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Inhalation Toxicity Assessment of Carbon-Based Nanoparticles

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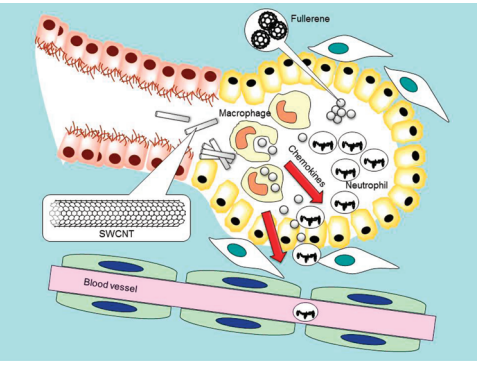
CONSPECTUS

Although the demand for nanomaterials has grown, researchers have not conclusively determined the effects of nanomaterials on the human body. To understand the effects of nanomaterials on occupational health, we need to estimate the respiratory toxicity of nanomaterials through inhalation studies, intratracheal instillation studies, and pharyngeal aspiration studies. The discrepancies observed among these studies tend to result from differences in the physiochemical properties of nanomaterials, such as aggregation and dispersion. Therefore, in all toxicity studies, identification of the physicochemical properties of nanomaterials is essential.



This Account reviews the inhalation toxicity of manufactured nanomaterials and compares them with inhalation and intratracheal instillation studies of well-characterized fullerene and carbon nanotubes. In many reports, pulmonary inflammation and injury served as pulmonary endpoints for the inhalation toxicity. To assess pulmonary inflammation, we examined neutrophil and macrophage infiltration in the alveolar and/or interstitial space, and the expression of the neutrophil and/or monocyte chemokines. We also reported the release of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) in the bronchoalveolar lavage fluid (BALF), the expression of oxidative stress-related genes characteristic of lung injury, and the presence of granulomatous lesion and pulmonary fibrosis.

In the inhalation and intratracheal instillation studies of well-characterized fullerenes, exposure to fullerene did not induce pulmonary inflammation or transient inflammation. By contrast, in an inhalation study, a high concentration of multiwall carbon nanotubes (MWCNTs) and single-wall carbon nanotubes (SWCNTs) induced neutrophil inflammation or granulomatous formations in the lung, and intratracheal instillation of MWCNTs and SWCNTs induced persistent inflammation in the lung.

Among the physicochemical properties of carbon nanotubes, the increased surface area is associated with inflammatory activity as measured by the increase in the rate of neutrophils measured in bronchoalveolar lavage fluid. Metal impurities such as iron and nickel enhanced the pulmonary toxicity of carbon nanotubes, and SWCNTs that included an amorphous carbon induced multifocal granulomas in the lung while purer SWCNTs did not. The aggregation state also affects pulmonary response: Exposure to well-dispersed carbon nanotubes led to the thickening of the alveolar wall and fewer granulomatous lesions in the lung, while agglomerated carbon nanotubes produced granulomatous inflammation.

The values of the acceptable exposure concentration in some countries were based on the data of subacute and subchronic inhalation and intratracheal instillation studies of well-characterized fullerene and carbon nanotubes. In Japan, the acceptable exposure concentration of fullerene is 0.39 mg/m^3. In Europe, the proposal concentration is 44.4 μg/m^3 for acute toxicity and 0.27 μg/m^3 for chronic toxicity. The proposal acceptable exposure concentrations of carbon nanotubes are 0.03, 0.05, and 0.007 mg/m^3 in Japan, Europe, and the United States, respectively.

Introduction

The demand for nanomaterials has expanded, but the effect of nanomaterials on the human body is inconclusive. If we consider the harmful effect of nanomaterials from the standpoint of occupational health, it is very important to estimate the toxicity of nanomaterials through the

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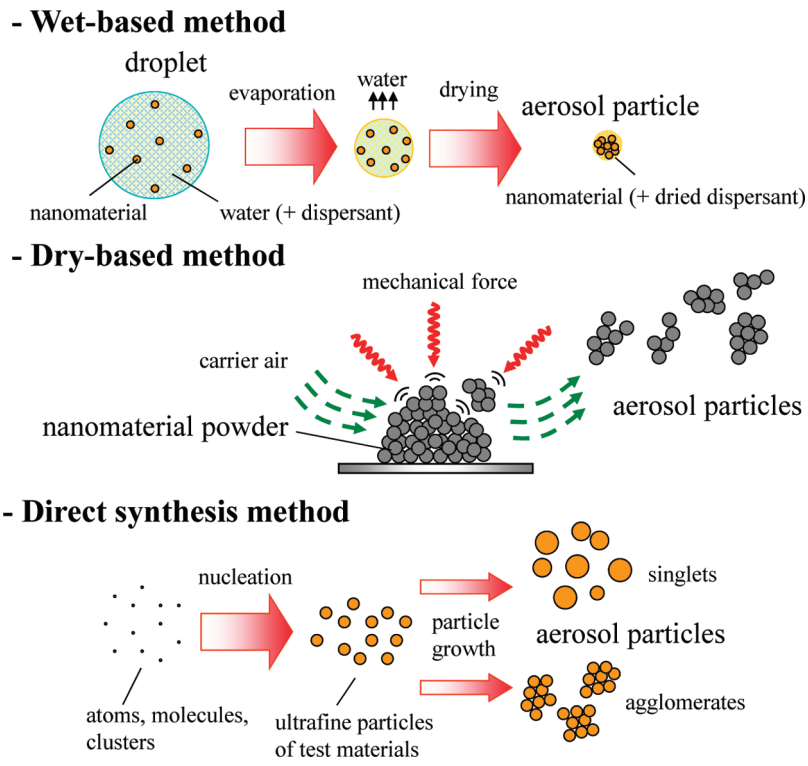

