3 MWCNTs at the end of the exposure period. At the end of the 4-week postexposure period, MWCNT deposition in the BALT and peritracheal lymph node was seen in the both the rats exposed to 1 and 5 mg/m^3 MWCNTs. The incidence of MWCNT deposition in the BALT and peritracheal lymph node of the rats exposed to 1 mg/m^3 MWCNTs was increased at end of the 4-week postexposure period compared

Table 1. Body Weight and Lung Weight of Rats at the End of the 2-week Exposure Period and the End of the 4-week Postexposure Period

Group (mg/m ³)	0	0.2	1	5
<Male>				
At the end of the 2-week exposure period				
Number of animals examined	5	5	5	5
Body weight (g)	180 ± 9	171 ± 9 *	174 ± 9	168 ± 6 **
		(95 %)	(97 %)	(93 %)
Relative lung weight (%)	0.192 ± 0.019	0.201 ± 0.004	0.206 ± 0.010	0.220 ± 0.011 **
At the end of the 4-week postexposure period				
Number of animals examined	5	5	5	5
Body weight (g)	255 ± 8	251 ± 10	249 ± 15	241 ± 14
		(98 %)	(98 %)	(95 %)
Relative lung weight (%)	0.159 ± 0.011	0.169 ± 0.018	0.174 ± 0.009	0.181 ± 0.011
<Female>				
At the end of the 2-week exposure period				
Number of animals examined	5	5	5	5
Body weight (g)	123 ± 4	116 ± 5 **	116 ± 4 **	116 ± 5 **
		(94 %)	(94 %)	(94 %)
Relative lung weight (%)	0.235 ± 0.018	0.244 ± 0.029	0.231 ± 0.015	0.270 ± 0.023
At the end of the 4-week postexposure period				
Number of animals examined	5	5	5	5
Body weight (g)	155 ± 9	147 ± 5	149 ± 6	147 ± 5
		(95 %)	(96 %)	(95 %)
Relative lung weight (%)	0.213 ± 0.012	0.214 ± 0.003	0.223 ± 0.017	0.223 ± 0.015

*: $p < 0.05$ by Dunnett's multiple comparison test. **: $p < 0.01$ by Dunnett's multiple comparison test. Values are means ± SD. The data in parenthesis indicate the percentage of the body weight in the control group.

Table 2. Deposition of MWCNTs in the Upper and Lower Respiratory Tracts and Lymph Nodes of Rats

Group (mg/m ³)	At the end of the 2-week exposure period				At the end of the 4-week postexposure period			
	0	0.2	1	5	0	0.2	1	5
Number of animals examined	5	5	5	5	5	5	5	5
<Male>								
Nasal cavity								
Respiratory epithelium	0	0	1	4	0	0	0	5
Lung								
Bronchiolar space	0	3	5	5	0	2	5	5
Alveolar space	0	5	5	5	0	5	5	5
Alveolar wall	0	0	5	5	0	2	5	5
BALT	0	0	0	5	0	0	5	5
Peritracheal lymph node	0	0	0	1	0	0	1	5
<Female>								
Nasal cavity								
Respiratory epithelium	0	0	2	5	0	0	0	5
Lung								
Bronchiolar space	0	4	5	5	0	2	5	5
Alveolar space	0	5	5	5	0	5	5	5
Alveolar wall	0	1	5	5	0	2	5	5
BALT	0	0	2	5	0	0	5	5
Peritracheal lymph node	0	0	0	3	0	0	1	5

Values indicate number of animals bearing the lesions.

with the incidence of MWCNT deposition at the end of the 2-week exposure period. Somewhat shorter MWCNT fibers were seen in the lymph node (Fig. 2).

