Table 3 Chemical intake, terminal body weight and organ weight of mice administered 2,4-DCNB in the diet for 2 years

Dose (ppm)	0 (control)	750	1,500	3,000
Male				
No. of animals examined	50	50	50	50
No. of surviving animals	35	38	29	23
2,4-DCNB intake (mg/kg bw/day)[a]	–	82 ± 21	172 ± 45	355 ± 80
Terminal body weight (g)[b]	50.9 ± 9.3	50.8 ± 8.5	45.9 ± 8.5	37.1 ± 4.9**
Organ weight				
Liver (g)	1.86 ± 0.77	2.15 ± 0.74	3.24 ± 2.17**	5.25 ± 3.28**
Liver (%)[c]	4.12 ± 2.67	4.68 ± 1.99	8.25 ± 6.56**	15.71 ± 9.67**
Dose (ppm)	0 (control)	1,500	3,000	6,000
Female				
No. of animals examined	49[d]	50	50	50
No. of surviving animals	28	28	18	19
2,4-DCNB intake (mg/kg bw/day)	–	203 ± 46	416 ± 86	942 ± 271
Terminal body weight (g)	40.3 ± 7.2	36.9 ± 4.9	30.8 ± 3.3**	24.2 ± 2.6**
Organ weight				
Liver (g)	1.54 ± 0.69	2.26 ± 1.30**	3.28 ± 1.53**	3.79 ± 1.32**
Liver (%)	4.21 ± 2.17	6.73 ± 4.37**	11.62 ± 5.56**	17.27 ± 6.65**

*, ** Significantly different from control at $p < 0.05$ and $p < 0.01$ by Dunnett's test, respectively

[a] Mean ± SD of the surviving animals averaged over the 2-year administration period

[b] Mean ± SD of the surviving animals averaged at the end of the 2-year administration period

[c] Relative organ weight was calculated with the following equation. absolute organ weight/fasted body weight × 100

[d] One female mouse died during the administration period

N-acetyl-*S*-(4-chloro-3-nitrophenyl)-*L*-cysteine is responsible for the renal tumors induced in rats by 2-year dietary administration 1,4-DCNB (Yamazaki et al. 2006). Similarly to 1,4-DCNB, 2,4-DCNB is metabolized to *N*-acetyl-*S*-(5-chloro-2-nitrophenyl)-*L*-cysteine by *β*-lyase in the kidney and excreted in the urine (Ohnishi et al. 2009). These data, taken in conjunction with the fact that 2,4-DCNB is mutagenic only when metabolically activated by S9, lead to the premises that genotoxic metabolites of 2,4-DCNB, such as *N*-acetyl-*S*-(5-chloro-2-nitrophenyl)-*L*-cysteine, are responsible for the renal carcinogenesis observed in the present study.

Hepatocarcinogenicity of 2,4-DCNB for male and female mice was clearly evidenced by dose-related increases in the incidences of hepatocellular adenomas, hepatocellular carcinomas and hepatoblastomas in male and female mice. A striking feature of 2,4-DCNB-induced hepatocarcinogenicity was the induction of historically rare hepatoblastomas; hepatoblastomas have a morphological structure completely different from that of hepatocellular carcinomas (Frith et al. 1994). The liver tumors induced by 2,4-DCNB were highly malignant as evidenced by metastasis of the hepatocellular carcinomas and hepatoblastomas to the lung, peritoneum, lymph node and stomach. Although neither hypertrophy of hepatocytes nor necrotic or regenerative changes were histopathologically observed in the 1,500 or 3,000 ppm female mouse livers of the present study, liver tumors were induced in the 1,500 and 3,000 ppm 2,4-DCNB-fed females. Since it is well known that centrilobular hypertrophy relates to a non-genotoxic mechanism of hepatocarcinogenesis in mice, a genotoxic mode of action is therefore likely to be responsible for the observed 2,4-DCNB-induced hepatocarcinogenesis.

Induction of carcinogenic peritoneal tumors by 2,4-DCNB was clearly evidenced by dose-related increases in the incidences of malignant peritoneal hemangiosarcomas in male and female mice. The incidence of hemangiosarcomas in the 1,500 ppm-fed females exceeded the maximum incidence of the JBRC historical control data and was significantly increased in the 3,000 and 6,000 ppm-fed females. In males, while the increase in the incidence of peritoneal hemangiosarcomas was not statistically significant by Fisher's exact test, the incidences of peritoneal hemangiosarcomas in the 3,000 ppm-fed males exceeded the maximum incidence of the JBRC historical control data.