FIGURE 1. Schematic illustration of methods for preparing aerosolized test nanomaterials. Wet-based method, dry-based method, and direct synthesis method are representative ones in inhalation systems.

respiratory route by inhalation studies, intratracheal instillation studies, and pharyngeal aspiration studies.[1,2] Among these in vivo studies, the inhalation study is the "gold standard" for inhalation toxicity assessments due to its similarity to the exposure in workers. However, there are only a few reports of inhalation studies of nanomaterials because of the high cost and high technique needed to maintain a stable exposure.[3−5] Therefore, it is necessary to gather lots of data on inhalation toxicology from not only inhalation but also intratracheal instillation studies, and to estimate the harmful effect of nanomaterials comprehensively.

On the other hand, there are discrepancies in the results among these studies. The main cause of the discrepancies is the discrepancies in the physiochemical properties of nanomaterials.[6−10] For example, the toxicity of carbon nanotubes was reported to be modified by their purification by acid and the removal of the metal as a catalyst.[6−9] There were also reports that the agglomeration of nanotubes altered the translocation of material,[10] that dispersed nanotubes induced bacterial toxicity and the proliferation of epithelial cells and fibroblasts compared with nondispersed ones, and that the difference in the fiber dimension or the surface area is also related to their bioactivity.[11,12] Therefore, it is important to identify the many physicochemical properties of nanomaterials in order to evaluate their toxicity regardless of the type of study.

In this Account, we review the assessments of the inhalation toxicity, focusing on in vivo studies through the respiratory route, especially inhalation studies, by well-characterized nanomaterials.

Methods of Preparing Test Nanomaterials for Inhalation Study

It is essential for nanomaterials to be dispersed and suspended in air to perform inhalation experiments. Various techniques have so far been employed to prepare such nanomaterials in air. These techniques can roughly be categorized into the three methods shown schematically in Figure 1. The outlines and features of them are as follows.

Wet-Based Method. In this method, an aqueous dispersion of test nanomaterials is sprayed into droplets, and the water is removed from the droplets to obtain aerosol particles of the nanomaterials.[13−15] Except for those originally available as a colloidal suspension, nanomaterials are sufficiently dispersed in water with the aid of sonication, and so forth. Depending on the materials, a dispersant needs to be added to the dispersion to ensure its stability. Atomizers or nebulizers using pressurized air, ultrasonic vibration,

or electrospray have been used to generate droplets. Airborne particles below 100 nm in size can be prepared by properly adjusting the state of the nanomaterials in the dispersion together with the spraying and drying conditions of the droplets. Fibrous materials can also be brought into air at a much more dispersed state than in the dry-based method mentioned below. However, the number and mass concentration of the dispersed particles are usually lower than in the other two methods.

Dry-Based Method. Mechanical forces are added to dry powder, and the resulting separated particles are entrained in air.[16−18] High-speed airflow, impaction with brushes or blades, mechanical or acoustic vibration, and shaking with coarser materials are the origin of such forces. This method has been employed more often than the others because of its simplicity. Test nanomaterials of a high mass concentration in air can be expected. However, dispersion reaching to individual nanoparticles is hardly possible; the particles in air are agglomerates of several hundreds of nanometers to several tens of micrometers in size. In particular, highly entangled, bulky particles are usually formed from fibrous materials. Therefore, a coarse fraction of the particles sometimes has to be removed to prevent obstruction of respirability.

Direct Synthesis Method. This denotes a process in which nanomaterials are synthesized in the gas phase and delivered to inhalation chambers as they remain suspended.[19−21] Liquid or solid raw materials are once converted into molecular or cluster level substances (vaporization or ablation) by the addition of energy, then led to the formation of solid nanomaterials via chemical reaction, nucleation, condensation, and/or coagulation. The typical size of a nanomaterial unit (i.e., primary particle) ranges from several to several hundreds of nanometers. Among the three methods, the highest number concentration in air is attainable by this one. However, the synthesized materials usually form agglomerates of several tens of nanometers to several micrometers in size. In addition, gas and particles other than test nanomaterials often coexist as byproduct from the chemical reaction. Sufficient separation or removal of such matter is usually difficult.

As mentioned above, each of the methods has advantages and disadvantages. A selection of the method should be made after careful consideration of the physicochemical properties of the nanomaterials to be tested, as well as the purpose of the inhalation experiment to be performed.