Inhalation of MWCNT fibers for 2 weeks effected changes in the upper and lower respiratory tract in both male and female rats (Table 3). Goblet cell hyperplasia in

the nasal cavity and nasopharynx were observed in the 1 and 5 mg/m³ groups at the end of the 2-week exposure period. Goblet cell hyperplasia was characterized by increased numbers of goblet (mucous) cells in the respiratory epithelium (Fig. 3). Goblet cell hyperplasia in the nasal cavity and nasopharynx had largely regressed by the end of the 4-week

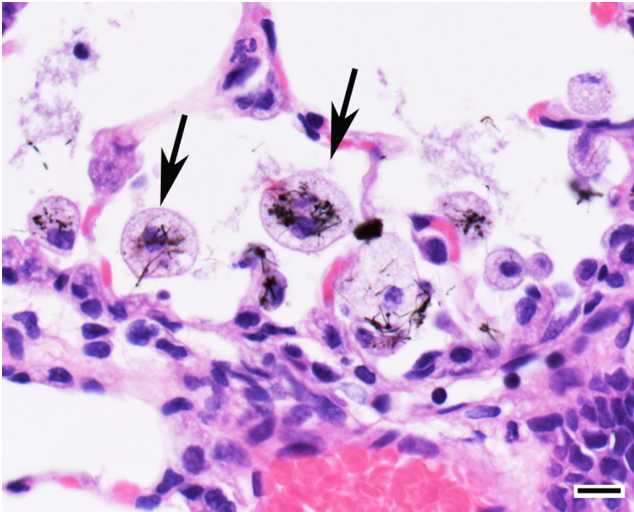


Fig. 1. MWCNTs phagocytosed by alveolar macrophages (arrows) in the alveolar space of the lung of a male rat exposed to 5 mg/m³ at the end of the 2-week exposure period. Bar indicates 10 µm. H&E stain.

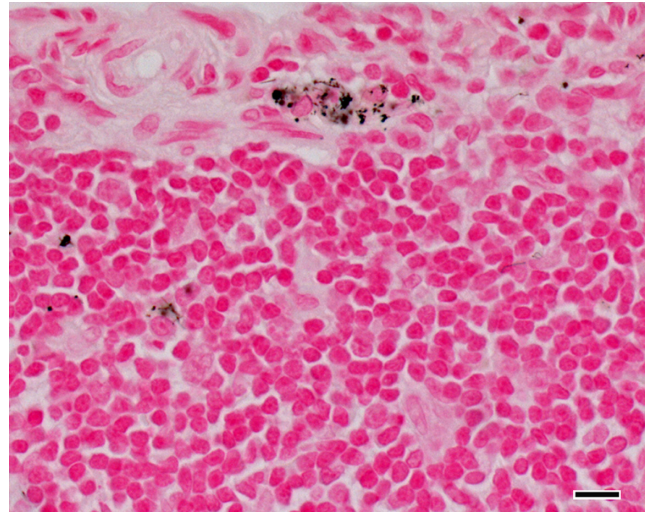


Fig. 2. MWCNT deposition in the peritracheal lymph node of a male rat exposed to 5 mg/m³ MWCNTs at the end of the 4-week postexposure period. Bar indicates 10 µm. Kernechtrot stain.

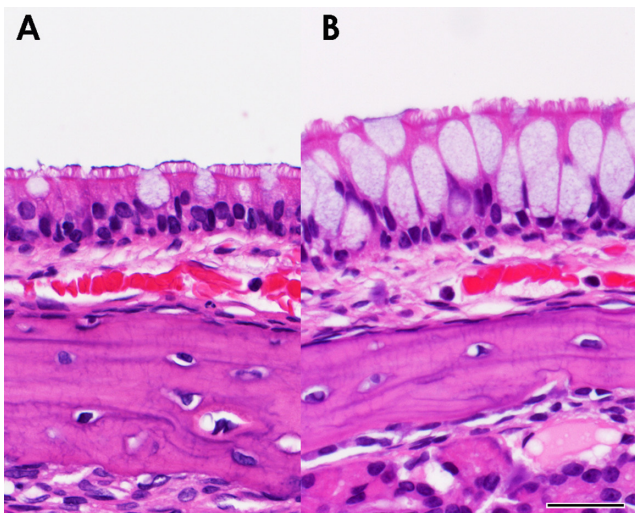


Fig. 3. A: Normal respiratory epithelium in the nasal cavity of a male rat in the control group at the end of the 2-week exposure period. B: Goblet cell hyperplasia in the nasal cavity of a male rat exposed to 5 mg/m³ MWCNTs at the end of the 2-week exposure period. Bar indicates 25 µm. H&E stain.