In addition, 2,4-DCNB also induced preputial gland adenomas as shown by a significant increase in the incidence of benign preputial gland adenomas in the 3,000 ppm-fed male rats and a slight increase in tumor incidence over the upper range of the JBRC historical control data.

Table 4 Incidences of neoplastic, pre-neoplastic and non-neoplastic lesions in mice administered 2,4-DCNB in the diet for 2 years

Dose (ppm)	Male					Female				
	0 (control)	750	1,500	3,000	Peto test	0 (control)	1,500	3,000	6,000	Peto test
No. of animals	50	50	50	50		49[a]	50	50	50	
Liver										
Hepatocellular adenoma	18	34 **	30 *	43 **	↑↑	8	25 **	42 **	45 **	↑↑
Hepatocellular carcinoma	7	7	11	15 *	↑↑	1	2	11 **	21 **	↑↑
Hepatoblastoma	1	5	16 **	27 **	↑↑	0	2	7 **	7 **	↑↑
Combined incidence[b]	19	39 **	41 **	45 **	↑↑	8	28 **	43 **	48 **	↑↑
Hepatocellular hypertrophy: central	0	7#	22##	29##		0	0	2	23##	
		(1.0)[c]	(1.0)	(1.8)				(1.0)	(1.7)	
Acidophilic cell focus	1	4	5	1		0	3	6#	8#	
	(1.0)	(1.0)	(1.2)	(1.0)			(1.3)	(1.2)	(1.0)	
Peritoneum										
Hemangiosarcoma	1	0	2	5	↑↑	0	3	7 **	17 **	↑↑
Nasal cavity										
Deposit of pigment	0	44##	40##	39##		0	43##	38##	47##	
		(1.0)	(1.0)	(1.0)			(1.0)	(1.0)	(1.0)	
Eosinophilic globules: olfactory epithelium	19	19	11	25		7	17	26##	45##	
	(1.1)	(1.2)	(1.2)	(1.4)		(1.1)	(1.2)	(1.2)	(1.5)	
Eosinophilic globules: respiratory epithelium	29	35	40	29		33	48##	50##	48##	
	(1.3)	(1.4)	(1.4)	(1.3)		(1.2)	(1.8)	(1.6)	(1.1)	
Respiratory metaplasia: olfactory epithelium	20	15	49##	50##		9	48##	50##	50##	
	(1.0)	(1.0)	(1.5)	(2.0)		(1.0)	(1.9)	(2.2)	(2.9)	
Respiratory metaplasia: gland	14	25#	47##	49##		6	47##	49##	50##	
	(1.0)	(1.3)	(2.0)	(2.9)		(1.0)	(2.2)	(3.0)	(3.0)	
Nasopharynx										
Eosinophilic globules	3	3	6	30##		3	35##	36##	36##	
	(1.0)	(1.7)	(1.7)	(1.2)		(3.0)	(1.7)	(1.6)	(1.6)	

Grade: *1* slight, *2* moderate, *3* marked, *4* severe

*, ** Significantly different from control at $p < 0.05$ and $p < 0.01$ by Fisher's exact test, respectively

↑,↑↑ Significantly increased at $p < 0.05$ and $p < 0.01$ by Peto's test, respectively

#, ## Significantly different at $p < 0.05$ and $p < 0.01$by Chi-square test, respectively

[a] One female mouse died during the administration period

[b] Combined incidence of hepatocellular adenoma, hepatocellular carcinoma and/or hepatoblastoma

[c] The values in parentheses indicate the average severity grade index of the lesion. The average severity grade was calculated using the following equation. Σ (grade × number of animals with grade)/number of affected animals

Chronic toxicity of dietary 2,4-DCNB was seen in both rats and mice. 2,4-DCNB-induced renal toxicity was evidenced by increased severity and progression of CPN as shown by a dose-dependent increase in the incidences of marked and severe grades of CPN in the 2,4-DCNB-fed male rats and an increase in the incidence of CPN in the 750 and 1,500 ppm-fed female rats. In addition, papillary mineralization and urothelial hyperplasia in the renal pelvis were noted in the male rats. Moreover, eosinophilic droplets in the proximal tubule and the serum levels of BUN were increased in the male and female rats.