Fullerene (Table 1)

There are three studies regarding short-term inhalation exposure to fullerene C_{60}.[3,5,22,23] These studies suggested that only minor and transient pulmonary inflammation was induced by C_{60} particles. To determine the acute effects of C_{60} exposure, Yokoyama et al.[22] measured electron paramagnetic resonance (EPR) in a 3 h nasal inhalation exposure test (3 h/day) using ICR mice with C_{60} nanoparticles (0.35 mg/m^3; 1.6 $\times$ 10^5/cm^3; 86 nm). As a result, no redox ability was found for C_{60} nanoparticles. To determine the subacute effects of C_{60} exposure, Baker et al.[3] observed the pulmonary inflammation and the cytokines in bronchoalveolar lavage fluid (BALF) and cytology in a 10 day nasal inhalation exposure test (3 h/day) using Fischer 344 rats with nanosize (2.22 mg/m^3; 55 nm) and micrometer size (2.35 mg/m^3; 930 nm) C_{60} particles. Since no pulmonary inflammation and minimal changes in BALF cytokines and neutrophil count were observed in both exposure groups until 7 days after the end of exposure, it was suggested that C_{60} has little acute inflammatory effect. Morimoto et al.[5] and Ogami et al.[23] conducted a 28 day whole-body inhalation exposure test (6 h/day; 5 days/week) using Wistar rats with C_{60} nanoparticles (0.12 mg/m^3; 4.1 $\times$ 10^4/cm^3; 96 nm). In the results, no persistent inflammation, no tumor, no granulomas, and no change in BALF cell counts and neutrophil count were observed until 3 months after the exposure; therefore, it can be considered that C_{60} does not induce inflammation at such an exposure level.

There are four studies regarding on intratracheal instillation tests of fullerene C_{60}.[5,23−26] These results indicate that C_{60} could induce transient pulmonary inflammation, but with very low toxicity. Sayes et al.[24] observed BALF and lung tissue in an intratracheal instillation test using SD CD rats with aqueous dispersion of C_{60} (0.2, 0.4, 1.5, and 3.0 mg/kg; 160 $\pm$ 50 nm) until 3 months after instillation. Since there was no persistent increase in BALF chemistry and neutrophil count, and no persistent pulmonary inflammation was observed, it is suggested that C_{60} can induce only transient inflammation. As the first intratracheal instillation test of nanosize C_{60} agglomerates, Morimoto et al.[5] and Ogami et al.[23] conducted intratracheal instillation tests using Wistar rats with C_{60} dispersion (0.33, 0.66, 3.3 mg/kg; 33 nm). A significant but very small increase in neutrophil count up to 3 months in the 3.3 mg/kg instilled group, only transient inflammation up to 1 week, and no tumor and no granulomas in lung tissue until 12 months were observed. Therefore, the inflammatory toxicity of C_{60} was considered to be low. In addition, genetic profiling was also tested.[27] Many representative genes involved in inflammatory response, such as the *Cxcl2*, *Cxcl6*, *Orm1*, and *Spp1* genes, were upregulated for over 6 months after an instillation of the

TABLE 1. Inhalation and Intratracheal Instillation of Fullerene

exposure	bulk size	size	BET surface area [m^2/g]	impurtities (%)	animal	exposure period	observation time point	biological end point	concentration/ dose	findings	ref
inhalation (wet-based)	–	86 nm (number-based GM size)	0.92 m^2/g (before grinding)	>99.5% (before disperse)	ICR mouse	3 h	0 h	redox ability	0.35 mg/m^3	no redox ability	22
inhalation (direct synthesis)	–	55 nm 930 nm	–	>99.5% (before sublimation and nebulization)	Fischer 344 rat	10 days (3 h/day)	0 days, 1 days, 5 days, 7day	lung pathology, BALF	2.22 mg/m^3 2.35 mg/m^5	no pulmonary inflammation and minimal changes in BALF cytokines and neutrophil count	3
inhalation (wet-based)	–	96 ± 5 nm (number-based GM size)	0.92 m^2/g (before grinding)	>99.5% (before disperse)	Wistar rat	4 weeks (6 h/day) (5 days/week)	3 days, 1 month, 3 months	lung pathology, BALF, gene expression	0.12 ± 0.03 mg/m^3	no persistent inflammation and no change in BALF cell counts and neutrophil count	5
							3 days, 1 month, 3 months	lung pathology		no tumor and no granulomas	23
							3 days, 1 month, 3 months	gene expression		little genes were up-regulated	28
intratracheal instillation	–	160 ± 50 nm	–	–	SD CD (Crl:CD (SD) IGS BR) rat	Single treatment	1 days, 1 week, 1 month, 3 months	lung pathology BALF	0.2, 0.4, 1.5, 3.0 mg/kg-bw	no persistent increase in BALF chemistry and neutrophil count and no persistent pulmonary inflammation	24
intratracheal instillation	–	33 nm (mass standard 50%ile diameter)	0.92 m^2/g (before grinding)	>99.5% (before disperse)	Wistar rat	single treatment	3 days, 1 week, 1 month, 3 months, 6 months, 12 months	lung pathology BALF	0.1, 0.2, and 1.0, mg/rat (0.33, 0.66, 3.3 mg/kg)	small increase in neutrophil count up to 3 months at 3.3 mg/kg, only transient inflammation up to 1 week	5
							3 days, 1 week, 1 month, 3 months, 6 months	lung pathology		no tumor and no granulomas	23
							3 days, 1 week, 1 month, 3 months, 6 months	gene expression		representative genes involved in inflammatory response, such as the *Cxcl2*, *Cxcl6*, *Orm1*, and *Spp1* genes, were upregulated for over 6-months	27
intratracheal instillation	–	211 nm (zeta size volume diameter)	–	–	apolipoprtein E knockout mice	single treatment	3 h, 24 h	lung cell BALF	5.4×10^{-2} mg/rat	very small BALF cytokines changes	25
intratracheal instillation	–	46.7 ± 18.6 nm	–	–	ICR mouse	single treatment	1 days, 7 days, 14 days, 28 days	cytokines and IgE in BALF and blood	0.5, 1.0, and 2.0 mg/kg	dose-responsive increase in some cytokine values and blood IgE	26