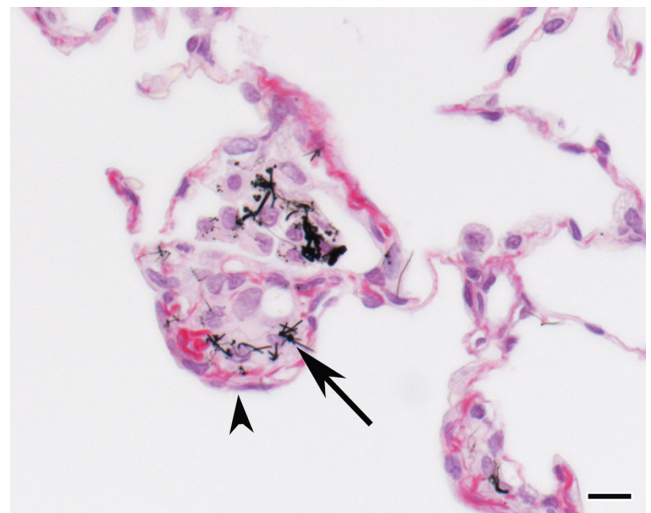


Fig. 4. Granulomatous change, early stage of granuloma formation, including MWCNT-phagocytosed alveolar macrophages (arrow) in the lung of a male rat exposed to 5 mg/m³ MWCNTs at the end of the 4-week postexposure period. The small amount of collagen fibers in the granulomatous change is stained with Sirius red stain (arrowhead). Bar indicates 10 µm.

postexposure period.

Granulomatous changes in the lung were observed in the male and female rats exposed to 5 mg/m³ MWCNTs at the end of the 2-week exposure period, and the incidence of granulomatous changes had increased in these rats by the end of the 4-week postexposure period. Granulomatous changes were characterized by aggregation of MWCNT-phagocytosed alveolar macrophages and included a small amount of collagen fiber deposition (Fig. 4). Multinuclear giant cells within the granulomatous changes or around the granulomatous changes were also found in the male and female rats exposed to 5 mg/m³ MWCNTs at the end of the

4-week postexposure period. Nuclei of the giant cells were characterized by a tendency to localize at the periphery of the cytoplasm.

Clear inflammatory cell infiltration in the regions of MWCNT deposition in the alveolar wall and lymph node were not observed in the MWCNT-exposed rats at the end of the 2-week exposure or 4-week postexposure periods. Similarly, granuloma formation was also not observed in the lymph node at the end of the 2-week exposure or 4-week postexposure period.

Table 3. Histopathological Findings of the Upper and Lower Respiratory System and Lymph Node of Rats

Group (mg/m ³)	At the end of the 2-week exposure period				At the end of the 4-week postexposure period			
	0	0.2	1	5	0	0.2	1	5
Number of animals examined	5	5	5	5	5	5	5	5
<Male>								
Nasal cavity								
Goblet cell hyperplasia	0	0	3	5	0	0	0	1
Nasopharynx								
Goblet cell hyperplasia	0	0	2	5	0	0	0	0
Lung								
Granulomatous change	0	0	0	1	0	0	0	4
<Female>								
Nasal cavity								
Goblet cell hyperplasia	0	0	1	5	0	0	0	0
Nasopharynx								
Goblet cell hyperplasia	0	0	0	5	0	0	0	0
Lung								
Granulomatous change	0	0	0	2	0	0	0	5

Values indicate number of animals bearing the lesions.

