Exposure to Multiwalled Carbon Nanotubes and Allergen Promotes Early- and Late-Phase Increases in Airway Resistance in Mice

Nobuaki Mizutani,*^a Takeshi Nabe,^b and Shin Yoshino^a

^aDepartment of Pharmacology, Kobe Pharmaceutical University; 4–19–1 Motoyamakita, Higashinada, Kobe 658–8558, Japan; and ^bDepartment of Pharmacology, Kyoto Pharmaceutical University; 5 Nakauchi, Misasagi, Yamashina, Kyoto 607–8414, Japan. Received April 19, 2012; accepted September 12, 2012

The facilitating effects of multiwalled carbon nanotubes (MWCNT) on allergic asthma have not been sufficiently examined, although MWCNT appear to significantly increase the risk of health problems from occupational or environmental exposure. In this study, we examined whether sensitization by the combination of MWCNT with ovalbumin (OVA) promotes allergic asthmatic responses. BALB/c mice administered vehicle, MWCNT, OVA, or MWCNT+OVA through an intranasal route were challenged with OVA intratracheally four times. In the MWCNT+OVA group, the fourth challenge caused not only early- but also late-phase increases in airway resistance, although these responses were not observed in the vehicle, MWCNT, or OVA group; furthermore, the extents of the early and late responses were comparable to those in mice systemically sensitized with OVA+alum. Sensitization with MWCNT and OVA promoted airway inflammation and goblet cell hyperplasia in the lung compared with the vehicle, MWCNT or OVA group. In addition, adjuvant activity for OVA-specific immunoglobulin E (IgE), IgG1, and IgG2a production in serum and increased levels of interleukin-4 (IL-4), IL-5, IL-13, and IL-17 in the lung tissue were observed. In conclusion, these results suggest that exposure to MWCNT and antigen can induce a biphasic increase in airway resistance, airway inflammation, goblet cell hyperplasia, and the production of antigen-specific antibodies. This study highlights the risk of exposure to a combination of MWCNT with antigen.

Key words carbon nanotube; allergic asthma; airway resistance; goblet cell hyperplasia; adjuvant activity

Allergic asthma is a chronic type of airway inflammation characterized by reversible airway obstruction and goblet cell hyperplasia associated with elevated levels of allergen-specific immunoglobulin E (IgE).^{1–3} The prevalence of asthma has increased markedly over the past 3 decades, and may be due to changes in environmental factors, including indoor and outdoor air pollution.^{4,5} Relatively few investigations on the environmental exacerbating factors of the pathology have been conducted, although intensive experiments have accumulated evidence regarding the molecular mechanisms of therapeutic targets. The former type of research should be focused on, since large environmental changes may occur throughout the world in the future.

Nanotechnology is now advancing at an incredible pace, such that it has created an alternative industrial revolution over the past few years.⁶ Consistent with this, the use of engineered nanomaterials has been rapidly increasing in commercial applications. As these materials have become more widespread, many questions have arisen regarding the effects of these materials as inhalable toxicants. Among several different types of nanomaterials, carbon nanotubes (CNT) are one of the most attractive materials because of their unique physical and chemical characteristics, including their size (1–20 nm width and many micrometers in length), strength, and surface chemistry.^{7,8} However, CNT show marked cytotoxicity, such as the induction of oxidative stress, cellular proliferation, and apoptosis/necrosis in several types of cell *in vitro*.^{9–12} There is *in vivo* evidence implicating CNT in the induction of inflammatory lung disorders, including oxidative tissue injury^{13,14}; however, less research has been conducted to examine the effects of CNT on allergic respiratory disorders, including asthma as an exacerbating factor.

