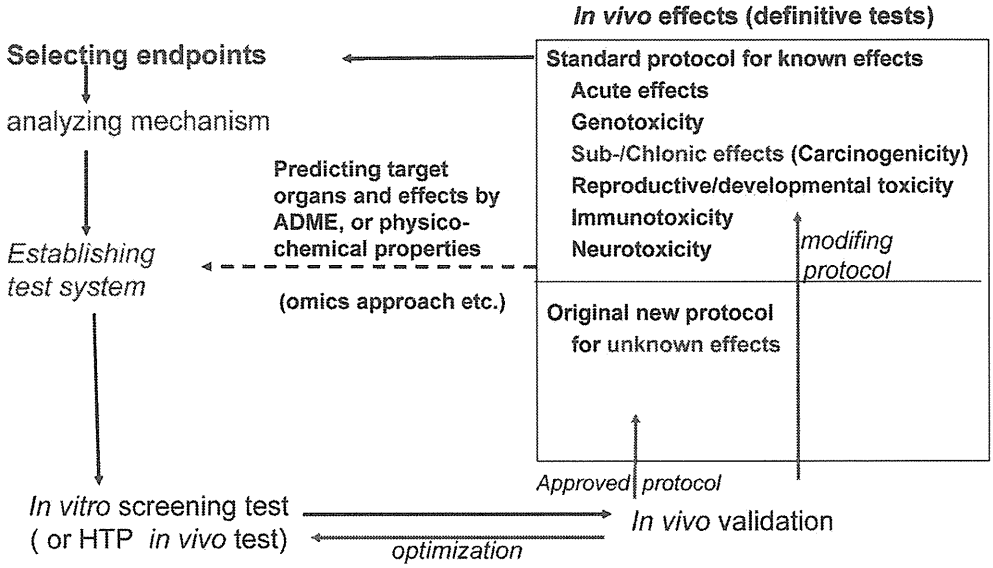

Fig. 4. The Schematic Development of Screening Tests and Definitive Tests

くとも代表的なナノマテリアルによる *in vivo* の慢性影響研究や，その影響を推定するためのナノマテリアルと生体成分との分子レベルでの相互作用や体内残留性様式の解析を進めていくべきであると考えられる.

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Original Article

Teratogenicity of multi-wall carbon nanotube (MWCNT) in ICR mice

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ABSTRACT — A possible teratogenicity of multi-wall carbon nanotube (MWCNT) was assessed using ICR mice. MWCNTs were suspended in 2% carboxymethyl cellulose and given intraperitoneally or intratracheally to pregnant ICR mice on day 9 of the gestation. All fetuses were removed from the uterus on day 18 of the gestation, and were examined for external and skeletal anomalies. In the intraperitoneal study, various types of malformation were observed in all MWCNT-treated groups (2, 3, 4 and 5 mg/kg body weight, intraperitoneal). In contrast, such malformations were observed in groups given 4 or 5 mg/kg body weight, but not in that treated with 3 mg/kg in the intratracheal study. In either study, the number of litters having fetuses with external malformation and that of litters having fetuses with skeletal malformations were both increased in proportion to the doses of MWCNT. The present results are the first to report that MWCNT possesses the teratogenicity at least under the present experimental conditions. Mechanism(s) to result such malformations is yet unclear and further experiment is necessary.

Key words: Multi-wall carbon nanotube, Nanomaterial, Teratogenicity, Hazard identification, Mice

INTRODUCTION

Carbon nanotube is a new form of the technological crystalline carbon and one of the most anticipated nanomaterials, because of its unique properties suitable for a variety of industrial products such as high strength materials, electronics and biomedical apparatuses (Martin and Kohli, 2003; Scott, 2005). On the other hand, potential hazards and/or risk for humans of carbon nanotube has been concerned, and large efforts have been internationally being made to investigate and evaluate them (Lam *et al.*, 2006; Pacurari *et al.*, 2010; Hubbs *et al.*, 2011). Among those, a possible carcinogenicity has been concerned most, assuming the structural similarity between carbon nanotubes and asbestos. Takagi *et al.* (2008) have first reported that multi-wall carbon nanotube (MWCNT) induces mesotheliomas, when intraperitoneally administered to male p53 gene deficient mice. Shortly afterwards, Sakamoto *et al.* (2009) have demonstrated that the carcinogenicity of MWCNT is a universal event and not specific to mice or genetically modified animals, by showing the mesothelioma development in male intact (not genetically modified) rats, intrascrotally administered the same MWCNT. Since then, carcinogenicity of MWCNT has enthusiastically been being studied but the mechanism(s) of such carcinogenicity is yet not clearly understood. Because the damage to DNA, directly or indirectly, by MWCNT is to be evaluated by prenatal stage, a possible teratogenicity must be another big issue for the risk assessment of MWCNT. To the best of our knowledge there have been no reports dealing with this issue in the literature. In this content, the present study was conducted to assess a possible teratogenicity of MWCNT.

MATERIALS AND METHODS

Ethical consideration of the experiments

An experimental protocol was approved by the Experiments Regulation Committee and the Animal Experiment Committee of the Tokyo Metropolitan Institute of Public Health prior to its execution and monitored at every step during the experimentation for its scientific and

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ethical appropriateness, including concern for animal welfare, with strict obedience to the National Institutes of Health Guideline for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law, Japanese Government Notification on Feeding and Safekeeping of Animals and other similar laws, guidelines, rules and *et cetera* provided domestically and internationally.

Animals

Specific pathogen free Crlj:CD1(ICR) mice, 5 weeks old, were purchased from Charles River Japan Inc., Kanagawa, Japan and were sufficiently acclimatized before use. Mice were housed individually in plastic cage (180 x 305 x 110mm^3) with cedar chip bedding and free access to the standard diet CE2 (Nihon Clea, Inc., Tokyo, Japan) and water. The animal room was maintained at 23-25°C with a relative humidity of 50-60%, with 10 ventilation per hour (drawing fresh air through an HEPA-filter, 0.3 μm, 99.9% efficiency) and on a 12 hr light/dark cycle. At 8 to 13 weeks old, a nulliparous female was housed overnight with a male and the next morning the female was checked for the presence of a vaginal plug. The day when vaginal-plug formation was observed was regarded as day 0 of the gestation.

Test chemicals

The presently utilized test chemicals, MWCNT (MITSUI MWCNT-7; lot number, 060125-01k) was exactly identical to those used in the carcinogenicity studies in *p53* gene deficient mice (Takagi *et al.*, 2008) and in intact rats (Sakamoto *et al.*, 2009). MWCNT was suspended in 2% carboxymethyl cellulose sodium (CMCNa; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) solution at concentrations of 0.2, 0.3, 0.4 or 0.5 mg/ml for the intraperitoneal study to achieve a uniform administration volume of 10 ml/kg body weight. In the case of the intratracheal study, 3, 4 and 5 mg/ml suspensions were prepared to achieve a uniform administration volume of 1 ml/kg. The control (0 mg/kg body weight) animals were received 2% CMCNa solution, intraperitoneally or intratracheally, respectively. These suspensions as well as a vehicle 2% CMCNa solution were sterilized by an autoclave at 120°C for 20 min and vigorously mixed by hand shaking immediately prior to the administration.

Animal treatment and assessments

Two independent experiments were performed. In experiment 1, pregnant female mice were given a single intraperitoneal administration of MWCNT at dosages of 2, 3, 4 or 5 mg/kg body weight on day 9 of the gestation. On the other hand, in experiment 2, mice were given a single intratracheal spray administration of 3, 4 or 5 mg/kg body weight using intratracheal aerosolizer (MicroSprayer Model IA-1B; Penn-Century, Inc., Philadelphia, PA, USA) on day 9 of the gestation.

In either experiment, body weights of mated females were measured daily, and clinical observations were recorded. All mice were killed on day 18 of the gestation under light ether anesthesia. The liver, lung, spleen, heart, kidney, thymus and tracheobronchial lymph node of each dam were removed and weighed. Peripheral blood was examined for the leukocyte counting by Sysmex KX-21NV. Blood films were made, stained by May/Grünwald;Giemsa and counted for the subtypes of leucocytes under the light microscopy.

The uterus was opened to examine for early and late fetal deaths, and to record the position of dead and live fetuses. The numbers of implantation sites and corpora lutea in the ovaries were also counted. Each live fetus was weighed and examined for external anomalies. Fetuses were fixed in 95% ethanol and stained with Alizarin Red S (Dawson, 1926) to examine skeletal anomalies.

Statistical analysis

Scheffe's multiple comparison was applied for the organ weights of dams, maternal body weights, number of implantations and live fetuses, and fetal body weights. The incidence of pregnant females and of litters with malformed fetuses, and the number of malformed fetuses were analyzed using the Chi square test. The rank sum test was used for data on the resorption and the percent incidence of malformations (Nishimura, 1976). The trend test (cumulative X2 test) was performed to evaluate the significance of the development of malformations by the administered doses of MWCNT.