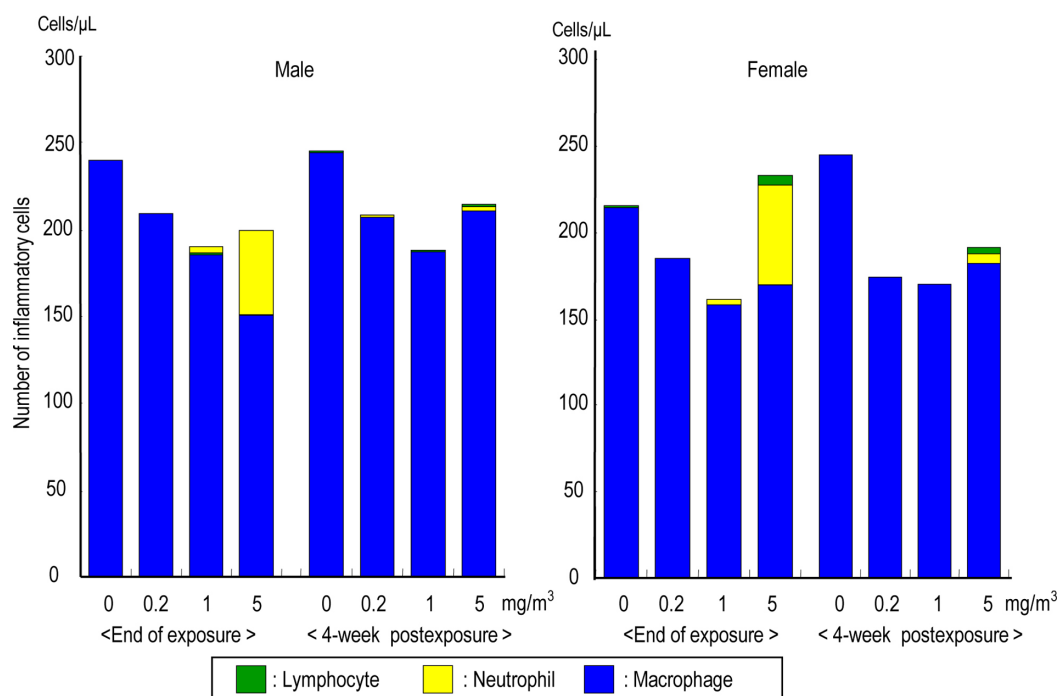


Fig. 5. Changes in the number of inflammatory cells in the BALF from rats at the end of the 2-week exposure and 4-week postexposure periods.

Cytological and biochemical analyses of BALF

There was a concentration-dependent decrease in the number of macrophages in the BALF of the MWCNT-exposed rats, and there were increases in the number of neutrophils in the BALF of the male and female rats exposed to 1 and 5 mg/m³ MWCNTs and in the number of lymphocytes in the BALF of the female rats exposed to 5 mg/m³ MWCNTs at the end of 2-week exposure period (Fig. 5). At the end of the 4-week postexposure period, the numbers of macrophages in the BALF of the MWCNT-exposed rats

tended to remain lower than in the controls, and the numbers of neutrophils and lymphocytes in the BALF of the rats exposed to 5 mg/m³ MWCNTs, although low, remained elevated compared to the controls. The percentage of bi- and multinucleated (three or more nuclei) macrophages increased mainly in the male and female rats exposed to 1 and 5 mg/m³ MWCNTs at the end of the 2-week exposure period (Figs. 6 and 7), although, slight increases in the percentage of multinucleated macrophages were observed in the female rats exposed to 0.2 mg/m³ MWCNTs. These increases, with