We have reported a mouse model showing early- and late-phase increases in airway resistance and goblet cell hyperplasia by systemic sensitization with Al(OH)₃ plus ovalbumin (OVA) as an allergen and repeatedly allergen challenge.^{15,16} In the present study, we hypothesized that sensitization by the co-administration of multiwalled CNT (MWCNT) with allergen promote allergic asthmatic responses in mice, indicating that this research has important implications for understanding the health risks of exposure to MWCNT and allergen. Therefore, we intranasally administered vehicle, MWCNT, OVA, or MWCNT+OVA to mice, which were then challenged repeatedly by the intratracheal instillation of OVA. Using this experiment protocol, we examined whether MWCNT exacerbated an antigen-induced biphasic increase in airway resistance, airway inflammation, goblet cell hyperplasia, cytokine production in the lung tissue, and the production of antigen-specific antibodies (IgE, IgG1, and IgG2a) in serum.

MATERIALS AND METHODS

Animals Male 7-wk-old BALB/c mice were obtained from Japan SLC. These mice were maintained in a temperature-controlled environment with free access to standard rodent chow and water. The first sensitization was started 1 wk after arrival.

All of the experimental procedures were approved by the Experimental Animal Research Committee at Kobe Pharmaceutical University.

Reagents MWCNT (diameter: 10–15 nm and length: 0.1–10 μ m) and OVA (grade V) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). MWCNT were suspended in 0.1% Tween 20 in saline, and then the solution was sonicated for 30 min immediately before use. It has been demonstrated that well-dispersed CNT are less toxic than agglomerated or

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* To whom correspondence should be addressed. e-mail: mizutani@kobepharm-u.ac.jp

Fig. 1. Effect of MWCNT on Early- and Late-Phase Increases in Airway Resistance after the Antigen Challenges

(A) Experimental protocol for sensitization with MWCNT and OVA and challenge with OVA in mice. (B) Changes in sRaw after the first (a) and fourth (b) challenges in mice sensitized with MWCNT and OVA. Results of Fig. 1Ba indicate those at 10 min after the first challenge. Each value is the mean $\pm$ S.E.M. of 5–7 animals. * $p < 0.05$ and ** $p < 0.01$ compared with control group. # $p < 0.05$ and ## $p < 0.01$ compared with OVA+Tween group. & $p < 0.05$ and && $p < 0.01$ compared with MWCNT group.

pellet CNT in a mesothelioma cell line¹⁷⁾; thus, the degree of agglomeration is important for evaluation of the adverse effects of CNT. Tween 20 is a stable and relatively nontoxic detergent and emulsifier used in a number of pharmacological applications; indeed, when three types of dispersion agent (phosphate-buffered saline (PBS), PBS+dimethyl sulfoxide, and PBS+Tween 20) were used, PBS+Tween 20 was the most effective dispersion agent for CNT¹⁸⁾; therefore, we used Tween 20 for dispersing MWCNT. Nevertheless, MWCNT occur, at least in part, in agglomerated forms due to van der Waals forces.

Sensitization and Challenge As shown in Fig. 1A, BALB/c mice were sensitized on days 0, 1, 2, 14, 15, and 16 under anesthesia with escain (Mylan, Osaka, Japan) with saline (Control), 0.01–1 mg MWCNT/mL in Tween 20 (MWCNT0.01, MWCNT0.1, and MWCNT1), 2.5 mg OVA/mL in saline (OVA+saline), 2.5 mg OVA/mL in Tween 20 (OVA+Tween), 2.5 mg OVA/0.01–1 mg MWCNT/mL in Tween 20 (OVA+MWCNT0.01, OVA+MWCNT0.1, and OVA+MWCNT1) in a volume of 30 μ L through an intranasal route. Subsequently, the mice were challenged on days 28, 29, 30, and 35 with 1% OVA in a volume of 20 μ L by intratracheal administration.¹⁶⁾

Furthermore, systemic sensitization with OVA+alum was performed according to a previously described method.¹⁶⁾ BALB/c mice were sensitized by intraperitoneal injection with OVA adsorbed to alum (Wako Pure Chemical Industries, Ltd., Osaka, Japan). OVA was used at a dose of 50 μ g adsorbed to

1 mg alum/0.2 mL/mouse on days 0 and 14. The sensitized mice were challenged on days 28, 29, 30, and 35 with 1% OVA in a volume of 20 μ L by intratracheal administration.