RESULTS

Experiment 1, the intraperitoneal study

The pregnant status is summarized in Table 1. No animals died after the MWCNT administration. While most of the mated mice were gestated regardless to treatments, 1, 1, 6 and 6 pregnant mice, which were dosed 2, 3, 4 and 5 mg/kg body weight of MWCNT respectively, did not have any living fetuses on 18 day of the gestation. The statistical significances of this change were obtained in the 4- and 5-mg/kg groups. Similarly, the rates of early resorption of fetuses were significantly increased, with the number of live fetuses per litter being decreased, in these groups. In addition, the body weights of live fetuses were significantly lower in the 2-, 3- and 4-mg/kg groups,

Table 1. Experiment 1; pregnant status

Reproductive parameters	MWCNT dose (mg/kg body weight)				
	0 (control)	2	3	4	5
Female mated[1)]	11	12	12	15	10
Female died[2)]	0	0	0	0	0
Female gestated[3)]	10	8	9	13	9
Female with >1 live fetus	10	7	8	7*	3**
Corpora lutea/litter#	15.8 ± 1.9	15.6 ± 1.6	16.0 ± 4.1	15.4 ± 1.8	14.4 ± 2.2
Implantations/litter#	14.5 ± 2.5	14.4 ± 1.5	12.3 ± 2.7	14.0 ± 2.1	12.7 ± 3.8
Resorption of fetuses(%)[4)#]					
Early	11.0 ± 13.5	35.3 ± 34.9	41.7 ± 34.8	67.1 ± 38.8**	81.7 ± 28.2***
Late	1.7 ± 3.7	2.4 ± 3.4	0	1.6 ± 3.1	0.9 ± 2.6
Live fetus /litter#	12.6 ± 2.6	9.5 ± 5.1	7.3 ± 4.1	4.8 ± 5.8**	1.4 ± 3.3***
Body wt of live fetus (g) #					
Male	1.48 ± 0.10	1.29 ± 0.08*	1.28 ± 0.10**	1.31 ± 0.08*	1.42 ± 0.12
Female	1.43 ± 0.13	1.23 ± 0.09*	1.24 ± 0.12*	1.21 ± 0.11*	1.33 ± 0.02

[1)] Number of animals with vaginal plug. [2)] Number of animals died before the scheduled sacrifice on day 18 of the gestation. [3)] Number of animals with implantation sites. [4)] 'Early' was defined as a case showing the implanted sites and amorphous mass, while 'Late' was defined as a case showing the head and limbs. # Values are the means ± S.D. The percent resorption and foetal body weight were obtained by averaging the value for each litter. Asterisks represent that the values are significantly different from the control value (*, ** or *** indicating $p < 0.05$, 0.01 or 0.001, respectively).

but not in the 5-mg/kg group, than in the control group.

Figure 1 illustrates changes of the maternal body weight, of which increment was retarded by MWCNT with a dose-dependent tendency. The body and organ weights and leucocyte typing and counting data of dams are summarized in Table 2. The final body weights were significantly decreased in the 4- and 5-mg/kg groups. The liver weight tended decreased in the dose groups but changes were not statistically significant. The weight of the spleen was significantly increased in the dose groups but no other adverse effect was evident. The numbers of total white blood cells, neutrophils, eosinophils and monocytes, lymphocytes as well but lesser degree, all tended increased in all MWCNT-treated groups. The statistical significances of these changes were obtained in the 3-mg/kg group for the total white blood cells and in 2- 3- and 4-mg/kg groups for the neutro- and eosinophils.

The incidences of malformations were summarized in Table 3. Various types of external and skeletal malformations, such as reduction deformity of limb, short or absent tail, cleft palate, fusion of vertebrae, hypophalangia and hyperphalangia, were observed not in the control group but in all MWCNT-treated groups. Whereas respective incidences of such malformations were a few, the ratio of litters with malformed fetuses, the percent incidence of malformations and the ratio of malformed fetuses were all increased in all MWCNT-treated groups, most of them being with the statistical significance. The trend test evaluated that the development of skeletal malformations by the administered doses of MWCNT was significant ($p < 0.05$).

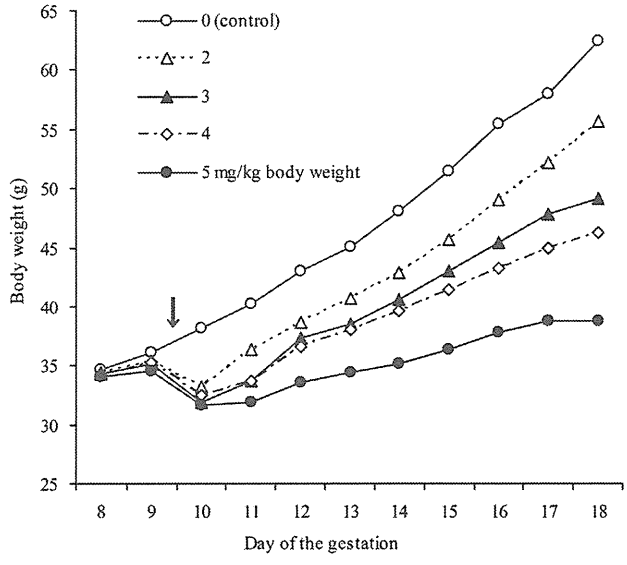

Fig. 1. Experiment 1; changes of the maternal body weights. The arrow represents the timing of the intraperitoneal administration of MWCNT.

Table 2. Experiment 1; body and organ weights, and leucocyte typing and counting of dams

Items	MWCNT dose (mg/kg body weight)				
	0 (control)	2	3	4	5
Number of dam	10	8	9	13	9
Body weight on day 9 of the gestation	36.1 ± 1.3	35.3 ± 1.9	35.2 ± 2.9	35.2 ± 2.6	34.6 ± 2.4
on day 18 of the gestation	62.4 ± 2.8	55.7 ± 12.0	49.2 ± 7.9	46.3 ± 11.9*	38.8 ± 9.4***
Organ weight					
Liver (g)	3.11 ± 0.40	3.17 ± 0.53	2.99 ± 0.49	2.80 ± 0.52	2.42 ± 0.64
Kidney (mg)	478 ± 133	503 ± 52	447 ± 48	472 ± 55	452 ± 55
Heart (mg)	179 ± 17	180 ± 23	167 ± 18	165 ± 16	157 ± 22
Lung (mg)	189 ± 8	181 ± 3	176 ± 20	188 ± 15	202 ± 20
Spleen (mg)	145 ± 40	297 ± 88*	323 ± 86**	333 ± 99**	372 ± 91***
Thymus (mg)	26.6 ± 12.9	22.6 ± 5.4	17.2 ± 9.2	25.3 ± 7.4	37.8 ± 15.0
Tracheobronchial lymph node (mg)	7.4 ± 8.3	7.3 ± 3.5	8.8 ± 4.5	6.2 ± 4.5	14.6 ± 5.1
Leucocyte count ($10^2/\mu l$)					
Total	47 ± 19	115 ± 34	124 ± 48*	109 ± 68	82 ± 38
Lymphocyte	28.9 ± 11.7	38.8 ± 12.0	33.6 ± 15.1	33.0 ± 35.7	23.9 ± 11.4
Neutrophil	15.0 ± 7.0	54.6 ± 19.2*	66.1 ± 23.0**	53.6 ± 37.0*	46.5 ± 23.9
Eosinophil	0.9 ± 0.5	17.5 ± 14.1**	16.1 ± 8.9**	15.5 ± 8.1**	6.7 ± 5.0
Monocyte	1.9 ± 1.7	4.1 ± 3.0	8.2 ± 7.8	7.0 ± 4.2	5.5 ± 4.0

Values are the mean ± S.D. Asterisks represent that the values are significantly different from the control value (*, ** or *** indicating $p < 0.05$, 0.01 or 0.001, respectively).

Table 3. Experiment 1; incidences of malformations

Items	MWCNT dose (mg/kg body weight)				
	0 (control)	2	3	4	5
External malformation					
Numbers of litters with malformed fetuses/examined (percentages in the parentheses)					
	0/10(0)	2/7(28.6)	2/8(25.0)	3/7(42.9)*	1/3(33.3)
Percent incidence of malformations#	0	9.2 ± 18.8	3.6 ± 6.8	4.6 ± 6.5	6.7 ± 11.5
Numbers of malformed fetuses/examined	0/126	3/76*	3/66*	3/63*	2/13***
Numbers of fetuses with					
short or absent tail	0	2	1	1	0
cleft palate	0	0	0	1	0
reduction deformity of limb	0	2	3	1	2
Skeletal malformation					
Numbers of litters with malformed fetuses/examined (percentages in the parentheses)					
	0/10(0)	4/7(57.1)**	3/8(37.5)*	3/7(42.9)*	2/3(66.7)**
Percent incidence of malformations#	0.0 ± 0.0	14.4 ± 18.1	11.1 ± 21.7	11.9 ± 19.2	40.0 ± 52.9
Numbers of malformed fetuses/examined	0/126	9/76***	7/66***	7/63***	5/13***
Numbers of fetuses with					
fusion of ribs	0	3	1	2	0
fusion of vertebral bodies and arches	0	6	7	0	3
hypophalangia	0	2	2	3	2
hyperphalangia	0	0	0	2	0

#Calculated by averaging the percentage in each litter (*i.e.* numbers of malformations/fetuses) and shown as the means ± S.D. Asterisks represent that the values are significantly different from the control value (*, ** or *** indicating $p < 0.05$, 0.01 or 0.001, respectively).

Experiment 2, the intratracheal study

The pregnant status is summarized in Table 4. No animals died after the MWCNT administration. Most of the treated mated mice were gestated, and all of them had living fetuses. The rates of early as well as late resorption of fetuses were increased in the 4- and 5-mg/kg groups, respectively, but these changes were not statistically significant because of a large dispersion. The numbers of live fetuses per litter in MWCNT-treated groups were well maintained, although slight decreases were seen in the 4- and 5-mg/kg groups. In contrast, the body weight of live fetuses was significantly lower in the 5-mg/kg group.

Figure 2 illustrates changes of the maternal body weights, of which increment was retarded in the 5-mg/kg group. The body and organ weights, and leucocyte typing and counting data of dam are summarized in Table 5. The final body weight was significantly decreased in the 5-mg/kg group. The weight of the lung and tracheobronchial lymph nodes tended increased in a dose-dependent tendency, and the statistical significance was achieved for the lung in the 5-mg/kg group. Lungs of dosed groups looked blackened. The numbers of total white blood cells tended increased in a dose-dependent tendency, and the statistical significance was achieved in the 4- and 5-mg/kg group, but the magnitude of this change was not so high. The numbers of all types of white blood cell looked increased in some MWCNT-treated group, but the changes were modest and lacked statistical significances.