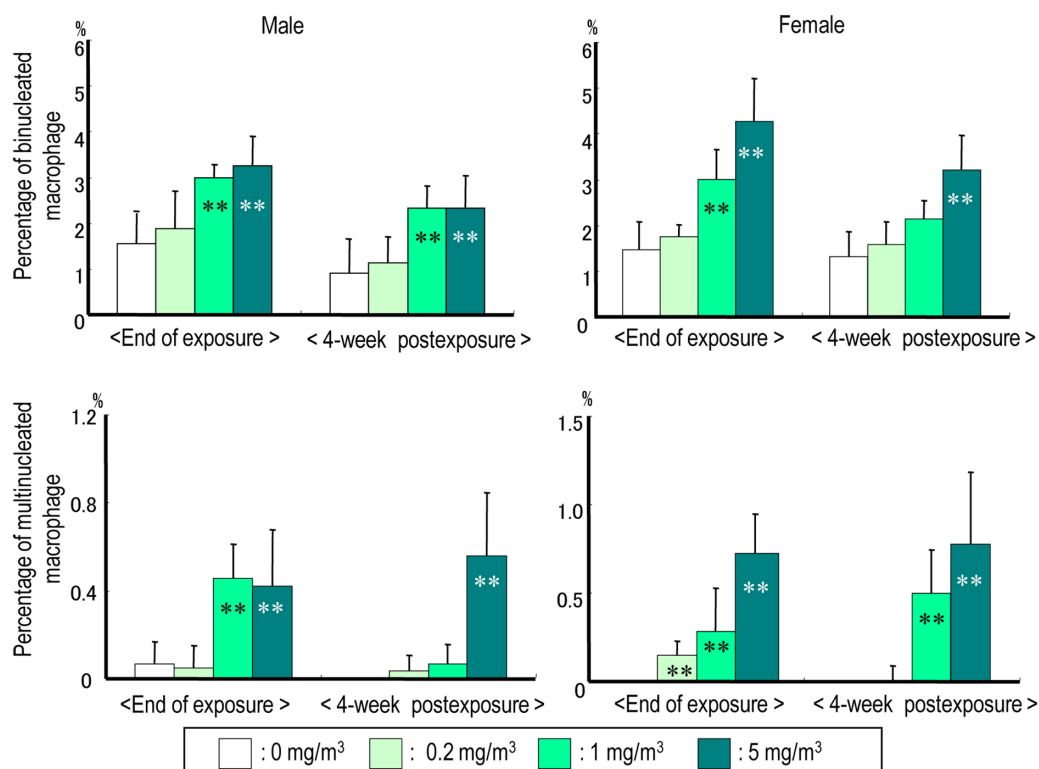


Fig. 6. Percentage of bi- or multinucleated macrophages in the BALF from rats at the end of the 2-week exposure and 4-week postexposure periods. Error bars indicate the SD of 5 rats. *: $p < 0.05$ by Dunnett's multiple comparison test. **: $p < 0.01$ by Dunnett's multiple comparison test.

the exception of multinucleated macrophages in the males exposed to 1 mg/m³ MWCNTs, were persistent in the males and females exposed to 1 and 5 mg/m³ MWCNTs.

Morphologically, MWCNT fibers phagocytosed by alveolar macrophages (Fig. 8) were observed in all the exposed groups at the end of both the 2-week exposure and 4-week postexposure periods. Variable sizes of alveolar macrophages with phagocytosed MWCNTs were present in the BALF. Notably, the cytoplasm of many of these alveolar macrophages was filled with numerous vacuole-like cavities and several macrophages that had phagocytosed MWCNTs appeared to have died and lost their cytoplasm (Fig. 8).

The results of biochemical analyses of the BALF are shown in Fig. 9. There was a concentration-dependent increase in the levels of total protein and albumin and the levels of ALP activity in the BALF of the MWCNT-exposed male and female rats at the end of the two-week exposure period. At end of the 4-week postexposure period, concentration-dependent increases in the levels of total protein and albumin and the levels of ALP activity in the BALF were still observed, although the values of these parameters were lower than at the end of 2-week exposure period.

Discussion

In this study, we used a whole-body exposure system to expose rats to dry MWCNT aerosols at doses of 0.2, 1

and 5 mg/m³. The highest dose, 5 mg/m³, is the same as the permissible exposure limit (PEL) for synthetic graphite. Inhalation of MWCNTs resulted in persistent deposition of MWCNTs in the lung, changes in alveolar macrophages consistent with prolonged reactivity toward MWCNT fibers in the lung, and slight toxicity to the lung and nasal cavity.

MWCNT fibers were deposited in the nasal cavity and lung of MWCNT-exposed rats. Deposition in the nasal cavity was relatively transient: At the end of the 2-week exposure period, non-phagocytosed MWCNTs were found only in the rats exposed to 1 and 5 mg/m³ MWCNTs, and at the end of the 4-week postexposure period, MWCNTs had been cleared from the nasal cavities of the rats exposed 1 mg/m³ MWCNTs. Importantly, MWCNT deposition in the nasal cavity was primarily in the non-ciliated respiratory epithelium. In contrast, MWCNT fibers in the lung were found in all MWCNT-exposed animals at the end of the 2-week exposure period, and MWCNT deposition persisted in all exposed animals to the end of the 4-week postexposure period. It is likely that mucociliary clearance of MWCNTs from the nasal cavity was a major factor accounting for the difference in MWCNT deposition in the nasal cavity and lung. Notably, MWCNT fiber deposition in the bronchiolar space appeared to be somewhat less than in the alveolar space (see Table 2).