Measurement of Airway Resistance To evaluate the degree of airway resistance, specific airway resistance (sRaw; $\text{cmH}_2\text{O} \times \text{mL}/(\text{mL}/\text{s})$) was measured in conscious mice before and 10 min to 4 h after the antigen challenges using a two-chambered, double-flow plethysmograph system (Pulmous-I; MIPS, Osaka, Japan) according to the method of Pennock *et al.*¹⁹⁾

Analysis of Cells Recovered by Bronchoalveolar Lavage (BAL) To evaluate airway inflammation, we examined the accumulation of inflammatory cells in BAL fluid (BALF). Animals were killed with diethyl ether. The trachea was cannulated, and the left bronchi were tied for histological examination. The right air lumen was then washed twice with 0.5 mL Hank's balanced salt solution (HBSS). The recovered lavage fluid was centrifuged at $120 \times g$ for 5 min at 4°C. The cell pellet was suspended with a defined volume (200 μ L/sample) of HBSS. The total leukocyte count in the lavage fluid was determined by staining with Turk's solution. For differential cell counts, BAL cells were stained with Diff-Quik solution (Sysmex International Reagents, Kobe, Japan). A minimum of 200 cells were counted under a microscope and, based on their morphological criteria, classified as macrophages, lymphocytes, neutrophils, or eosinophils. Furthermore, the numbers of MWCNT-positive macrophages in BALF were counted relative to the total number of macrophages to estimate the

persistence of MWCNT in the lung phagocyte population.

Histological Analysis The left lungs were fixed in 10% neutral-buffered formalin, then dissected, embedded in paraffin, and cut 4- μ m thick. Sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS).

Each section was scored on scale of 0 to 4 with increments of 0.5 by a blinded observer for inflammation (H&E) and goblet cell hyperplasia (PAS).

Measurement of OVA-Specific IgE, IgG1, and IgG2a OVA-specific IgE, IgG1, and IgG2a antibodies in serum were measured by enzyme-linked immunosorbent assay (ELISA), as described.²⁰⁾ OVA-specific IgE antibody in serum was detected using plates coated with anti-mouse IgE antibody and adding biotin-labeled OVA. Streptavidin-horseradish peroxidase was added, the plate was developed with 3,3',5,5'-tetramethylbenzidine solution (Wako Pure Chemical) as the substrate, and measurements were made at 450 nm using a microplate reader after stopping the reaction with sulfuric acid. Values for serum OVA-specific IgE were expressed as absorbance units. Serum samples were diluted at 1:5.

OVA-specific IgG1 or IgG2a was detected using plates coated with OVA and adding alkaline phosphate-conjugated anti-mouse IgG1 or IgG2a, respectively. The plates were developed with *p*-nitrophenyl phosphate and read at 405 nm using a microplate reader. Values for serum OVA-specific IgG1 and IgG2a were expressed as absorbance units. Serum samples were diluted at 1:1000 and 1:20 for IgG1 and IgG2a, respectively.

Measurement of Cytokines and C3a Levels of cytokines in lung tissue and C3a in BALF 24 h after the fourth challenge were measured by ELISA as described.²¹⁾ The frozen right lobe (after BAL) was homogenized in 1 mL T-PER tissue protein extraction reagent (Thermo Scientific, Rockford, IL, U.S.A.) containing a Complete Mini Protease Inhibitor Cocktail tablet (Roche, Mannheim, Germany; 1 tablet/10 mL T-PER stock reagent). Lung homogenates were centrifuged at 9000 $\times g$ for 10 min at 4°C. IL-4, IL-5, IL-13 (R&D Systems, Minneapolis, MN, U.S.A.) and IL-17 (BioLegend, San Diego, CA, U.S.A.) in supernatants of lung homogenates were measured using quantitative colorimetric sandwich ELISA kits.