The incidences of malformations were summarized in Table 6. Various types of external and skeletal malformations, as seen in experiment 1, were observed not in the control group and scarcely in the 3-mg/kg group. In the 4- and 5-mg/kg groups, however, such malformations occurred frequently and significantly. Typical features of the reduction deformity of limb and the fusion of vertebrae and ribs are demonstrated in Figs. 3 and 4, respectively. The ratio of litter with malformed fetuses, the percent incidence of malformations and the ratio of malformed fetuses were all increased in 4- and 5-mg/kg group, most of them being with the statistical significance.

DISCUSSION

The present results clearly elicit that MWCNT is teratogenic in mice, at least under the present experimental conditions. This is the first report demonstrate the teratogenicity of this nanomaterials. Also, there is no report on teratogenicity of other exogenous fibers such as single wall nanotubes, asbestos and glass fibers. It is sometimes difficult to judge teratogenicities of chemicals, especially when the maternal toxicity is present. Because the maternal toxicity was in fact observed in some MWCNT-treated groups of the present study, one might consider the malformation of the fetuses only reflected and thus did not necessarily indicate the "true" teratogenicity of MWC-

Table 4. Experiment 2; pregnant status

Reproductive parameter	MWCNT dose (mg/kg body weight)			
	0 (control)	3	4	5
Female mated[1)]	11	12	16	6
Female died[2)]	0	0	0	0
Female gestated[3)]	10	10	15	5
Female with >1 live fetus	10	10	15	5
Corpora lutea/litter	14.6 ± 1.5	16.0 ± 1.8	15.1 ± 1.8	15.8 ± 2.3
Implantations/litter	12.8 ± 1.6	14.8 ± 2.2	13.8 ± 2.7	11.8 ± 2.9
Resorption of fetuses(%) [4)#]				
Early	9.8 ± 13.4	8.8 ± 8.4	21.0 ± 29.8	20.0 ± 17.7
Late	2.0 ± 4.6	0.6 ± 1.8	0.8 ± 2.2	6.3 ± 10.1
Live fetus/litter[#]	11.3 ± 2.1	13.3 ± 1.5	10.5 ± 4.4	8.8 ± 2.9
Body weight of live fetus (g) [#]				
Male	1.41 ± 0.14	1.36 ± 0.12	1.23 ± 0.19	1.07 ± 0.20*
Female	1.35 ± 0.13	1.31 ± 0.11	1.19 ± 0.19	1.06 ± 0.18*

[1)] Number of animals with vaginal plug. [2)] Number of animals died before the scheduled sacrifice on day 18 of the gestation. [3)] Number of animals with implantation sites. [4)] 'Early' was defined as a case showing the implanted sites and amorphous mass, while 'Late' was defined as a case showing the head and limbs. [#] Values are the means ± S.D. The percent resorption and fetal body weight were obtained by averaging the value for each litter. Asterisks represent that the values are significantly different from the control value (* indicating $p < 0.05$).

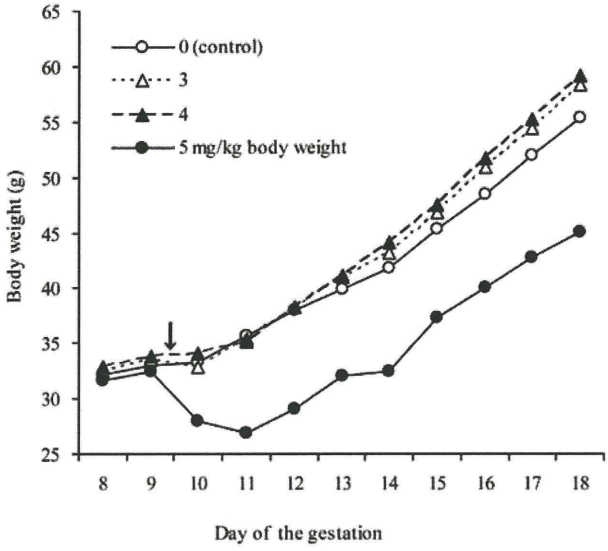

Fig. 2. Experiment 2; changes of the maternal body weights. The arrow represents the timing of the intratracheal administration of MWCNT.

Fig. 3. Experiment 2; an 18-day-old fetus, showing the reduction deformity of the limb, from a dam intratracheally administered MWCNT at a dose of 4 mg/kg body weight on day 9 of the gestation.

NT. Malformations were, however, induced even in the 4-mg/kg group of the intratracheal study, in which MWCNT did not apparently cause the maternal toxicity. In addition, the malformations induced by the MWCNT administration belonged to a reduction type, such as the reduction

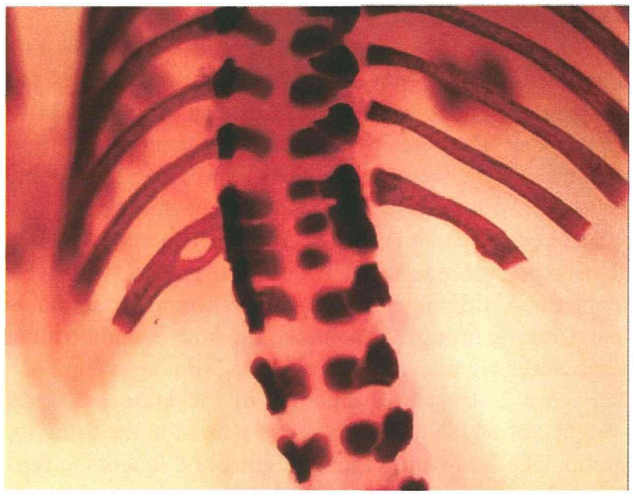

Fig. 4. Experiment 2; an 18-day-old fetus, showing the fusion of vertebrae and ribs, from a dam intratracheally administered MWCNT at a dose of 4 mg/kg body weight on day 9 of the gestation.

deformity of limbs and the short or absent tail. The malformations in this type have not been found among about 7,000 fetuses of ICR mice historically examined so far in our laboratory (Ogata *et al.*, 1984, 1987, 1989 and 1999). Also in other laboratories, the spontaneous incidence of the reduction deformity of limb of ICR mice is usually very low. For instance, the incidence of amelia and oligoductilia has both been reported to be 0.02% among 5,000 fetuses in another laboratory and no deformities have observed among 4,335 fetuses in another laboratory (Kameyama *et al.*, 1980). The malformations observed in this study are uncommon in merely by the maternal toxicity. It is thus safe to say that the teratogenicity of MWCNT demonstrated in the present study is true with a biological significance.

The reasons why we at first conducted the intraperitoneal study and used very high doses were to avoid missing a teratogenicity of MWCNT, if it is present, under the experimental condition as sensitive as possible form the point of the hazard identification. This is the same strategy that was adopted in the studies identifying the carcinogenic hazard of MWCNT (Sakamoto *et al.*, 2009; Takagi *et al.*, 2008). The relatively severe maternal toxicity in the high doses is thus rather expected. Nevertheless, the present intraperitoneal study can demonstrate the teratogenicity of MWCNT as stated above, witch then led us to confirm this hazard using a more human-relevant exposure route. In the intratracheal study, MWCNT was administered into the trachea of mice in a spray or mist shape, witch well mimics the most plausible human

Table 5. Experiment 2; body and organ weights, and leucocyte typing and counting of dams

Items	MWCNT dose (mg/kg body weight)			
	0 (control)	3	4	5
Number of dam	10	10	15	5
Body weight on day 9 of the gestation	33.0 ± 2.0	33.6 ± 2.8	33.8 ± 2.8	32.5 ± 2.2
on day 18 of the gestation	55.4 ± 3.1	58.4 ± 5.5	59.1 ± 6.9	45.1 ± 4.5*
Organ weight				
Liver (g)	2.80 ± 0.27	2.73 ± 0.36	3.05 ± 0.31	2.44 ± 0.11
Kidney (mg)	454 ± 52	431 ± 60	457 ± 54	422 ± 28
Heart (mg)	155 ± 10	161 ± 14	162 ± 15	150 ± 9
Lung (mg)	157 ± 14	168 ± 10	197 ± 51	228 ± 47**
Spleen (mg)	136 ± 22	122 ± 29	149 ± 40	158 ± 35
Thymus (mg)	19.9 ± 7.5	16.4 ± 5.3	18.9 ± 5.5	13.9 ± 8.9
Tracheobronchial lymph node (mg)	4.2 ± 3.6	6.8 ± 5.2	6.2 ± 4.3	8.7 ± 2.3
Leucocyte count ($10^2/\mu l$)				
Total	37.5 ± 6.4	49.5 ± 11.3	51.6 ± 11.5*	51.3 ± 10.6*
Lymphocyte	21.0 ± 4.4	30.0 ± 8.2	26.5 ± 7.4	22.5 ± 6.4
Neutrophil	14.7 ± 4.5	17.4 ± 9.7	20.3 ± 11.2	25.4 ± 11.5
Eosinophil	0.7 ± 0.9	1.1 ± 0.7	2.7 ± 2.5	1.6 ± 1.1
Monocyte	1.2 ± 0.7	1.0 ± 0.5	2.2 ± 1.4	1.7 ± 0.4

Values are the means ± S.D. Asterisks represent that values are significantly different from the control value (* or ** indicating $p < 0.05$ or 0.01, respectively).