3.3 mg/kg group. In that inhalation exposure test, few genes were upregulated.[28] As a test for a sensitive model of pulmonary effects, Jacobsen et al.[25] observed the effects on lung cells and BALF at 3 and 24 h after the instillation in an intratracheal instillation test using apolipoprotein E knockout mice with C_{60} particles (5.4×10^{-2} mg/rat, 211 nm (zeta size volume diameter)). Because the changes in BALF cytokines in the C_{60} instillation group were much smaller than those in the quantum dot, SWCNT, or CB instillation groups, the authors concluded that the results of this test showed an extremely low toxicity of C_{60} on the lungs. To determine the effect of C_{60} particles on allergy in addition to inflammation, Park et al.[26] measured cytokines and IgE in BALF and blood until 28 days after instillation in an intratracheal instillation test using ICR mice with C_{60} dispersion (0.5, 1.0, and 2.0 mg/kg) prepared by mixing C_{60} toluene solution with water and removing the toluene. Since a dose-responsive increase in some cytokine values and blood IgE were observed, the authors concluded that lung inflammatory reaction may be triggered by the intratracheal instillation of C_{60}.

Multiwall Carbon Nanotube (MWCNT) (Table 2)

Carbon nanotubes (CNTs) are fibrous materials formed from honeycomb crystal lattice layers of graphite wrapped into a tube shape either as a single layer or multiple layers, which are, respectively, called single-wall carbon nanotubes (SWCNTs) and multiwall carbon nanotubes (MWCNTs). Since CNTs have many outstanding physical and chemical properties, their applications and uses in various fields are being explored all over the world. The main concerns regarding the hazards of CNTs arise not only from their small sizes but also from their fibrous shapes. Specifically, there is a concern that carbon nanotubes may pose hazards similar to asbestos.

First, we review inhalation exposure studies using MWCNTs. There are two reports regarding short-term inhalation studies with MWCNTs.[17,29] Mitchell et al.[17] conducted an inhalation exposure of MWCNTs produced by Shenzehn Nanotech Port in mice at three levels of air concentration of MWCNTs (0.3, 1.0, and 5.3 mg/m^3) for 7 or 14 days. No pulmonary inflammation was observed in all groups; however, immune responses such as a decrease in T-cell mitogen and an increase of interleukin-10 in the spleen were observed in a 14 day inhalation exposure for all CNT concentrations. In this study, there was no clear dose–response relationship; changes in biomarkers were greater in the 1 mg/m^3 exposed group than in the 5 mg/m^3 exposed group. On the other hand, an inhalation study with the same MWCNTs in mice at 32.61 mg/m^3 for 5, 10, or 10 days showed a thickening of alveolar wall in all groups.[29] After publication of these studies, two 3 month inhalation exposure studies of MWCNTs according to the Organization for Economic Co-operation and Development (OECD) testing guideline #413 were published. Ma-Hock et al.[30] performed a 3 month inhalation study with MWCNTs (NC7000 produced by Nanocyl) in rats at 0.1, 0.5, and 2.5 mg/m^3. No systemic toxicities were observed in all groups; however, increased lung weights, pronounced multifocal granulomatous inflammation, diffuse histiocytic and neutrophilic inflammation, and intra-alveolar lipoproteinosis were observed in lung and lung-associated lymph nodes at 0.5 and 2.5 mg/m^3. Even at 0.1 mg/m^3, there was still minimal granulomatous inflammation in the lung and in lung-associated lymph nodes. Therefore, no observed effect concentration (NOEC) was not established in the study. Rats were exposed to MWCNTs (Baytube produced by Bayer) for 3 months at 0.1, 0.4, 1.5, and 6 mg/m^3.[31] Exposure-related lesions were observed at 0.4 mg/m^3 and above in the upper and lower respiratory tract; however, all end points examined were unremarkable at 0.1 mg/m^3. Therefore, the authors concluded that 0.1 mg/m^3 was a no-observed adverse effect level (NOAEL) of the MWCNTs, and then suggested an occupational exposure limit of 0.05 mg/m^3 in the workplace.[32] The biological responses to MWCNTs were considerably different in each of these two studies. Some characteristics of MWCNTs (e.g., tube length and diameter, surface area, impurities) may affect their toxicity. Both studies used large nondispersed MWCNT agglomerates. At present, there is only one inhalation study that used well dispersed MWCNTs. Rats were exposed to MWCNTs (produced by Nikkiso Co., Ltd.) for 4 weeks at 0.37 mg/m^3 by the "wet" aerosolization method.[15] The MWCNTs were dispersed in liquid, then this MWCNT dispersion was sprayed and dried in the air.[15] This method allowed to produce well-dispersed MWCNTs (70% of them were individually dispersed) in the aerosol. BALF neutrophils were significantly more increased than in the control group only at 3 days after the exposure period; no other toxicologically significant changes were observed. Therefore, 0.37 mg/m^3 was judged as the NOAEL of the MWCNTs.[33]