Although the 2,4-DCNB was administered by the oral route, it is noteworthy that the 2,4-DCNB-induced pathological changes were observed in the upper respiratory tract in mice: The incidences of pigment deposition and respiratory metaplasia in the olfactory epithelium and submucosal gland were increased in males and females, and the incidences of eosinophilic globules in the olfactory and respiratory epithelia were increased in females. In addition, the incidence of eosinophilic globules in the nasopharynx were increased in males and females.

2,4-DCNB is a genotoxic carcinogen, and the hepatocellular adenomas and the combined incidence of total hepatic tumors were increased at the lowest dose level in male and female mice. Therefore, we used a non-threshold approach to calculate the benchmark dose associated with

10 % risk over background ($BMDL_{10}$). Based on the dose–response relationships between 2,4-DCNB-intake and incidences of tumors obtained in the present study, a 95 % lower confidence limit of the $BMDL_{10}$ was calculated using the linearized multistage model for total hepatic tumors of male and female mice with US EPA's benchmark dose software (Ver. 2.1.1) (US EPA 2009). The $BMDL_{10}$ value for the endpoint of total hepatic tumors in male mice was 12.3 mg/kg per day and in female mice was 23.5 mg/kg per day.

Acknowledgments This work was contracted and supported by the Ministry of Health, Labour and Welfare, Japan. We wish to express our thanks to Dr. David B. Alexander, Nanomaterial Toxicology Project Laboratory, Nagoya City University, for proofreading this manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

References

Anden CC, Kanerva RK, Ridder G, Stone LS (1984) The pathogenesis of nephrotoxicity of volatile hydrocarbons in the male rats. In: Mehlman MA, Hemstree CP III, Thorpe JJ, Weaver NK (eds) Renal effects of petroleum hydrocarbons. Princeton Scientific Publishers Inc., Princeton, pp 107–120

Bannasch P, Griesemer RA, Anders F, Becker R, Cabral J R, Della Porta G, Feron VJ, Henschler D, Ito N, Kroes R, Magee P N, McKnight B, Mohr U, Montesano R, Napalkov NP, Pegg AE, Rao GN, Turusov VS, Wilbourn J (1986) Selection of doses. In: Montesano R, Bartsch H, Vainio J, Wilbourn J, Yamasaki H (eds) Long-term and short-term assays for carcinogens: a critical appraisal. IARC scientific publications No. 83, Lyon, pp 34–36

Beratergreimium für Umweltrelevant Altstoffe (BUA) (1991) 3-Dichloro-4-nitrobenzene. In: Syuttgart S (ed) GDCh-Advisory committee on existing chemicals of environmental relevance. Report 64, Hirzel Wissenschaftliche Verlagsgesellschaft, Germany

Charbonneau M, Strasser J Jr, Lock EA, Turner MJ Jr, Swenberg JA (1989) Involvement of reversible binding to α2u -globulin in 1,4-dichlorobenzene-induced nepthropathy. Toxicol Appl Pharmacol 99:122–132

Dekant W, Metzler M, Henschler D (1986) Identification of *S*-1,2-dichlorovinyl-*N*-acetyl-cysteine as a urinary metabolite of trichloroethylene: a possible explanation for its nephrocarcinogenicity in male rats. Biochem Pharmacol 35:2455–2458

Dekant W, Berthold K, Vamvakas S, Henschler D, Anders MW (1988) Thioacylating intermediates as metabolites of *S*-(1,2-dichlorovinyl)-L-cysteine and *S*-(1,2,2-trichlorovinyl)-L-cysteine formed by cysteine conjugate β-lyase. Chem Res Toxicol 1:175–178

Elfarra AA, Jakobson I, Anders MW (1986) Mechanism of S-(1,2-dichlorovinyl) glutathione-induced nephrotoxicity. Biochem Pharmacol 35:283–288

Frith CH, Ward JM, Turusov VS (1994) Tumours of the liver. In: Turusov VS, Mohr U (eds) Pathology of tumours in laboratory animals. Vol. II Tumours of the mouse. IARC Scientific Publication No. 111, Lyon, pp 223–248

Hard GC, Alden CL, Stula EF, Trump BF (1995) Proliferrative lesions of the kidney in rats. In: Guides for toxicologic pathology. STP/ARP/AFIP, Washington, DC, pp 1–19