Table 6. Experiment 2; incidences of malformations

Items	MWCNT dose (mg/kg body weight)			
	0 (control)	3	4	5
External malformation				
Number of litters with malformed fetuses/examined (percentages in the parentheses)				
	0/10(0)	0/10(0)	5/14(35.7)*	2/5(40.0)*
Percent incidence of malformations#	0	0	15.6 ± 27.9	5.6 ± 8.2
Number of malformed fetuses/examined	0/113	0/133	15/158***	3/44**
Number of fetuses with				
short or absent tail	0	0	12**	3**
reduction deformity of limb	0	0	7*	0
Skeletal malformation				
Number of litters with malformed fetuses/examined (percentages in the parentheses)				
	0/10(0)	1/10(10.0)	6/14(42.8)*	4/5(80.0)*
Percent incidence of malformations#	0	0	39.9 ± 48.4*	61.9 ± 38.2*
Number of malformed fetuses/examined	0/113	1/133	56/158***	31/44***
Number of fetuses with				
fusion of ribs	0	0	8*	10***
fusion of vertebral bodies and arches	0	0	54***	25***
hypophalangia	0	0	10*	1
hyperphalangia	0	1	0	1

Calculated by averaging the percentage in each litter (*i.e.*, number of malformations/fetuses) and shown as the means ± S.D. Asterisks represent that the values are significantly different from the control value (*, ** or *** indicating $p < 0.05$, 0.01 or 0.001, respectively).

exposure situation of the inhalation. The highest dose of 5 mg/kg body weight must have been too high, because it caused the apparent maternal toxicity, and it agglomerated in the lung (data not shown). It is clearly indicated, however, that MWCNT is teratogenic, because the malformations in the fetuses were significantly induced by the middle dose of 4 mg/kg body weight that did not cause the apparent maternal toxicity.

It is known that methyl cellulose of a certain length has nephrotoxicity but, in this study, no adverse effect on kidney of dam given 2% CMCNa solution (control) nor suspension of MWCNT in 2% CMCNa (dosed groups) was observed.

Mechanisms underlying the teratogenicity of MWCNT are still obscure. Sargent *et al.* (2009) has demonstrated that single-wall carbon nanotube induces aneuploidy in cultures primary and immortalized human airway epitherial cells by the disruption of the mitotic spindle. In that study, the association of nanotubes with cellular and mitotic tubulins as well as chromatins within the nuclei is demonstrated, and the similarity of nanotube bundles to microtubules in size of microtubules is considered to play roles, because it may make nanotubes incorporated into the mitotic spindle apparatus. Recently, Takahashi *et al.* (2010) has reported that MWCNT also induces polyploidy, suggesting that MWCNT may exert similar effects on microtubules to the situation of single-wall carbon nanotube. If it is a case, the disruption of the mitotic spindle and the fragmentation of the centrosomes may inhibit subsequent cell division, which results in the embryonic death in early phase and the malformation of the reduction type. Further studies are apparently warranted, and especially a passage of MWCNT through the placenta and the reach to the fetus should be evidenced.

Another possible factor involved in the teratogenicity may be the chronically persisting inflammation caused by the exposure to MWCNT, which is frequently considered to participate in the biological effects of nanomaterials (Takagi *et al.*, 2008; Sakamoto *et al.*, 2009; Erdely *et al.*, 2009; Hubbs *et al.*, 2011). The present results of the increments of the numbers of leucocyte and related hemocytes, and of the weight of the spleen might support this possibility.

The present intratracheal study gives no-observed-adverse-effect level (NOAEL) of 3 mg/kg body weight for external and skeletal malformations. Although the human exposure level of MWCNT has not as yet clearly determined, the interim report for the risk assessment of MWCNT by the National Institute of Advanced Industrial Science and Technology (AIST, 2009) roughly estimated the quantity of MWCNT exposure of workers as 0.53- 6.20 μg/kg/day. Comparing with these values, the above NOAEL for external and skeletal malformations are approximately 480-5,660 times high. It is thus tentatively evaluated that the present results may not necessarily or immediately indicate a human risk. Needless to say, however, more detailed and careful investigations including those for the teratogenicity must be conducted to complete the risk assessment of MWCNT.

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Original Article

Effects of sustained stimulation with multi-wall carbon nanotubes on immune and inflammatory responses in mice

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ABSTRACT — Possible effects of multi-wall carbon nanotubes (MWCNTs) on immune and inflammatory responses were examined in mice. Female ICR mice were given a single intraperitoneal administration (2 mg/kg body weight) of either MWCNTs, carbon black (CB), or crocidolite (blue asbestos) and controls received a vehicle of 2% sodium carboxymethyl cellulose (CMC Na). In the peritoneal cavity of MWCNT-administered mice, the liver had changed to a rounded shape and fibrous adhesions were seen on internal organs. Peritoneal cells overexpressed mRNA for genes of T helper (Th)2 cytokines (*interleukin [IL]-4*, *IL-5*, and *IL-13*), Th17 cytokine (*IL-17*), pro-inflammatory cytokines/chemokines (*IL-1β*, *IL-33, tumor necrosis factor α,* and *monocyte chemotactic protein-1*), and *myeloid differentiation factor 88* for at least 2 weeks after the administration of MWCNTs, while those of Th1 cytokine genes (*IL-2* and *interferon γ*) were overexpressed several weeks later and expression levels remained high up to 20 weeks. In MWCNT-treated mice, the numbers of leukocytes, monocytes, and granulocytes in the peripheral blood and the expression of the leukocyte adhesion molecules, cluster of differentiation (CD)49d and CD54, on granulocytes were increased 1 week after administration and remained high up to week 20. Production of ovalbumin-specific IgM and IgG_1 was enhanced by MWCNTs. These changes were not observed after CB or crocidolite administration. Thus, this study showed that MWCNTs exhibited sustained stimulating effects on immune and inflammatory responses, unlike the other mineral fibers with structural similarities.

Key words: Multi-wall carbon nanotube, Nanomaterial, Inflammation, Immunotoxicity, Hazard characterization

INTRODUCTION

Rapid progress in nanotechnology in recent years has made it possible to produce and apply numerous new and useful nanomaterials, such as nano-TiO_2, nano-SiO_2, nano-ZnO and nano-carbon materials. These are believed to be biologically inert, although inhalation of small-sized nanomaterials can cause pulmonary inflammation and fibrosis. (Mossman and Churg, 1998; Yazdi *et al.*, 2010). Carbon forms exist in many different shapes as both elementary substances and compounds, for example, diamond, charcoal, carbon black, graphite, fullerene, and carbon nanotubes are all carbon allotropes, while graphene is a single-wall product of graphite, whose structure consists of one-atom-thick planar sheets of hexagonal-bonded carbon atoms densely packed in honeycomb crystal lattices. Carbon nanotubes are seamless cylindrical structures comprising single or multiple graphene sheets. Both single-wall carbon nanotubes (SWCNTs) and multi-wall carbon nanotubes (MWCNTs) are several micrometers in length and approximately 1-20 nanometers in diameter. These needle-like structures resemble asbestos.

It is well known that asbestos inhalation causes pulmonary inflammation and fibrosis, lung cancer, and malignant mesothelioma after relatively long latency periods (Mossman *et al.*, 1990; Hei *et al.*, 1992). However, the signaling pathways that lead to the development of these asbestos-associated diseases remain largely unknown. If

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carbon nanotubes can create the hazards and risks similar to those associated with asbestos, they must be appropriately assessed and managed to protect human health.

In vivo effects of MWCNTs have been studied using animal models with a variety of exposure methods, such as inhalation, intratracheal instillation, pharyngeal aspiration, and intraperitoneal injection, and their effects on inflammatory responses have been described. Mice exposed to MWCNTs by inhalation caused platelet-derived growth factor (PDGF) overexpression, inflammatory cell aggregation, and recruitment of macrophages that phagocytosed MWCNTs in the lung within 1 day, followed by the subsequent development of subpleural fibrosis during weeks 2-6 (Ryman-Rasmussen *et al.*, 2009a, 2009b). Pharyngeal aspiration of MWCNTs in mice caused the rapid development of fibrosis within 7 days and a persistent granulomatous inflammation throughout a 56-day post-exposure period (Porter *et al.*, 2010). Intratracheal instillation of MWCNTs in mice caused an increase in the number of neutrophils and the levels of cytokines in bronchoalveolar lavage (BAL) fluid within 1 day, and granulomatous lesions developed and persisted until day 14 of these experiments (Park *et al.*, 2009). Intraperitoneal injection of MWCNTs given to rats (Sakamoto *et al.*, 2009) or *p53* gene heterozygously deficient mice (Takagi *et al.*, 2008) induced a long-lasting inflammation and resulted in fibrous thickening and granuloma formation in the peritoneum in association with the induction of mesothelioma.

However, despite evidences from these studies, the potential immunotoxicity of MWCNTs has not been sufficiently established till date. Thus, the present study was conducted to assess a possible involvement of MWCNTs in immune and inflammatory responses of ICR mice. Intraperitoneal administration was chosen as the exposure route for MWCNTs. Although it may not be directly relevant to humans, intraperitoneal administration in a rodent model is sensitive enough to detect weak effects of MWCNTs, which was why this strategy was adopted to identify a possible carcinogenic hazard of MWCNTs (Sakamoto *et al.*, 2009; Takagi *et al.*, 2008). In addition, intraperitoneal administration can control and ensure the relationship between administration doses and agent exposure. Furthermore, some reports have clearly indicated the detection of inhaled MWCNTs in the subpleura (Ryman-Rasmussen *et al.*, 2009a), pharyngeally-aspirated MWCNTs in the pleura (Porter *et al.*, 2010), and intraperitoneally-administered MWCNTs in the liver and mesenteric lymph nodes (Sakamoto *et al.*, 2009). These results suggest that MWCNTs are distributed to a certain extent in the entire body, regardless of the exposure route used.