Next, we review studies regarding the relationships between CNT characteristics and biological responses by intratracheal instillation of MWCNTs. Rats were intratracheally instilled with 0.5, 2, and 5 mg/rat of raw MWCNTs and MWCNTs ground by a ball mill.[34] The ground MWCNTs caused greater inflammatory responses than the raw MWCNTs at the same dose. Based on the characterization

TABLE 2. Inhalation and Intratracheal Instillation of Multiwall Carbon Nanotubes

exposure	bulk size (diameter × length)	secondary size	BET surface area [m^2/g]	impurities (%)	animal	exposure period	observation time point	biological end point	concentration/ dose	findings	ref
inhalation (dry-based)	10−20 nm × 0.5− 1.5 μm	0.7−1.8 μm[a]	100	–	mouse	7, 14 days (6 h/day)	0 days p.e.	lung pathology, BALF	0.3, 1.0, 5.3 mg/m^3	no effect (7 days exposure); immune suppression (14 days exposure)	17
inhalation (dry-based)	50 nm ×10 μm	–	280	–	mouse	5, 10, 15 days	0 days p.e.	lung pathology, BALF	32.61 mg/m^3	thickening of alveolar walls	29
inhalation (dry-based)	5−15 nm ×0.1−1 μm	0.5−2 μm[a]	250−300	–	rat	13 weeks (6 h/day)	0 days p.e.	systemic response, lung pathology	0.1, 0.5, 2.5 mg/m^3	minimal granulomatous inflammation at 0.1 mg/m^3; Multifocal granulomatous inflammation at 0.5 and 2.5 mg/m^3	30
inhalation (dry-based)	–	10 nm ×200−300 nm	253−259	Co (0.46−0.53%)	rat	13 weeks (6 h/day)	9 months p.e.	lung pathology, BALF	0.1, 0.4, 1.5, 6.0 mg/m^3	no adverse effect at 0.1 mg/m^3; pulmonary inflammation at 0.4 mg/m^3 and above	31
inhalation (west-based)	44 nm	63 nm ×1.1 μm	69	Fe <0.01%	rat	4 weeks (6 h/day)	3, 28, 60 days p.e.	BALF	0.37 mg/m^3	minimal transient change	15
intratracheal instillation	9.7 nm ×5.9 μm, 11.3 nm ×0.7 μm (ground)	–	378 307 (ground)	–	rat	single treatment	3, 15, 60 days p.e.	lung pathology, BALF	0.2, 2.5 mg/rat	ground MWCNT showed greater inflammation than bulk MWCNT	34
intratracheal instillation	60 nm × <20 μm	60 nm × 1.5 μm	37	total metal (<0.21%)	rat	single treatment	3, 7 days, 1, 3, 6 months p.e.	lung pathology, BALF	0.04, 0.2, 1 mg/kg	no inflammation (0.04 mg/kg); lung inflammation recovered at until week (0.2, 1 mg/kg)	35
intratracheal instillation	44 nm × >1 μm	48 nm × 0.94 μm	69	Fe (0.0053%)	rat	single treatment	3, 7 days, 1, 3, 6 months, 1, 2 years p.e.	lung pathology, BALF	0.2, 1 mg/rat	lung inflammation recovered at until week (0.2 mg/rat) or at 3 months (1 mg/rat)	15

results of the MWCNT samples, the lengths of individual MWCNTs were greatly altered by grounding, from 5.9 to 0.7 μm; however, other main characteristics, such as tube diameter and specific surface area, were not changed by the treatment.