Japan Chemical Industry Ecology-Toxicology and Information Center (JETOC) (1996) 2,4-Dichloro-1-nitrobenzene. In: Mutagenicity test data of existing chemical substances. Based on toxicity investigation system of the Industrial Safety and Health Law. JETOC, Tokyo, p 60 (323–324, 551–552, in Japanese)

Japan Industrial Safety and Health Association (2004) A list of mutagenic chemicals. In: General guidebook on industrial health 2004. Japan Industrial Safety and Health Association, Tokyo, pp 127–132

Kawai K (1980) Report from working group on long-term holding of experimental animals. Exp Anim 29:181–231 (in Japanese)

MacFarland HM, Ulrich CE, Holdsworth CE, Kitchen DN, Halliwell WH, Blum SC (1984) A chronic inhalation study with unleaded gasoline vapor. J Am Coll Toxicol 3:231–248

Montgomery CA, Seely JC (1990) Kidney. In: Boorman GA, Eustis SL, Elwell MR, Montgomery CA Jr, MacKenzie WF (eds) Pathology of the fischer rat. Academic Press Inc., San Diego, pp 127–153

National Research Council (NRC) (1996) Guide for the care and use of laboratory animals. Institute of Laboratory Animal Resources Commission on Life Sciences. NRC. National Academy Press, Washington, DC

National Toxicology Program (NTP) (1990) NTP technical report on the toxicology and carcinogenesis studies of *d*-limonene (CAS No. 5989-27-5) in F344/N rats and $B6C3F_1$ mice (gavage studies). NTP TR 347. NIH Publication No. 90-2802

Ohnishi M, Yamazaki K, Yamamoto S, Matsushima T (2004) Characterization of *N*-acetylcysteine conjugate in yellow urine by oral administration of 1,4-dichloro-2-nitrobenzene in rats. J Health Sci 50:319–322

Ohnishi M, Take M, Yamamoto S, Fukushima S, Yajima H (2009) Identification of an *N*-acetylcysteine conjugate in the urine after oral administration of 2,4-dichloro-1-nitrobenzene to rats. J Toxicol Sci 34:233–237

Organisation for Economic Co-operation and Development (OECD) (1981) OECD guideline for testing of chemicals 451 for "Carcinogenicity studies" Adopted 12 May 1981. OECD, Paris

Organisation for Economic Co-operation and Development (OECD) (1996) 2,4-Dichloronitrobenzene. In: Screening Information Data Set (SIDS): initial assessment report for SIAM4. OECD, Paris

Organisation for Economic Co-operation and Development (OECD) (1998) OECD series on principles of good laboratory practice. Series on principles of good laboratory practice and compliance monitoring No. 1. ENV/MC/CHEM (98), vol 17, OECD, Paris

Organisation for Economic Co-operation and Development (OECD) (2004) The 2004 OECD list of high production volume chemicals. Environment directorate, OECD, Paris. http://www.oecd.org/dataoecd/55/38/33883530.pdf Accessed 23 May 2012

Sontag JM, Page NP, Saffiotti U (1976) Guidelines for carcinogen bioassay in small rodents. NCI-CG-TR-1. DHHS Publication (NIH) 76-801. National Cancer Institute, Bethesda (MD)

United States Environmental Protection Agency (US EPA) (2009) Benchmark dose software version 2.1 user's manual. 53-BMDS-RPT-0028, US EPA. Washington, DC

Vambakas S, Elfarra AA, Dekant W, Henschler D, Anders MW (1988) Mutagenicity of amino acid and glutathione *S*-conjugates in the Ames test. Mutat Res 206:83–90

Yamazaki K, Aiso S, Matsumoto M, Kano H, Arito H, Nagano K, Yamamoto S, Matsushima T (2006) Carcinogenicity and chronic toxicity of 1,4-dichloro-2-nitorobenzene in rats and mice by two years feeding. Ind Health 44:230–243

J Toxicol Pathol 2013; 26: 131–140

Original Article

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

Yumi Umeda[1*], Tatsuya Kasai[1], Misae Saito[1], Hitomi Kondo[1], Tadao Toya[1], Shigetoshi Aiso[1], Hirokazu Okuda[1], Tomoshi Nishizawa[1], and Shoji Fukushima[1]

[1] Japan Bioassay Research Center, Japan Industrial Safety and Health Association, 2445 Hirasawa, Hadano, Kanagawa 257-0015, Japan