C3a in BALF was detected using plates coated with rat anti-mouse C3a antibody (BD Biosciences, San Diego, CA, U.S.A.) and adding biotin-labeled rat anti-mouse C3a antibody (BD Biosciences). Alkaline phosphatase-conjugated anti-biotin was added, the plate was developed with *p*-nitrophenyl phosphate, and measurements were made at 405 nm using a microplate reader. The value for C3a in BALF was expressed as absorbance units.

Statistical Analyses Statistical analyses were performed with one-way analysis of variance (ANOVA). If significant differences were detected, individual group differences were determined by a Bonferroni–Dunn test. A *p* value of <0.05 was considered significant.

RESULTS

Effect of MWCNT on Early- and Late-Phase Increases in Airway Resistance after the Antigen Challenges We have previously reported that the first antigen challenge caused a swift elevation of sRaw that peaked at 10 min, but no late response was induced in mice systemically sensitized

with OVA+alum.¹⁶⁾ Therefore, we investigated the effect of sensitization by the combination of MWCNT and OVA on the first antigen-induced early-phase increase in airway resistance by measuring sRaw at 10 min. The first administration of antigen dose-dependently significantly induced the early-phase increase in airway resistance in OVA+MWCNT0.1 and OVA+MWCNT1 groups compared with in the control, MWCNT (0.1 or 1), and OVA+Tween groups, although the response in mice sensitized with OVA alone or OVA+MWCNT0.01 was only slightly induced after the challenge (Fig. 1Ba). Furthermore, MWCNT-alone administration did not induce a response at any dose. The extent of early responses in OVA+MWCNT1 groups was comparable to that in mice systemically sensitized with OVA+alum (Fig. 1Ba).

Fourth antigen challenge significantly induced not only early but also late increases in airway resistance, peaking 10 min and 3 h later, in OVA+MWCNT0.1 and OVA+MWCNT1 groups (Fig. 1Bb), although no responses were observed in other groups except for the OVA+alum group (Fig. 1Bb). Furthermore, the extent of early and late increases in airway resistance in the OVA+MWCNT1 group was comparable to that in mice systemically sensitized with OVA+alum.

sRaw values before the fourth challenge were 2.444 ± 0.045 in control, 2.440 ± 0.050 in MWCNT0.01, 2.450 ± 0.048 in MWCNT0.1, 2.422 ± 0.065 in MWCNT1, 2.411 ± 0.052 in OVA+saline, 2.405 ± 0.062 in OVA+Tween, 2.467 ± 0.065 in OVA+MWCNT0.01, 2.436 ± 0.067 in OVA+MWCNT0.1, and 2.495 ± 0.057 in OVA+MWCNT1 groups. Throughout the above experiment, no significant differences in sRaw before the challenges were found among the groups.

Effect of MWCNT on Inflammatory Cells in BALF after the Fourth Antigen Challenge Figure 2A shows inflammatory cells in BALF of mice sensitized with MWCNT and OVA. Consistent with inflammatory cells in BALF of the OVA+alum group, numbers of inflammatory cells, such as macrophages, lymphocytes, neutrophils, and eosinophils were significantly increased 24 h after the fourth challenge in the OVA+MWCNT0.1 and OVA+MWCNT1 groups compared with control and OVA+Tween groups, and the increase was in a dose-dependent manner; furthermore, the number of lymphocytes and eosinophils in OVA+MWCNT1 groups was higher than in MWCNT1 groups. Additionally, MWCNT 0.1 and MWCNT1 groups showed an increase in macrophages and neutrophils, but not lymphocytes and eosinophils, compared with the control group.

On the other hand, MWCNT-negative and -positive macrophages in BALF were easily identified by light microscopy, as shown in Figs. 2Ba,Bb, respectively. In MWCNT0.1 and MWCNT1 groups, 40–70% of the total number of macrophages contained MWCNT on day 36; however, there were no differences in the relative percentages of MWCNT-positive macrophages between MWCNT and OVA+MWCNT groups (Fig. 2Bc).