Rats were intratracheally instilled with 0.04, 0.2, and 1 mg/kg of individually dispersed MWCNT (MWNT-7 produced by Mitsui) suspension.[35] Transient pulmonary inflammatory responses were observed only in the lungs of the rats exposed to 1 mg/kg of MWCNTs. Light microscopy examination revealed that the MWCNTs deposited in the lungs of the rats were typically phagocytosed by the alveolar macrophages, and these macrophages consequently accumulated in the alveoli until 6 months after instillation. The 400 TEM images obtained showed that all the MWCNTs were located in the alveolar macrophages or macrophages in the interstitial tissues, and no MWCNTs were located in the cells of the interstitial tissues. These results suggest that MWCNTs were being processed and cleared by alveolar macrophages. Morimoto et al.[15] conducted an intratracheal instillation study with 0.2 and 1 mg/rat of well-dispersed MWCNTs, which was the same as in their inhalation study. Dose-dependent pulmonary inflammation was observed in this study. Minimal inflammation was observed in the 0.2 mg/rat group, whereas infiltration in BALF neutrophils, eosinophils, and macrophages was persistently observed until 6 months after instillation in the 1 mg/rat group. However, the inflammatory responses were gradually recovered from, and there were no significant differences from the control group in histopathological findings and number of BALF cells at 1 and 2 years after instillation. Further, no benign or malignant tumors were observed during 2 year observation period. A dose of 0.2 mg/rat is equal to a dose of approximately 0.67 mg/kg, assuming a rat body weight of 300 g. Meanwhile, the pulmonary retention after 1-month exposure to 0.37 mg/m^3 is calculated to be 0.12 mg/kg, assuming a clearance rate of 0 (no clearance in the lung) and a pulmonary deposition rate of 0.1 (10%). Based on these assumptions, the pulmonary retention after a 3 month exposure can be virtually calculated to be 0.36 mg/kg. This pulmonary retention (0.36 mg/kg) is lower than the minimum dose in the intratracheal instillation study (0.67 mg/kg). Therefore, Nakanishi[33] concluded that there would be no adverse effect even when exposed to 0.37 mg/m^3 of MWCNTs for 3 months.

Single-Wall Carbon Nanotube (SWCNT) (Table 3)

There are two reports about the inhalation of SWCNTs.[4,36] In these studies, one study showed that SWCNTs had an inflammation and fibrosis induction ability, although, another study did not show that. The first inhalation test of SWCNTs was reported by Shvedova et al.[4] The inhalation of aggregated SWCNTs by mouse at a dose of 5.0 mg/m^3 for 4 days induced transient neutrophil infiltration, and then the infiltration of macrophage and oxidative stress were observed after the decrease of neutrophil. Morimoto et al.[36] performed an inhalation test of SWCNTs with rats. Although the dose in their examination was low, they used very pure and well-dispersed SWCNTs. The rats inhaled aerosol containing SWCNTs for 4 weeks, and the mass concentrations of inhaled SWCNTs were 0.03 and 0.13 mg/m^3. There were no observations of any pulmonary toxicity, neutrophil inflammation, or oxidative stress at this dose.

Physical and Chemical Properties of CNT

It is suggested that various physical and chemical properties are involved in the pulmonary toxicity of CNTs. The difference in the physical and chemical properties of CNTs leads to different biological responses in their pulmonary toxicity. Particularly, the content of impurities, the agglomeration state, length of fiber, and specific surface area affect their biological activity.

Specific surface area is an important factor in the pulmonary toxicity of CNTs. The inflammatory activity of CNTs depends on the specific surface area. Nakanishi[33] indicated that inflammatory activity of CNTs , of which the index is the increased rate of BALF neutrophil depending on the specific surface area. This observation can apply regardless of SWCNT or MWCNT. Association of the toxicity and the specific surface area is also indicated in other nanoparticles such as metal oxide nanoparticles,[37,38] and therefore, specific surface area might be considerable also in the toxicity of CNT.

In CNTs, which are a fibrous material, it is suggested that the fiber length is also involved in their toxicity. Poland et al.[39] reported the intraperitoneal administration to mouse of MWCNTs with different length. As a result, longer MWCNTs showed stronger inflammation activity than short ones. On the other hand, Mühlfeld et al.[40] also reported that the pulmonary inflammation responses differed by length of MWCNT fiber. Although the shorter CNTs showed stronger induction potential of polymorphonuclear cells, the longer CNTs showed stronger induction of IL-6. These results suggest that the length of CNT fibers is also one of the important factors in pulmonary toxicity. As the possibility of the cause of a different response by different length of CNT, the length of CNT fiber affects efficiency of phagocytosis by macrophage and thus longer CNTs will stay in the lung for longer.

TABLE 3. Inhalation and Intratracheal Instillation of Single-Wall Carbon Nanotubes