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/m^3 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/m^3 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/m^3 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/m^3 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/m^3. (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 1991[1], 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 Japan[2]. 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 exposure[3] 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 toxicity[6]. However, a more recent study using mice reported pulmonary inflammation and damage in mice after a single whole-body exposure to aerosolized dry MWCNTs[7]. 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 mice[9] and in F344 rats[10]. These studies demonstrated that the type

Received: 29 November 2012, Accepted: 4 January 2013
*Corresponding author: Y Umeda (e-mail: y-umeda @jisha.or.jp)

of fibers formed by MWCNTs have the potential to present a risk similar to that of asbestos and induce inflammation and mesothelioma in the pleura[11]. 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 system[15]. 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 m^2/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 ± SD width of 88 ± 5 nm and length of 5.0 ± 4.5 μm with 38.9% >5 μm length[16].

Animals

Five-week-old F344/DuCrlCrlj 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 Experimentation[17]. 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 × 216 D × 176 H mm) that were placed in stainless steel inhalation exposure chamber with a volume of 1.24 m^3. 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/m^3 for 6 hrs/day, 5 days/week for 2 weeks. Five mg/m^3 was selected as the highest concentration of MWCNTs because it is the Occupational Safety and Health Administration (OSHA) permissible exposure limit (PEL) value for synthetic graphite[18], 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 previously[15]. In the present study, MWCNT aerosols of 0.2, 1 or 5 mg/m^3 were generated using the cyclone sieve method (sieve of 200 mm in diameter and 53 μm 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 report[15]. 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 previously[19]. 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 × 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 × 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 ± 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 * (95 %)	174 ± 9 (97 %)	168 ± 6 ** (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 (98 %)	249 ± 15 (98 %)	241 ± 14 (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 ** (94 %)	116 ± 4 ** (94 %)	116 ± 5 ** (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 (95 %)	149 ± 6 (96 %)	147 ± 5 (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

	At the end of the 2-week exposure period				At the end of the 4-week postexposure period			
Group (mg/m^3)	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/m^3 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

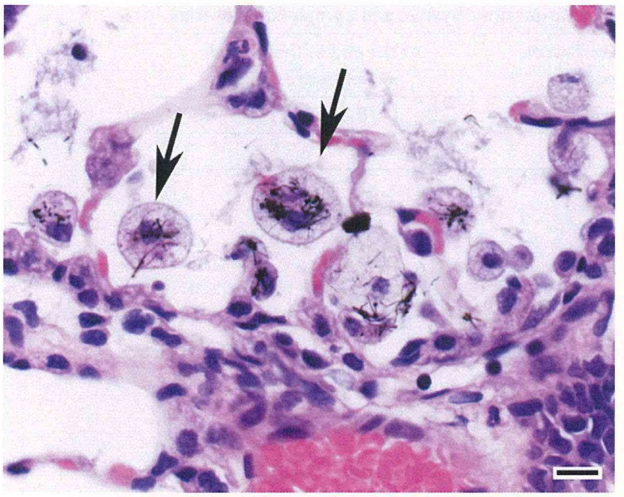

Fig. 1. MWCNTs phagocytosed by alveolar macrophages (arrows) in the alveolar space of the lung of a male rat exposed to 5 mg/m^3 at the end of the 2-week exposure period. Bar indicates 10 μm. H&E stain.

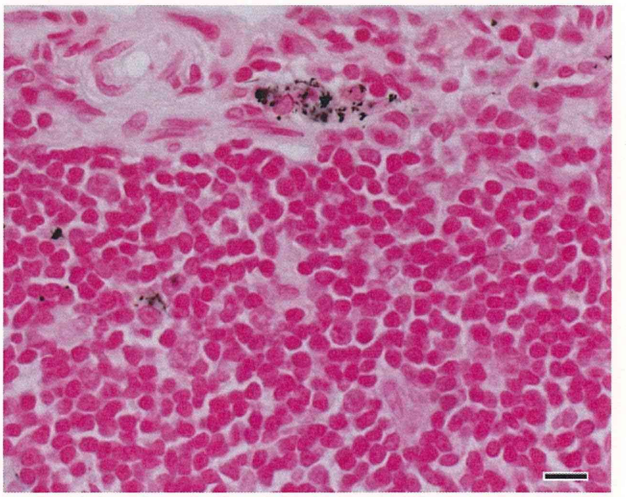

Fig. 2. MWCNT deposition in the peritracheal lymph node of a male rat exposed to 5 mg/m^3 MWCNTs at the end of the 4-week postexposure period. Bar indicates 10 μm. Kernechtrot stain.