Effect of MWCNT on Airway Inflammation and Goblet Cells in the Lung after the Fourth Antigen Challenge As defined by histological analysis using H&E staining, little leukocyte recruitment was observed 24 h after the fourth challenge in the control group (Fig. 3Aa). In contrast, there was marked infiltration of leukocytes around the blood vessels and airways 24 h after the fourth challenge in MWCNT1, OVA+Tween, and OVA+MWCNT1 groups

Fig. 2. Effect of MWCNT on Inflammatory Cells in BALF after the Fourth Antigen Challenge

(A) Changes in inflammatory cell numbers in BALF 24h after the fourth challenge in mice sensitized with MWCNT and OVA. (B) Persistence of MWCNT in macrophages 24h after the fourth challenge in MWCNT and OVA+MWCNT groups. Each value is the mean $\pm$ S.E.M. of 5–7 animals. * p <0.05 and ** p <0.01 compared with control group. # p <0.05 and ## p <0.01 compared with OVA+Tween group. & p <0.05 and && p <0.01 compared with MWCNT group. Total, all cells; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

(Figs. 3Ab–d). Furthermore, the infiltration of leukocytes in OVA+MWCNT0.1 and OVA+MWCNT1 groups was observed compared with control, MWCNT (0.1 or 1), and OVA+Tween groups, and the increase was dose-dependent (Fig. 3Ae).

Additionally, we performed a histological examination with PAS staining for the detection of goblet cells. In the lungs of MWCNT1, OVA+Tween, and OVA+MWCNT1 groups, goblet cell hyperplasia was observed 24h after the fourth challenge (Figs. 3Bb–d), although goblet cell hyperplasia was not observed in the control group (Fig. 3Ba). Furthermore, more increased goblet cell hyperplasia in OVA+MWCNT0.1 and OVA+MWCNT1 groups was observed compared with the control, MWCNT (0.1 or 1), and OVA+Tween groups, and the increase was in a dose-dependent manner (Fig. 3Be).

Effect of MWCNT on Production of Antigen-Specific Antibodies (IgE, IgG1, and IgG2a) and Cytokines after the Fourth Antigen Challenge Figures 4A–C show the production of OVA-specific IgE, IgG1, and IgG2a in the serum of mice sensitized with MWCNT and OVA, respectively. The production of IgE and IgG2a was not increased in OVA+Tween group in comparison with the control group, although IgG1 was slightly significantly increased. In contrast, consistent with the findings in mice systemically sensitized with OVA+alum, the fourth administration of antigen in the

OVA+MWCNT1 group significantly increased the production of IgE, IgG1, and IgG2a compared with control, MWCNT1, and OVA+Tween groups (Fig. 4).

IL-4, IL-5, IL-13 (Fig. 5A), and IL-17 (Fig. 5B) in lung tissue, and C3a (Fig. 5C) in BALF 24h after the fourth challenge in OVA+MWCNT1 groups were significantly increased in comparison with control, MWCNT1, and OVA+Tween groups. In addition, the levels of IL-13 in MWCNT1 and OVA+Tween groups were significantly increased compared with the control group (Fig. 5Ac).

DISCUSSION

In the present study, when mice sensitized by a combination of MWCNT and OVA received repeated antigen challenges, the early increase in airway resistance at the first challenge, and not only the early but also a late response at the fourth challenge were potentially aggravated; furthermore, airway inflammation and goblet cell hyperplasia in the lungs, and increased levels of OVA-specific IgE, IgG1, and IgG2a in serum were observed. These findings suggest that sensitization with MWCNT and antigen can exacerbate allergic asthmatic responses associated with increased levels of antigen specific antibodies.