The total amounts of MWCNTs in the 5 mg/m³ group were approximately 43.4 μg/lung at the end of the 2-week

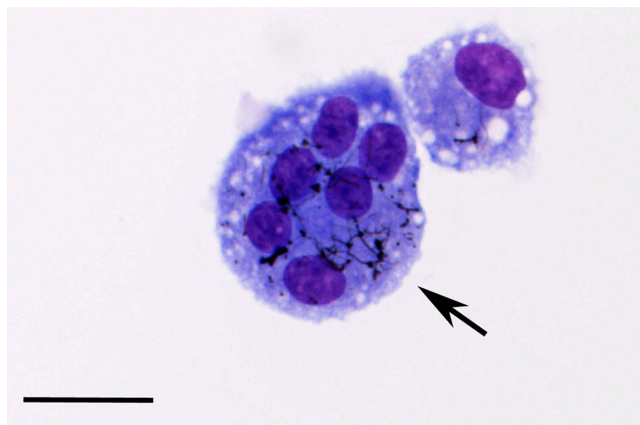


Fig. 7. Alveolar macrophages in the BALF from a rat exposed to 5 mg/m³ MWCNTs at the end of the 4-week postexposure period. A multinucleated macrophage with phagocytosed MWCNTs is shown (arrow). Bar indicates 30 μm. May-Grünwald-Giemsa stain.

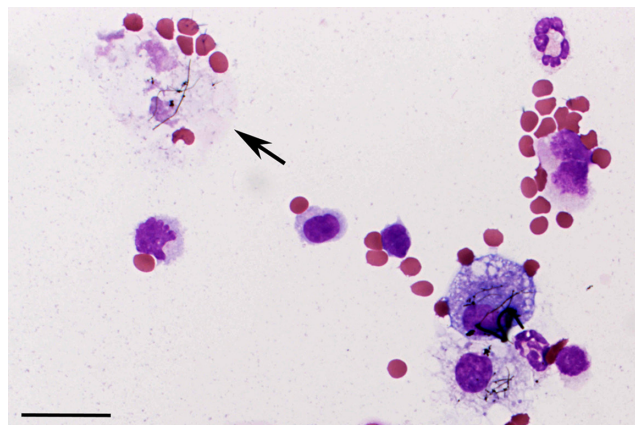


Fig. 8. Alveolar macrophages in the BALF from a rat exposed to 5 mg/m³ MWCNTs at the end of the 2-week exposure period. The arrow indicates a macrophage with phagocytosed MWCNTs that has lost its cytoplasmic contents. Bar indicates 30 μm. May-Grünwald-Giemsa stain.

exposure period and approximately 41.2 μg/lung at the end of the 4-week postexposure period: this data was obtained using an MWCNT-measuring method in our research center (unpublished data). Therefore, most MWCNTs remained in the lung, and only a small amount of MWCNTs was delivered out of the lungs during the 4-week postexposure period.

The incidence of MWCNT deposition in the peritracheal lymph node increased after the 4-week postexposure period, suggesting that MWCNTs are transported to lymph nodes outside the lung. In our previous study using intratracheally instilled MWCNTs (MWNT-7), MWCNT deposition in the posterior mediastinal lymph node (peritracheal lymph node) was observed 7 days after intratracheal instillation, and MWCNT deposition in the parathymic lymph node was observed 91 days after intratracheal instillation²⁰. These data suggest that MWCNTs deposited in the alveoli migrated through the lymphatic drainage systems for pulmonary dust clearance: the deep-set drainage system consisting of the periarterial, perivenous and peribronchiolar lymph vessels and the pleural drainage system following the surface of the lung segments and lobes.

Deposition of MWCNT fibers in the respiratory tract caused foreign body reactions to occur. In the nasal cavity and nasopharynx of rats exposed to 1 or 5 mg/m³ MWCNTs, goblet cell hyperplasia occurred. Goblet cell hyperplasia regressed when exposure to MWCNTs was discontinued. It is likely that the enhanced mucus secretion by goblet cells played a role in the clearance of MWCNTs from the nasal cavity.