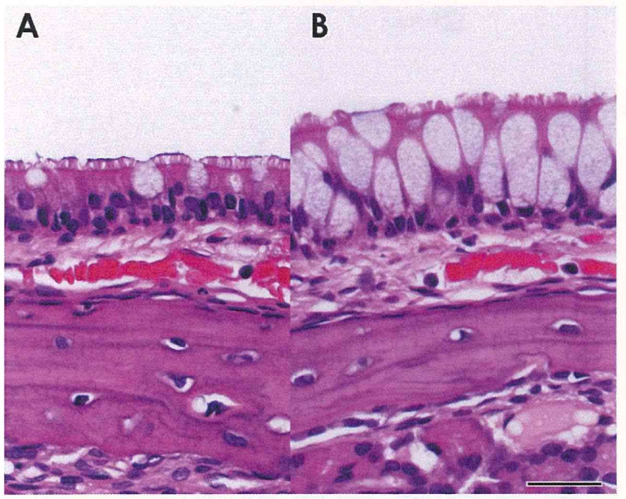

Fig. 3. A: Normal respiratory epithelium in the nasal cavity of a male rat in the control group at the end of the 2-week exposure period. B: Goblet cell hyperplasia in the nasal cavity of a male rat exposed to 5 mg/m^3 MWCNTs at the end of the 2-week exposure period. Bar indicates 25 μm. H&E stain.

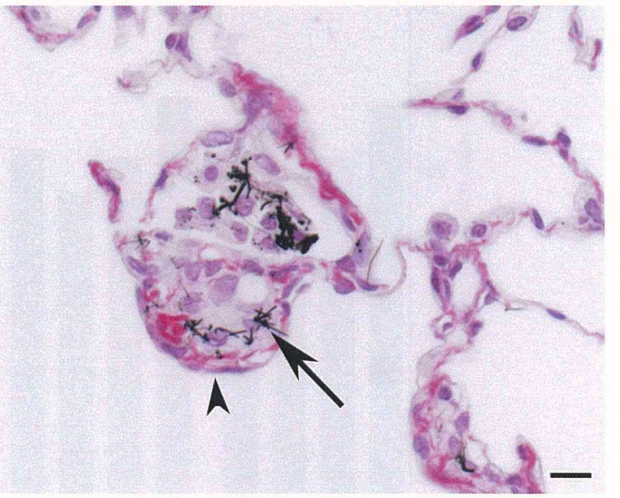

Fig. 4. Granulomatous change, early stage of granuloma formation, including MWCNT-phagocytosed alveolar macrophages (arrow) in the lung of a male rat exposed to 5 mg/m^3 MWCNTs at the end of the 4-week postexposure period. The small amount of collagen fibers in the granulomatous change is stained with 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/m^3 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/m^3 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

	At the end of the 2-week exposure period				At the end of the 4-week postexposure period			
Group (mg/m^3)	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.