MATERIALS AND METHODS

Ethical approval

Our experimental protocols were approved by the Experiments Regulation Committee and the Animal Experiment Committee of the Tokyo Metropolitan Institute of Public Health prior to beginning of these experiments and were monitored at each step of experimentation for scientific and ethical appropriateness, including concerns for animal welfare, with strict adherence to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law, Japanese Government Notification on Feeding and Safekeeping of Animals, and other similar laws, guidelines, and rules provided domestically and internationally.

Animals

Specific pathogen-free female Crlj:CD1(ICR) mice, 6-7 weeks old, were purchased from Charles River Japan, Inc. (Kanagawa, Japan) and acclimatized for 1 week. Mice were housed individually in plastic cages (22 × 15 × 12 cm) with cedar chip bedding and had free access to a standard diet CE2 (Nihon Clea, Inc., Tokyo, Japan) and water. The animal room was maintained at 24°C-26°C with a relative humidity of 50%-60%, with 10 ventilations per hour (drawing fresh air through a high-efficiency particulate air filter, 0.3 μm, 99.9% efficiency), and on a 12 hr light/dark cycle.

Chemicals, reagents, and kits

MWCNTs (MITSUI MWCNT-7; lot number 060125-01k) were provided by National Institute of Health Science, Tokyo, Japan. These were exactly identical to those used in carcinogenicity studies with male Fisher 344 rats (Sakamoto *et al.*, 2009) and male C57BL/6-originated mice that were heterozygously deficient in the *p53* gene (Takagi *et al.*, 2008); these reports describe their physicochemical properties. Carbon black (CB; 22 nm in diameter) was purchased from Showa Chemical Industry Co., Ltd. (Tokyo, Japan). UICC-grade crocidolite was provided by the Tokyo Metropolitan Institute of Public Health.

Ovalbumin (OVA) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse IgM and IgG_1 antibodies were purchased from Zymed Laboratories (South San Francisco, CA, USA). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) tablets, a substrate for HPR-conjugated antibodies, were purchased from Roche

Diagnostics Division (Basel, Switzerland). Monoclonal anti-OVA-IgG_1 antibody (clone OVA-14) was purchased from Sigma-Aldrich. Phycoerythrin-conjugated (PE-) anti-CD3 (derived from T cell clone 145-2C11), fluorescein isothiocyanate-conjugated (FITC-) anti-CD45R (B220) (derived from B cell clone RA3-6B2), PE- anti-CD8 (clone 53-6.7), and FITC- anti-CD4 (clone GK1.5) were purchased from Beckman Coulter, Inc. (Fullerton, CA, USA). PE-Cy5.5--anti-CD3 (clone 145-2C11), PE-Cy5.5--anti-CD45 (derived from leukocyte clone 30-F11), PE--anti-CD14 (derived from monocyte clone: Sa2-8), PE--anti-Ly-6G (derived from granulocyte clone RB6-8C5), FITC--anti-CD54 (intercellular adhesion molecule [ICAM]-1; clone YN1/1.7.4), FITC--anti-CD49d (integrin α4; clone R1-2), FITC-anti-CD11b (integrin αM; clone 1/70), and anti-CD16/CD32 (Fcγ receptor III/II; clone 93) antibodies were purchased from eBioscience, Inc. (San Diego, CA, USA). RNAprotect Cell Reagent, RNeasy Mini kit, High Capacity RNA-to-cDNA kit, TaqMan Gene Expression Master Mix, and TaqMan Gene Expression Assays Inventoried were purchased from Life Technologies Co. (Carlsbad, CA, USA).

Animal experiments

Three independent animal experiments were conducted; protocols for handling test chemicals were identical in each. MWCNTs, CB, or crocidolite was suspended in 2% sodium carboxymethyl cellulose (CMC Na) to a concentration of 0.2 mg/ml. A single intraperitoneal dose (2 mg/kg body weight) of each of these was administered to mice. In a vehicle control group, CMC Na was administered with a single intraperitoneal volume of 10 ml/kg body weight.

The first animal experiment included histopathological examination and real-time polymerase chain reaction (PCR) assays for mRNA expression of cytokine/chemokine genes. Within 32 weeks, 2 of 6 mice that were administered MWCNT died; hence, the last experiment was conducted at the end of 34 weeks after administration. Mice were maintained up to 34 weeks after their exposure to test chemicals or vehicle. From each treatment or vehicle group, 3-6 animals were chosen for assays at the end of 2, 4, 10, 20, and 34 weeks. Under light ether anesthesia, cells were collected from the abdominal cavity and suspended in 5 ml of phosphate-buffered saline (PBS), centrifuged at 1,200 rpm for 10 min, and stored in RNAprotect Cell Reagent until RNA extraction for the real-time PCR assay. Tissues and organs were harvested for histopathological examinations. Samples were fixed in neutrally buffered formalin, embedded in paraffin, and stained with sirius red for collagen or hematoxylin-eosin.

The second animal experiment included flow cytometry analysis of the peripheral blood cells. Mice were maintained up to 20 weeks after their exposure to test chemicals or vehicle. From each treatment or vehicle group, 4 animals were chosen for the assays on day 2 and at the end of 1, 2, 4, and 20 weeks. Under light ether anesthesia, approximately 1 ml of blood was collected through cardiac puncture into a syringe with 20 μl of an anticoagulant, ethylenediaminetetraacetic acid, and used for flow cytometry.

The third animal experiment included determinations of OVA-specific immunoglobulins. After their exposure to test chemicals or vehicle, mice were immunized with OVA/alum intraperitoneally administered at a dose of 100 μg/mouse on days 2 and 10 as previously described (Ito *et al.*, 2002). Under light ether anesthesia, blood samples of approximately 0.1 ml were collected from a tail vein. Samples were taken from 10 to19 animals from each treatment or vehicle group from the tail vein 8 days after the last immunization for IgM and from 15 animals from each group 20 days after the last immunization for IgG_1. Serum was stored at -80°C until assayed.

Real-time PCR assays for mRNA expression of cytokine/chemokine gene

Total RNA was isolated from 5 x 10^4 peritoneal cells obtained in the first animal experiment as described above, using RNeasy Mini kit. RNA from untreated 8-16-week-old female ICR mice were prepared separately, pooled, and used as a basal expression control. First-strand cDNA was prepared from 0.9 μg of RNA using a High Capacity RNA-to-cDNA kit. PCR used TaqMan Gene Expression Master Mix for genes (*IL-1β*, Mm01336189_m1; *IL-2*, Mm00434256_m1; *IL-4*, Mm99999154_m1; *IL-5*, Mm99999063_m1; *IL-6*, Mm99999064_m1; *IL-8*, Mm00436450_m1; *IL-10*, Mm99999062_m1; *IL-13*, Mm00434204_m1; *IL-17*, Mm00439619_m1; *IL-18*, Mm00434225_m1; *IL-33*, Mm00505403_m1; *IFNγ*, Mm99999071_m1; *MCP-1*, Mm00441242_m1; *MyD88*, Mm00440338_m1; *TGFβ1*, Mm03024053_m1; *TNFα*, Mm99999068_m1; *TATA box binding protein [TBP]*, Mm00446973_m1; *hypoxanthine phosphoribosyltransferase [HPRT]*, Mm00446968_m1), cDNA-specific TaqMan Gene Expression Assays, and an ABI 7500 Real-Time PCR System (Life Technologies). All PCR reactions were performed in duplicates. The quantity of PCR product was determined by the Comparative Ct Method as described by the manufacturer, in which each sample was normalized against the value of a housekeeping gene, *HPRT*. Fold-changes were expressed as either an increase or decrease compared with the basal expression control level.

Flow cytometry analysis of the peripheral blood cells

After 15 minute pre-incubation with an anti-CD16/32 monoclonal antibody to prevent non-specific binding, a peripheral blood sample (100 μl) obtained in the second animal experiment described above was reacted with various combinations of antibodies. After a 30-min incubation in the dark, erythrocytes were lysed with 4 ml of Tris (1 g/500 ml)plus NH_4Cl (2.8 g /500 ml) for 10 min, suspended in 4 ml of PBS, and centrifuged at 1,200 rpm for 10 min. The cell pellet was washed in PBS with 0.5% BSA. Fluorescence intensity and cell numbers were determined using a Cell Lab Quanta SC (Beckman Coulter). The number of leucocytes was counted as positive cells of PE-Cy5.5- anti-CD45 antibody. The number of lymphocytes was distinguished based on CD45 fluorescence and side scatter. T and B cells were distinguished based on PE and FITC fluorescence from PE-Cy5.5- CD45 positive cells. CD4 and CD8 cells were distinguished based on PE and FITC fluorescence from PE-Cy5.5- CD3 positive cells. Percent of CD11b, CD49d, and CD54 positive cells was measured based on FITC fluorescence from CD45 and CD14 or CD45 and Ly6G positive cells.

Serum OVA-specific immunoglobulin concentrations

Concentrations of OVA-specific IgM and IgG_1 in serum were determined using ELISA. We added 100 μl of 100 μg/ml of OVA to wells of a microtest plate and incubated the plates overnight at 4°C. Wells were washed 6 times with 0.05% Tween20/PBS (0.05T/PBS) and blocked with 5% BSA in PBS (5B/PBS) for 2 hr at room temperature. Diluted serum (IgM: 1/150, IgG_1: 1/5 x 10^6) was then added to each well and incubated for 2 hr. After 6 washes with 0.05T/PBS, the wells were blocked with 5B/PBS for 1 hr at room temperature. HRP-labeled anti-mouse IgM and IgG_1 antibodies were added to each well, and the plates were incubated for 2 hr at room temperature. After 6 washes with 0.05T/PBS, a substrate solution prepared using ABTS Tablets according to the manufacturer's instructions was added and the color reaction was allowed to develop in the dark at room temperature for 30 min. Optical density (OD) at 405 against 492 nm was determined using a microplate reader (SUNRISE REMOTE; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Values for anti-OVA antibody were used as basal expression control.