In the lung, exposure to the highest levels of MWCNTs, 5 mg/m³, caused the formation of granulomatous changes. These granulomatous changes were characterized by aggregation of macrophages containing phagocytosed MWCNTs with a small amount of fibrosis. The low amounts of collagen formation in these lesions indicate that they were early stage granulomas. However, granuloma formation increased slightly and was progressing toward a chronic lesion with

giant cells during the 4-week postexposure period. Thus, if the postexposure period was lengthened, persistent MWCNT deposition could have effects not seen in this short-term study. As discussed above, during the 4-week postexposure period, very few MWCNTs were removed from the lung; one possible factor could be that granuloma formation by macrophages with phagocytosed MWCNTs would hinder clearance of MWCNTs from the lung.

Increased levels of multinucleated macrophages were observed in rats exposed to 1 or 5 mg/m³ MWCNTs. Since MWCNT deposition persisted in the lung, the levels of these large multinucleated macrophages phagocytosing MWCNTs tended to remain elevated throughout the 4-week postexposure period. It is known that multinucleated giant cells, which are frequently found in conditions of granulomatous inflammation, are formed by amitotic nuclear division or by fusions of macrophages^{21,22}. Asakura *et al.* (2010) reported that exposure of Chinese hamster lung cells to MWCNTs resulted in the formation of polyploidy, bi- and multinucleated cells and suggested that MWCNTs physically interfere with biological processes during cytokinesis²³. Therefore, it is possible that the increases in multinucleated macrophages in the present study were caused by changes associated with granulomatous inflammation and the mitotic inhibition of alveolar macrophages containing phagocytosed MWCNTs. In addition, morphological examination of the BALF showed toxic effects on alveolar macrophages in the rats exposed to 1 and 5 mg/m³ MWCNTs. Taken together, the decrease of alveolar macrophages in the BALF could reflect the cytotoxicity of phagocytosed MWCNTs and the formation of multinucleated macrophages. The toxicity of phagocytosed MWCNTs could also be a factor hindering clearance of MWCNT fibers from the lung.

Finally, inflammatory parameters in the BALF were elevated in rats exposed to 1 and 5 mg/m³ MWCNT: Elevated neutrophil and lymphocyte counts and elevated levels of total protein and albumin in the BALF were observed. While