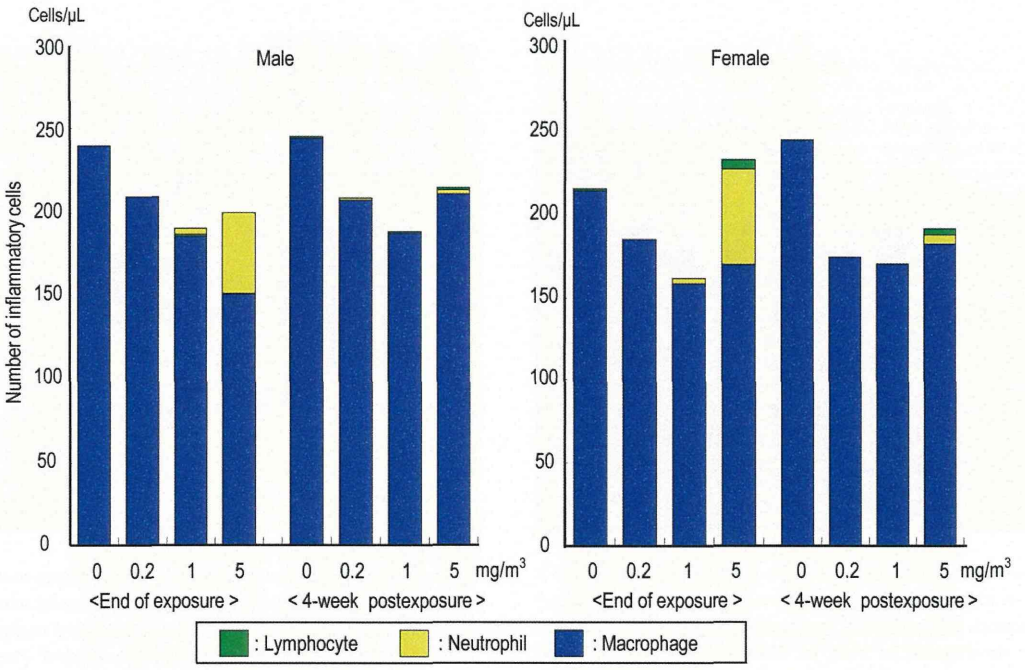

Fig. 5. Changes in the number of inflammatory cells in the BALF from rats at the end of the 2-week exposure and 4-week postexposure periods.

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/m^3 MWCNTs and in the number of lymphocytes in the BALF of the female rats exposed to 5 mg/m^3 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/m^3 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/m^3 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/m^3 MWCNTs. These increases, with

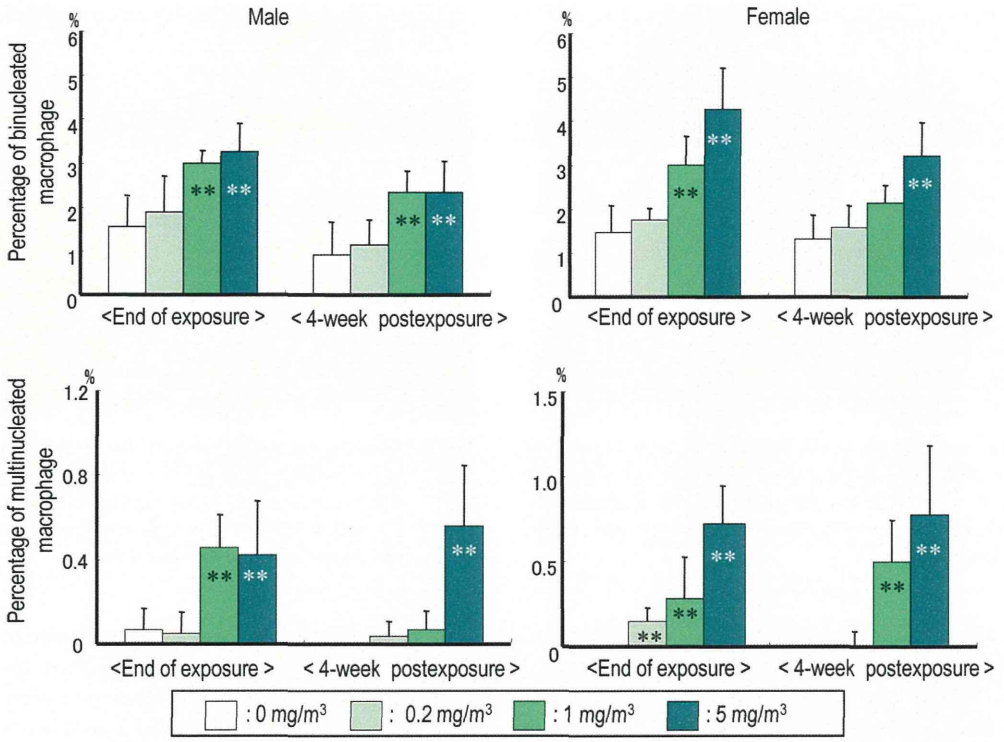

Fig. 6. Percentage of bi- or multinucleated macrophages in the BALF from rats at the end of the 2-week exposure and 4-week postexposure periods. Error bars indicate the SD of 5 rats. *: $p<0.05$ by Dunnett's multiple comparison test. **: $p<0.01$ by Dunnett's multiple comparison test.

the exception of multinucleated macrophages in the males exposed to 1 mg/m^3 MWCNTs, were persistent in the males and females exposed to 1 and 5 mg/m^3 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/m^3. The highest dose, 5 mg/m^3, 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/m^3 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/m^3 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/m^3 group were approximately 43.4 μg/lung at the end of the 2-week