Statistical analysis

Intergroup comparisons were made using Student's *t*-test. Significance level was set at p value < 0.05 .

RESULTS

General findings

During the course of the experiments, the body weights of mice increased within the same range after intraperitoneal administration of CMC Na, MWCNTs, CB, or crocidolite. In the first animal experiment, morphological assessments were conducted for mice that were given a single intraperitoneal administration of MWCNT, CB, or crocidolite. In the abdominal cavities of MWCNT-treated mice as compared with CMC Na-treated mice, liver edges had lost their sharpness, fibrous adhesions were seen on internal organs, and deposits were observed on the surfaces of the liver and diaphragm (Figs. 1a and 1b). In CB-treated mice, deposits were scattered in the abdominal cavity, especially on intestinal surfaces (Fig. 1c). No noteworthy changes were observed in the abdominal cavities of the CMC Na- or crocidolite-treated mice (Figs. 1a and 1d), or anywhere outside of the abdominal cavity in any of the groups.

Peritoneal cells obtained from MWCNT-treated mice contained small amounts of erythrocytes as compared with CMC Na-treated mice (Figs. 2a and 2b), whereas numerous erythrocytes were found for the crocidolite-treated mice (Fig. 2d). The peritoneal cells obtained from the CB-treated mice looked black, presumably because of the engulfment of the test chemical (Fig. 2c).

Fig. 3 showed the micrograms of liver, and Fig. 3a and 3b were the liver of CMC Na treated-mice that had thin layered mesothelium. Histopathological examinations revealed that the hepatic visceral peritoneum had fibrous thickening along with mesothelial cell hypertrophy in the MWCNT-treated mice (Figs. 3c and 3d). Inflammatory cells had infiltrated into this fibrously thickened visceral peritoneum. The majority of these infiltrating cells were macrophages containing MWCNTs, along with eosinophils, plasma cells, and immature myeloid cells (Fig. 3e) that occasionally formed a granulation (Fig. 3c). These changes were not observed in the CB- or crocidolite-treated mice.

No tumorigenic changes were observed either macroscopically or histopathologically in any of the mice treated with any of the test chemicals within the 34-week experimental period.

Expression of cytokine mRNA in peritoneal cells

mRNA expression levels of certain cytokine genes were substantially increased in the peritoneal cells obtained from MWCNT-treated mice, and these high levels were maintained up to the ends of 20 and 34 weeks. Th2 cytokine gene mRNA levels for *IL-4*, *IL-5,* and *IL-13*

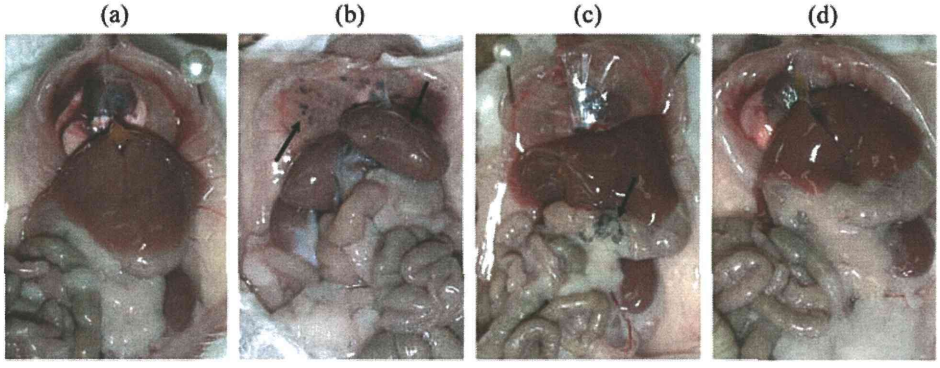

Fig. 1. Representative macroscopic appearances of the mouse abdominal cavity in the first animal experiment. Observations were made at 10 weeks after exposure to (a) CMC Na, (b) MWCNTs, (c) CB, or (d) crocidolite. Arrows indicate deposits of test chemicals.

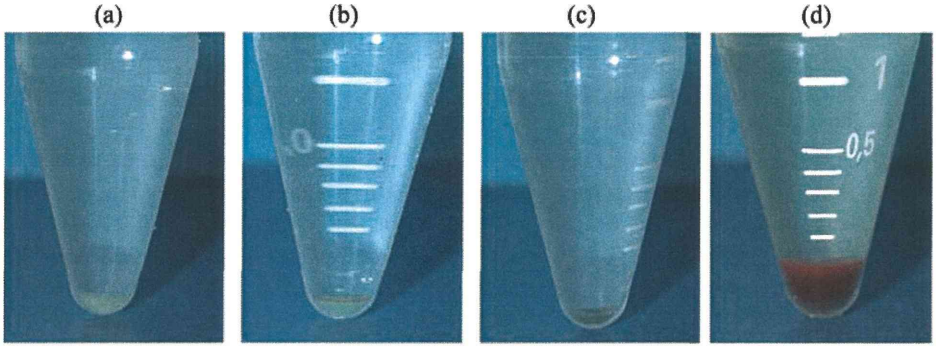

Fig. 2. Representative macroscopic appearances of peritoneal cells obtained from mice in the first animal experiment. Observations were made at 10 weeks after exposure to (a) CMC Na, (b) MWCNTs, (c) CB, or (d) crocidolite.

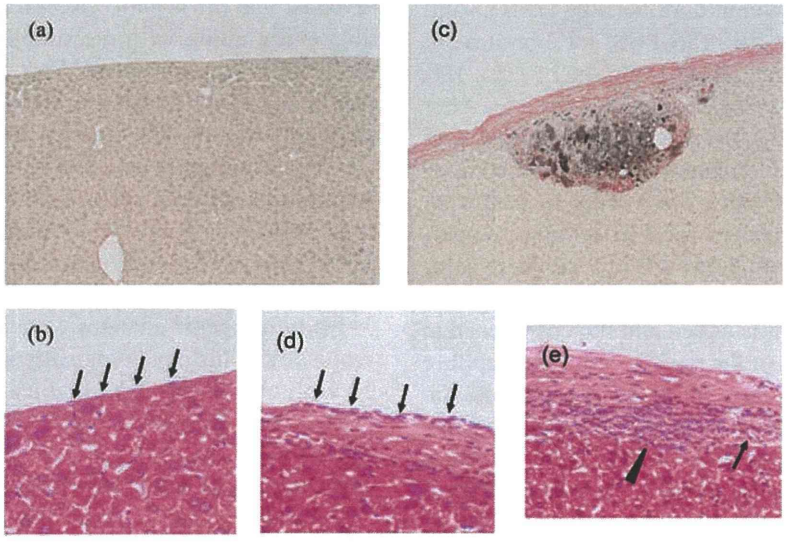

Fig. 3. Representative histology of the mouse liver in the first animal experiment. Examinations were made at 20 weeks after exposure to (a, b) CMC Na or (c-e) MWCNTs. Arrows indicate mesothelial cells and arrowheads indicate the infiltrations of eosinophils and immature myeloid cells. (a) and (c) were stained with sirius red staining, x 100, and (b), (d), and (e) were stained with hematoxylin-eosin, x 400.

were significantly increased from 2 to 20 weeks after administering MWCNTs. When compared with the basal expression control value for untreated animals, mRNA levels of *IL-4* were 34, 43, 24, 60, and 3 times higher, those of *IL-5* were 110, 127, 63, 226, and 69 times higher, and those of *IL-13* were 55, 38, 11, 28, and 3 times higher at 2, 4, 10, 20, and 34 weeks, respectively, after administering MWCNTs (Fig. 4).

Overexpression of mRNA of Th1 cytokine genes, *IL-2* and *IFNγ*, were delayed compared with mRNA of Th2 cytokine genes, but were also sustained; however, these were not significantly higher than basal expression levels except for *IL-2* at the end of 34 weeks. mRNA expression levels of *IL-2* were 0.3, 0.6, 1.5, 5.4, and 4.8 times higher, and those of *IFNγ* were 0.5, 0.3, 0.6, 1.6, and 4.3 times higher at the end of 2, 4, 10, 20, and 34 weeks, respectively (Fig. 4). Sustained mRNA overexpression was also found for a Th17 cytokine gene, *IL-17*, and these increases were significant at the end of 10 to 20 weeks.

mRNA for genes of proinflammatory cytokines, *IL-1β*, *IL-33*, and *TNFα*, and an inflammatory chemokine, *MCP-1*, were increased significantly at the end of 2 to 20 weeks (*IL-1β* and *TNFα*) and at 2 to 34 weeks (*IL-33* and *MCP-1*). mRNA level of an adapter protein of Toll-like receptors (TLR), *MyD88*, was also increased significantly at the end of week 2 to 20. mRNA levels of *IL-17* were 9, 13, 9, 26, and 25 times higher, those of *IL-1β* were 29, 23, 32, 28, and 19 times higher, those of *IL-33* were 13, 20, 5, 13, and 20 times higher, those of *TNFα* were 3, 2, 2, 3, and 2 times higher, those of *MCP-1* were 17, 28, 41, 49, and 42 times higher, and those of *MyD88* were 3, 3, 2, 2, and 1 time higher at 2, 4, 10, 20, and 34 weeks, respectively, after MWCNT administration (Figs. 4 and 5). mRNA levels of other inflammatory cytokine genes (*IL-6*, *IL-8,* and *IL-18*), anti-inflammatory cytokines (*IL-10* and *TGFβ*), and a housekeeping gene (*TBP*) were not affected by exposure to MWCNTs (Figs. 4 and 5).