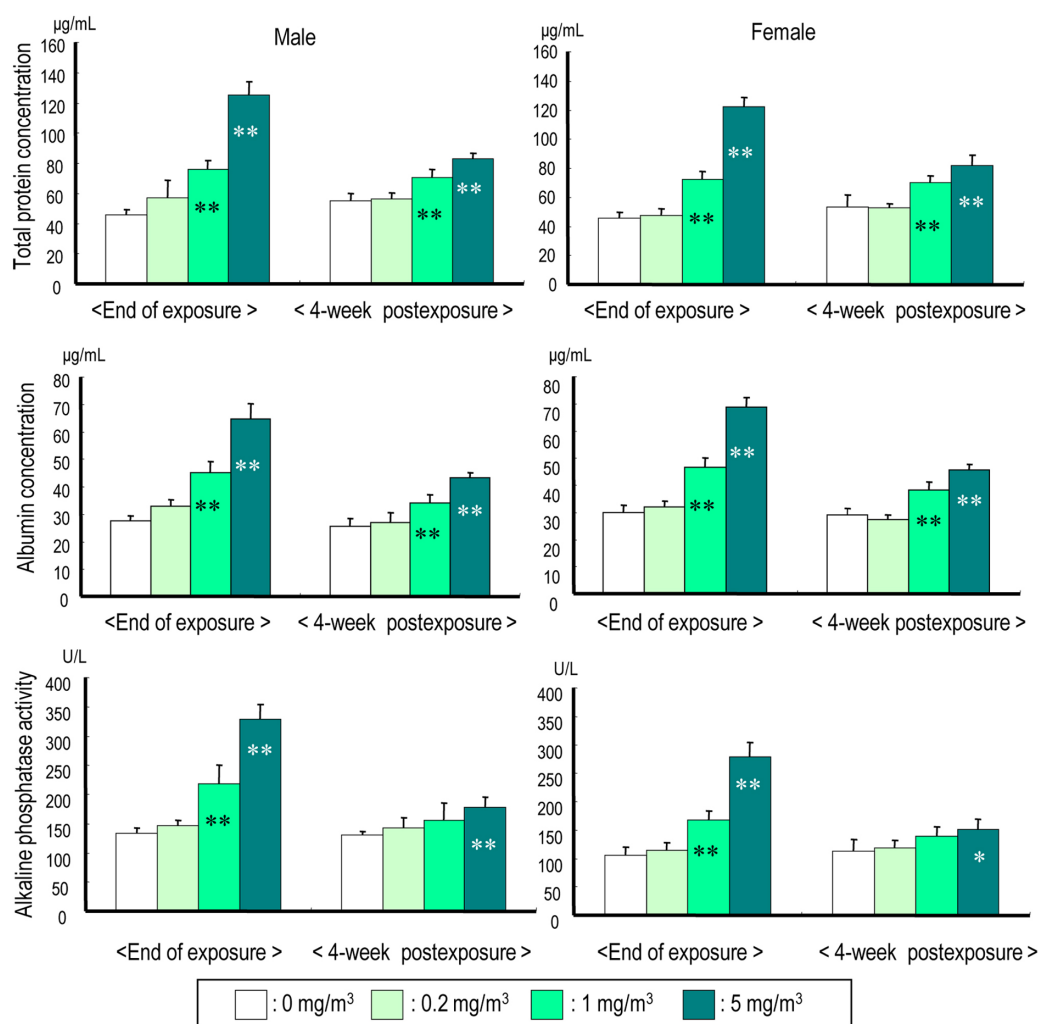


Fig. 9. Total protein and albumin concentrations and ALP activity in the BALF from rats at the end of the 2-week exposure and 4-week postexposure periods. Error bars indicate the SD of 5 rats. *: $p < 0.05$ by Dunnett's multiple comparison test. **: $p < 0.01$ by Dunnett's multiple comparison test.

the changes in these inflammatory parameters were comparatively weak, and clear inflammatory cell infiltration was not found in the lower or upper pulmonary tracts by histopathological examination, it is notable that changes in the inflammatory parameters were still present at the end of the 4-week postexposure period. These results indicate that the inflammatory changes at the end of the 4-week postexposure period were caused by the persistence of MWCNT deposition in the lung. In addition, ALP activity in the BALF was elevated in rats exposed to 1 and 5 mg/m³ MWCNTs and was still present at the end of the 4-week postexposure period. Therefore, MWCNT exposure induced persistent pulmonary damage, although its severity was weak.

In conclusion, F344 rats of both sexes were exposed by inhalation to 0.2, 1 or 5 mg/m³ MWCNT aerosol for 6 h/day, 5 days/week for 2 weeks using a whole-body exposure system. We found persistent deposition of MWCNTs in the lungs of all MWCNT-exposed groups, and MWCNTs migrated to the lymphatic drainage systems for pulmonary

dust clearance. Foreign body reactions included goblet cell hyperplasia in the nasal cavity and the nasopharynx and granulomatous formation with slight fibrosis in the alveoli. Macrophage levels in the BALF were decreased, and the levels of multinucleated macrophages were increased, possibly in response to the toxicity of phagocytosed MWCNTs. There was also a persistent pulmonary inflammatory reaction to MWCNT exposure. No histopathological or inflammatory changes in the rats exposed to 0.2 mg/m³ MWCNTs were observed; therefore, the NOAEL for inhaled MWCNTs in this study was 0.2 mg/m³. However, our results suggest that MWCNTs might persist in the lung for long periods of time and could eventually cause severe pulmonary toxicities. Moreover, migration of MWCNTs to the lymphatic system outside the lung raises concern about toxic effects of inhaled MWCNTs on the visceral pleura. Therefore, further, long-term studies are essential to reveal the toxicities in the lung, pleura and other organs caused by inhalation exposure to MWCNTs.

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