exposure	bulk size (diameter × length)	secondary size	BET surface area [m^2/g]	impurtities (%)	animal	exposure period	observation time point	biological end point	concentration/ dose	findings	ref
inhalation (wet-based)	3.0 nm	2.0 nm × 0.32 μm	1064	Fe (0.0145%) Ni (0.0103%)	rat	4 weeks (6 h/day, 5days/week)	3 days, 1, 3 months	lung pathology, BALF	0.03, 0.13 mg/m^3	no neutrophil inflammation in the lung	36
inhalation (dry-based)	0.8−1.2 nm × 0.1−1.0 μm	<4.2 μm	508	Fe 17.7%) Ni (0.046%)	mouse	4 consective days 5 h/day	1, 7, 28 days	lung pathology, BALF	5.0 mg/m^3	PNM, total protein, and LDH were increased at 1 day after postinhalation and then decreased; macrophage and total cells were increased at 7 and 28 days after postinhalation; collagen was increased with time; GSH was decreased at 7 and 28 after	4
intratracheal instillation	1.83 nm	43.6 nm × 0.69 μm	878	Fe (1.37%)	rat	single treatment	3 days, 1 week, 1, 3, 6 months	lung pathology, BALF	0.2, 0.4 mg/rat (0.66, 1.32 mg/kg)	BALF and pathological features revealed that the dose of SWCNT induced persistent neutrophil infiltration in rat lungs; in the CINCs family the BALF increased persistently in the SWCNT-exposed groups; concentration of HO-1 in the BALF was also upregulated persistently in the exposed groups	48
intratracheal instillation	–	–	–	raw: Fe (26.9%), Ni (0.78%); purified: Fe (2.4%), Ni (0.0%); CarboLex:Fe (0.53%), Ni (25.99%)	mouse	single treatment	7, 90 days	lung pathology, BALF	0.1, 0.5 mg/mouse	Ni-included SWCNT killed half or more animals at dose of 0.5 mg/mouse. Both Fe-includied and purified SWCNT showed inflammation and granuloma at dose of 0.1 mg/mouse	9
intratracheal instillation	1.4 nm × >1.0 μm	formed <30 nm aggregated "ropes"	–	Ni (5%) pathology, amorphous carbon (30−40%)	rat	single treatment	24 h, 1 week, 1, 3 months	lung pathology, BALF	1.5 mg/kg	inflammation and lung injury were observed in high dose (5 mg/kg) animals; dose indipendent multifocal granulomas was observed; pulmonary toxicity of SWCNT was lower than quartz particles	41

TABLE 3. Continued

exposure	bulk size (diameter × length)	secondary size	BET surface area [m^2/g]	impurtities (%)	animal	exposure period	observation time point	biological end point	concentration/ dose	findings	ref
pharyngeal aspiration	0.4−1.2 nm ×0.5- 1.0 μm	–	1040	Fe (0.23%)	mouse	multiple treatment	3 weeks, twice/week	lung pathology, BALF	240 μg/mouse (total)	SWCNT incduced inflammation in lung (total cells and PMN were increased); pathology examination showed multifocal and mnoderate pyogranulomatous, mild fibrosis and collagen accumulation; IL-12, MDC and MMP-2 were significantly increased	49
pharyngeal aspiration	–	–	–	95% purif	mouse	single treatment	3 days, 2 weeks	lung pathology, BALF	0.5 mg/mouse	significant inflammation response was shown; foamy-like macrophage was observed at 3 days after aspiration; multifocal macrophage-containing granuloma was observed around the SWCNT aggregate at 2 weeks after aspiration	50
pharyngeal aspiration	0.9−1.7 nm × <1.0 μm	–	–	–	mouse	single treatment	3, 24 h	lung pathology, BALF	54 μg/mouse	gene expression of Mip-2, Mcp-a, IL-6 were signigficantly higher than control; transient inflammation was observed at 24 h after instillation	25
intratracheal instillation	1.2 nm × 2−10 μm	average length in PBS dispersion: 0.76 μm	–	metal contents: 10 wt %	mouse	single treatment	1, 7, 14, 28 days	lung pathology, BALF	100 μg/kg	SWCNT induced inflammation in lung; total cells in the BALF were increased with time; neutrophil increased at 1 day after instillation, and then decreased; instead of neutrophil, lymphocyte increased from 7 days after	51
pharyngeal aspiration	mean diameter: 1−4 nm	–	1040	Fe (0.23%)	mouse	single treatment	1, 3, 7, 28, 60 days	lung pathology, BALF	0, 10, 20, 40 μg/mouse	aspiration of SWCNT induced inflammation and oxidative stress in lung; aspiration of SWCNT showed formation of granuloma and thickness of alveolar connective tissue. PMN, total cells and proteins in the BALF were increased; neutrophil increased at 1 day after instillation, and then decreased; instead of neutrophil, lymphocyte increased from from 3 days to 7days after aspiration	52

TABLE 3. Continued

exposure	bulk size (diameter × length)	secondary size	BET surface area [m^2/g]	impurities (%)	animal	exposure period	observation time point	biological end point	concentration/ dose	findings	ref
intratracheal instillation	1–2 nm, 100- 2000 nm (mode length 500 nm)	–	–	Fe: law; 20.6 wt %, well-dispersed SWCNT; 8.5 wt %	mouse	single treatment	24 h, 30, 90 days	lung pathology, BALF	10, 40 μg/mouse	no observation of granulomatus inflammation in highly dispersed nanoscale SWCNT exposed animals; highly dispersed SWCNT did not show acute toxicity; total lung collagen tended to increase in aggregated SWCNT; aggregated SWCNT showed granulomatus inflammation, macrophage activation	44
intratracheal instillation	3.0 nm × <1.2 μm	12.0 nm × 0.32 μm	1064	Fe (0.0145%), Ni (0.0103%)	rat	single treatment	24 h, 3 days, 1 week, 1, 3 months (0.2, 2.0 mg/kg) ; 3 days, 1week, 1, 3, 6 months (0.04, 0.2, 1.0 mg/kg)	lung pathology, BALF	0.04, 0.2, 1.0, 2.0 mg/mL (0.012, 0.06, 0.3, 0.6 mg/rat)	instillation of the SWCNT induced acute lung inflammation; there was no fibrosis; histpathological changes due to SWCNT were observed only in the lung and lung-associated lymph node	42