CB did not affect these cytokine mRNA expressions in peritoneal cells. For crocidolite-treated mice, sustained mRNA overexpression was observed only for an inflammatory cytokine gene, *IL-6* (4, 5, 6, and 8 times higher at the end of 2, 4, 10, and 20 weeks, respectively), which

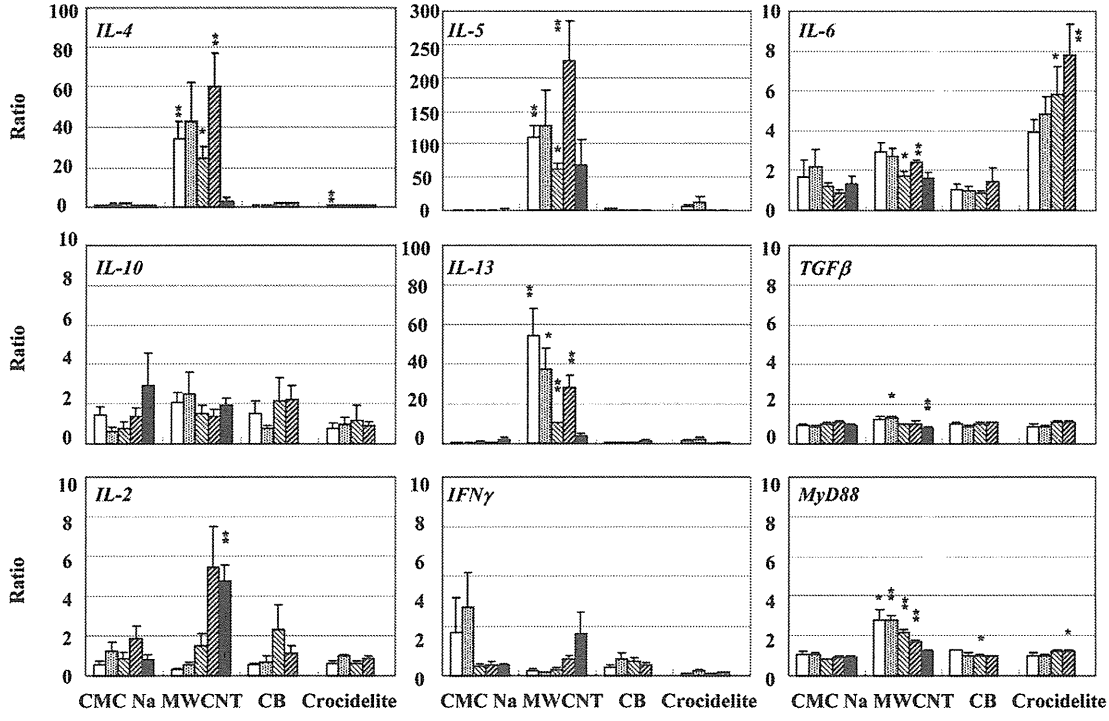

Fig. 4. mRNA expression of Th2 cytokine genes (*IL-4*, *IL-5*, *IL-10*, *IL-13,* and *TGFβ*), Th1 cytokine genes (*IL-2* and *IFNγ*), and a TLR adaptor protein (*MyD88*) in peritoneal cells obtained from mice in the first animal experiment. For each group of mice exposed to a test chemical or vehicle, columns from the left to the right are average values (n = 3-6) at 2, 4, 10, 20, and 34 weeks after exposure. These determinations were not made at the end of week 34 for the CB- and crocidolite-treated groups. (*$p < 0.05$, **$p < 0.01$).

was a more pronounced change than that with MWCNTs (Fig. 4). In addition, mRNA levels of *IL-5* were 5, 11, 1, and 1 times higher (Fig. 4), and those of *MCP-1* were 14, 9, 2, and 4 times higher (Fig. 5) at the end of 2, 4, 10, and 20 weeks, respectively; however, these changes were faint and transient.

Effects on the peripheral blood cells

MWCNT treatment increased the total number of leukocytes, granulocytes, and monocytes in the peripheral blood as early as 1 week after its administration, and these high levels were maintained up to the end of week 20 (Figs. 6a, 6b and 6c). The number of total lymphocytes was also increased, but only at the end of week 20. B and T cells were increased, although not significantly, within the 20-week experimental period in the MWCNT-treated mice (Figs. 6d, 6e and 6f). In the crocidolite treatment mice, the numbers of leukocytes, granulocytes, and monocytes exhibited a statistically significant, although minimal, transient increase at the end of week 1 (Figs. 6a, 6b and 6c). CB and crocidolite treatment increased the numbers of lymphocytes, B, and T cells at the end of day 2 and 1 week, but not significantly, and then decreased (Figs. 6d, 6e and 6f).

Expression of leukocyte adhesion molecules on the peripheral blood cells

MWCNT treatment induced overexpression of CD49d and CD54, but not CD11b, on granulocytes as early as 2 and 1 weeks, respectively, after its administration, and these high levels were maintained up to the end of week 20 (Fig. 7a). The expression of adhesion molecules was not altered on monocytes, with the exception that a statistically significant, although minimal, transient overexpression was observed for CD49d at the end of week 4 (Fig. 7b). CB and crocidolite did not induce overexpression of any of the leukocyte adhesion molecules on the peripheral blood cells, and in fact their expression was transiently decreased in some cases (Fig. 7).

OVA-specific immunoglobulins in serum

Figure 8 summarizes the results for the serum con-

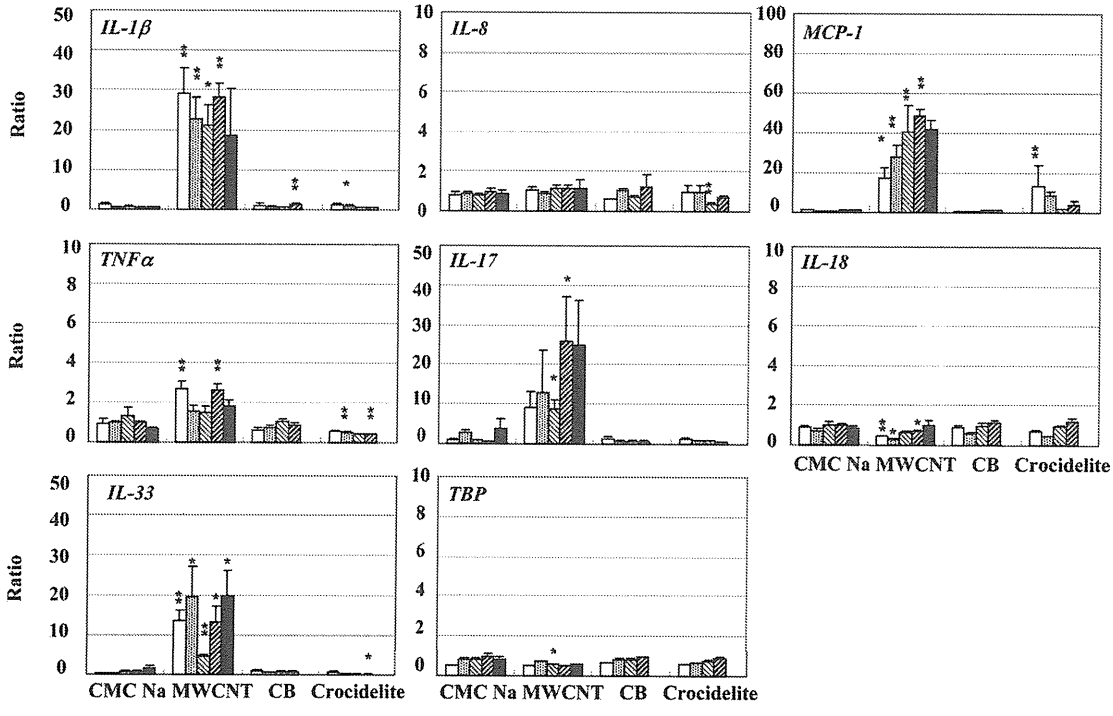

Fig. 5. mRNA expression of inflammatory cytokine genes in peritoneal cells obtained from mice in the first animal experiment. For each group of mice exposed to a test chemical or vehicle, columns from the left to the right are average values (n = 3-6) at 2, 4, 10, 20, and 34 weeks after exposure. These determinations were not made at the end of week 34 for the CB- and crocidolite-treated groups. (*$p < 0.05$, **$p < 0.01$).

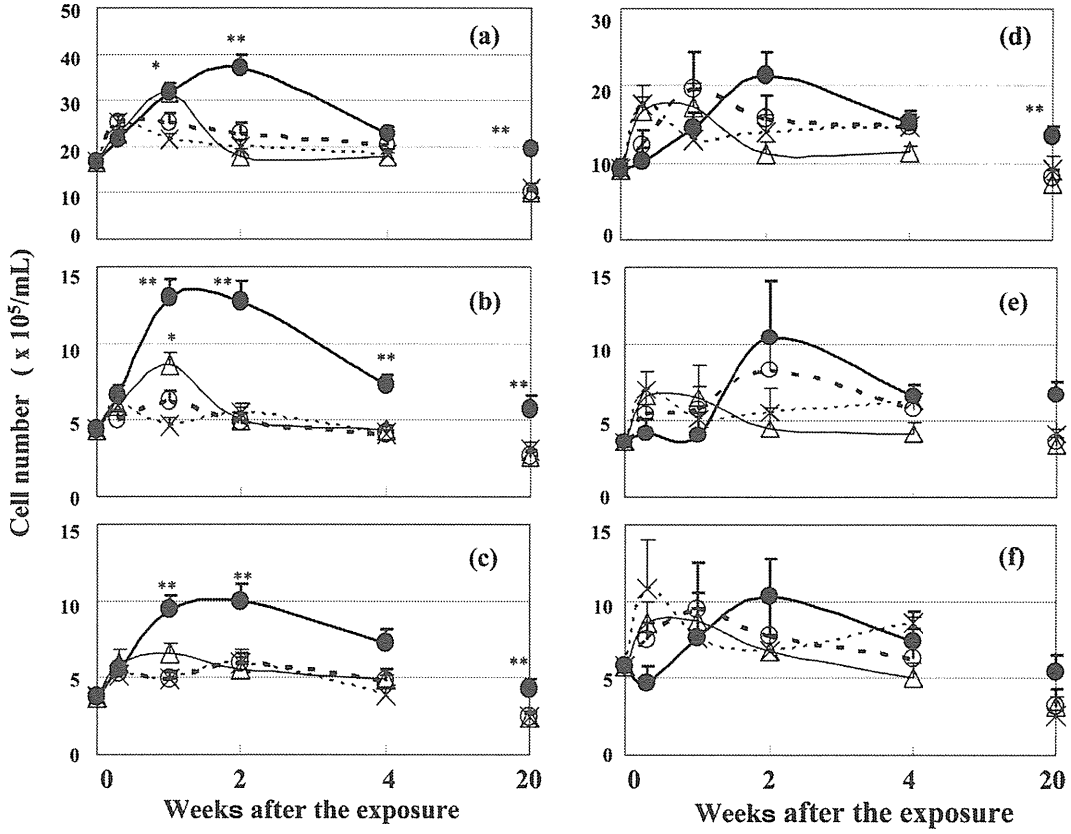

Fig. 6. Flow cytometry results for the peripheral blood cells obtained from mice in the second animal experiment. Changes in the numbers of (a) leukocytes, (b) granulocytes, (c) monocytes, (d) lymphocytes, (e) B cells, and (f) T cells after exposure to CMC Na (open circles), MWCNTs (closed circles), CB (crosses), and crocidolite (open triangles). Results are means ± standard deviations (n = 4). Asterisks indicate that results are significantly different from those of controls (*$p < 0.05$, **$p < 0.01$).

centrations of OVA-specific IgM (Fig. 8a) and IgG_1 (Fig. 8b). For mice treated with MWCNTs, CB, crocidolite, and CMC Na, the relative amounts (arbitrary units; AU) of OVA-specific IgM were, 1.33 ± 0.20, 1.07 ± 0.20, 1.07 ± 0.15, and 0.79 ± 0.12 AU, respectively, while those for OVA-specific IgG_1 were, 3.68 ± 0.57, 2.49 ± 0.29, 2.13 ± 0.32, and 2.28 ± 0.35 AU, respectively. Thus, MWCNT and not CB or crocidolite, significantly enhanced the production of OVA-specific immunoglobulins in mice.

DISCUSSION

The present study clearly shows that MWCNTs stimulated immune and inflammatory responses in mice and these effects sustained until the mice died. It has been previously shown in other animal models that a single intraperitoneal administration of MWCNT caused severe inflammation throughout the abdominal cavity and mesothelioma. Male Fisher 344 rats died at 37-52 weeks after administration (Sakamoto *et al.*, 2009) and and male C57BL/6-originated mice heterozygously deficient in the *p53* gene died within 25 weeks of administration (Takagi *et al.*, 2008).

The toxicity caused by MWCNTs in the present study did not involve tumor formation, but did induce severe inflammation, and 2 of 6 mice had died by the end of 32 weeks. The differences in the magnitudes of MWCNT toxicity between the present and previous studies was apparently because of differences in species, strains, and/or genders. To extrapolate the animal toxicity data to information important for human health concerns, further investigations are required. The most aggressive morphological change we observed was the infiltration of macrophages, eosinophils, plasma cells, and immature myeloid cells into the fibrously thickened visceral peritoneum of the liver with occasional granulation, and severe fibrous adhesions to the internal organs.

Light microscopic examination revealed that MWCNT

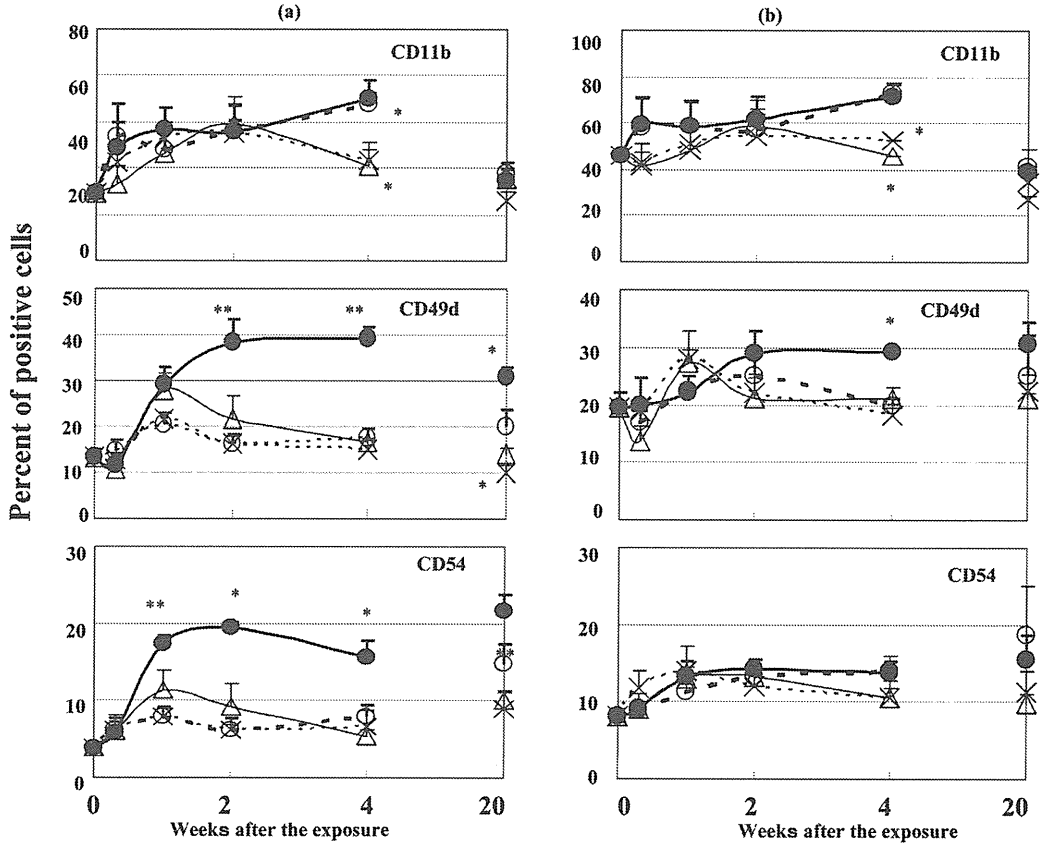

Fig. 7. Flow cytometry results for the peripheral blood cells obtained from mice in the second animal experiment. Changes in the expression of adhesion molecules on the surfaces of (a) granulocytes and (b) monocytes after exposure to CMC Na (open circles), MWCNT (closed circles), CB (crosses), and crocidolite (open triangles). Results are means ± S.D. (n = 4). Asterisks indicate that values are significantly different from those of controls ($*p < 0.05$).

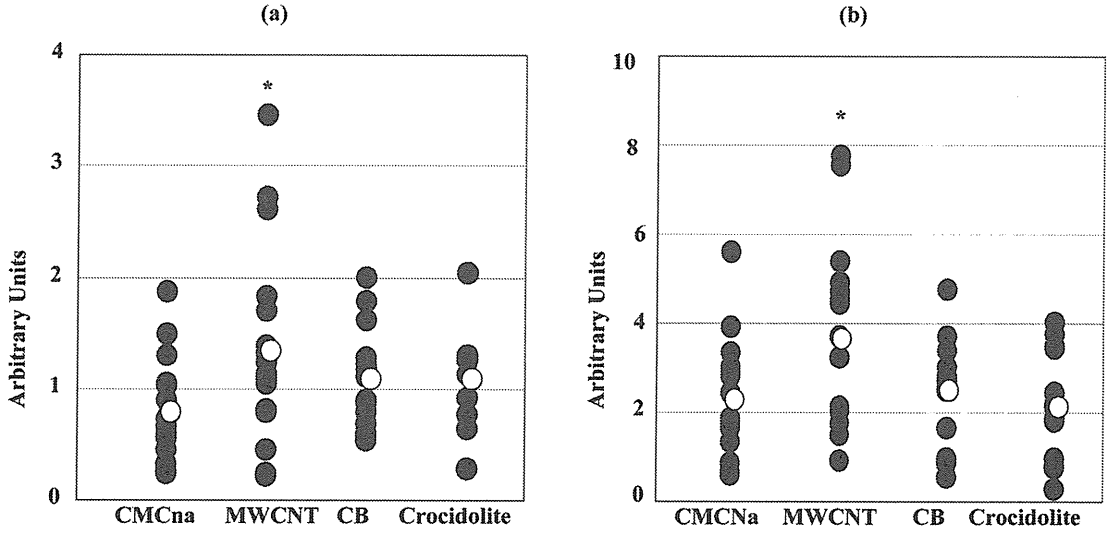

Fig. 8. Production of OVA-specific antibodies by mice in the third animal experiment. Serum concentrations of OVA-specific (a) IgM and (b) IgG_1 Open circles are average values, and closed circles are individual values (n = 10-19 for IgM, and n = 15 for IgG_1). Asterisks indicate that values are significantly different from those of controls ($*p < 0.05$).