As for impurities, the content of the metal used as a catalyst is important. Lam et al.[9] compared lung influences on the lung between a raw SWCNT which included 26.9% Fe and a purified SWCNT which included 2.14% Fe. According to the intratracheal instillation of the two types of SWCNT, inflammation and formation of granulomas in the tested animals tended to be more in the raw CNT (3 or 5/5 mice) than in the purified SWCNT (2/5 mice). On the other hand, another SWCNT including 26% Ni induced the formation of granulomas. Thus, there is a possibility that impure metals enhance the pulmonary toxicity of SWCNTs.

The content of amorphous carbon is also involved in toxicity. Besides a metallic content, it is necessary to determine the purity as "CNTs". A high content of amorphous carbon in CNTs leads to inaccurate results of biological responses by "SWCNTs". Contamination of amorphous carbon in the CNT might affect their toxicity.[41,42] In ceramic fiber, which is an alternative fiber for asbestos, the presence of nonfibrous particulates increased polymorphonucleocytes (PMNs) and showed a retardation of clearance from lung compared with the inhalation of pure ceramic fiber.[43]

Additionally, it is suggested that an agglomerated state of SWCNTs also affects their biological activity. Different aggregation states of SWCNT showed different biological responses such as collagen deposition,[10] collagen production,[11] glanulomatus inflammation,[15,42,44] and cytokine production.[33,42] As just described, it is suggested that the agglomeration state of SWCNTs affects qualitative responses in the lung.

As described above, the content of impurity and many physical and chemical properties are involved in the toxicity of SWCNTs.

Occupational Exposure Limit (OEL) of C_{60} and CNT Is Suggested by Europe, the United States, and Japan

About the C_{60}, in Japan, the period-limited OEL and the period-limited ambient air quality criteria for fullerene nanoparticles with a geometric average of 96 nm (GSD 2.0) was proposed to be 0.39 mg/m^3 and 1.4×10^{-2} mg/m^3, respectively.[45,46] Since the lung deposition fraction depends on the secondary particle size, the period-limited OEL and period-limited AAQC for C_{60} particles were proposed to be 0.39×0.0913/(deposition fraction) mg/m^3 and $1.4 \times 10^{-2} \times 0.107$/(deposition fraction) mg/m^3, respectively.[45,46] These values were estimated based on the results of intratracheal instillation tests[15,24] and clearance parameters.[47] In Europe, the proposed Derived-No-Effect-Levels (DNEL) of

C_{60} for workers was 44.4 $\mu g/m^3$ for acute toxicity and 0.27 $\mu g/m^3$ for chronic toxicity in the ENRHES (Engineered Nanoparticles - Review of Health & Environmental Safety) project of Joint Research Center. These values were estimated based on the results of gene expression in an inhalation exposure test.[28] The differences in the levels proposed by the two groups were mainly induced by the differences in the end point. The European group considered that some of the inflammation-related gene expression was the toxicity effect of C_{60}, while the Japanese group did not consider the gene expression to be a toxicity effect because the gene expression could not mean toxicity itself but only suggested the mechanism of toxicity.

In Japan,[33] NOAEL of SWCNT which was used by studies of Morimoto et al.[15] and Kobayashi et al.[42] in rat was 0.065 mg/m.[3] This value was adjusted by the interspecies difference in respiratory volume, exposure time, particle deposition, and body weight. And particle deposition rate per alveolar surface area was adopted as the metrics of the exposure dose to be used for converting NOAEL in animal experiments to exposure limit amounts of humans. The OEL extrapolated from test animal data to humans is 0.03 mg/m^3. This value applies to not only SWCNT but also to MWCNT. However, this value is time-limited. Because many investigations about risk assessment of SWCNT are advancing, it is necessary to update the value within 10 years. Pauluhn[31] suggested 0.05 mg/m^3 as the OEL, based on the data of NOAEL (0.1 mg/m^3) estimated from 13 weeks inhalation of MWCNT to rats. He considered respiratory volume, particle deposition, retained amount, and alveolar macrophage volume. NIOSH suggested 0.007 mg/m^3 based on the data of Pauluhn[31] and Ma-Hock et al.[30] The estimated OEL value of NIOSH was based on a multiple path particle dosimetry (MPPD2) model. The value 0.007 mg/m^3 is the upper limit in quantification of the current measurement method.

These OEL values were indicated by weight concentration until now. Hereafter, if physical and chemical factors which involve the toxicity of CNTs are clarified by the development of inhalation and intratracheal instillation tests, we can possess higher reliability administration using toxicity-sensitive and specific parameters.

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FOOTNOTES

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