Fig. 3. Effect of MWCNT on Airway Inflammation and Goblet Cell Hyperplasia in Lungs after the Fourth Antigen Challenge

(A) Changes in inflammation (H&E) in the lungs 24h after the fourth challenge in mice sensitized with MWCNT and OVA. Bar shows 100 μ m. Histological appearance was scored for inflammation (e). (B) Changes in goblet cell (PAS) in the lungs 24h after the fourth challenge. Bar shows 100 μ m. Histological appearance was scored for goblet cell hyperplasia (e). Each value is the mean $\pm$ S.E.M. of 5–7 animals. * p <0.05 and ** p <0.01 compared with control group. # p <0.05 and ## p <0.01 compared with OVA+Tween group. & p <0.05 and && p <0.01 compared with MWCNT group.

Fig. 4. Effect of MWCNT on the Production of Antigen-Specific IgE, IgG1, and IgG2a after the Fourth Antigen Challenge

(A–C) Levels of OVA-specific IgE (A), IgG1 (B), and IgG2a (C) in serum 24h after the fourth challenge in mice sensitized with MWCNT and OVA. Each value is the mean $\pm$ S.E.M. of 5–7 animals. * p <0.05 and ** p <0.01 compared with control group. # p <0.05 and ## p <0.01 compared with OVA+Tween group. & p <0.05 and && p <0.01 compared with MWCNT group.

It has been reported that nano-sized particles, including TiO₂ and carbon black, have more prominent adjuvant effects on allergen-specific responses with Ig production^{22–24}), however, latex nanoparticles do not facilitate these responses.²⁵) These previous observations suggest that each nano-level particle has different effects on this pathology, which may depend on their characteristics. Based on these reports, we proposed that MWCNT exposure may be a risk factor for the development of allergic asthma. In this study, sensitization with MWCNT and antigen increased the production of antigen-specific IgE, IgG1, and IgG2a in serum in comparison with mice administered with antigen or MWCNT alone. Thus, MWCNT exhibited adjuvant activity for antigen-specific antibodies.

Antigen-presenting cells (APC), including dendritic cells and macrophages, play fundamental roles in the pathogenesis of asthma.^{26,27}) APC-mediated phagocyte and subsequent antigen presentation for T-cells is the first step in both primary and secondary immune responses; in particular, dendritic

cells are recognized to be professional APC, exhibiting potent antigen-presenting ability.^{28,29}) It has been reported that carbon black nanoparticles enhance the maturation and function of mouse bone marrow-derived dendritic cells *in vitro*,³⁰) and intrapulmonary APC activation with asthma pathophysiology *in vivo*,³¹) indicating that the effects of MWCNT on allergic asthma can be mediated, at least in part, through the activation of APC. On the other hand, we demonstrated that the same levels of macrophages containing MWCNT in both MWCNT and MWCNT+antigen groups were observed. In addition, the amount of allergen carried on the particles is dependent on the particle surface area³²) and MWCNT share particle characteristics, such as a nano-sized diameter and a large surface area, with ultrafine particles of urban air pollution,³³) suggesting that the large particle surface area of MWCNT is likely to augment the carried amount of allergen and thus the cellular response.

As expected, sensitization with MWCNT and antigen

Fig. 5. Effect of MWCNT on the Production of Cytokines (IL-4, IL-5, IL-13, and IL-17) in the Lungs and C3a in BALF after the Fourth Challenge (A) Levels of IL-4 (a), IL-5 (b), and IL-13 (c) in lung tissue 24h after the fourth challenge in mice sensitized with MWCNT and OVA. (B) Levels of IL-17 in lung tissue 24h after the fourth challenge. (C) Levels of C3a in BALF 24h after the fourth challenge. Each value is the mean $\pm$ S.E.M. of 5–7 animals. * p <0.05 and ** p <0.01 compared with control group. # p <0.05 and ## p <0.01 compared with OVA+Tween group. & p <0.05 and && p <0.01 compared with MWCNT group.

induced airway inflammation, such as the infiltration of eosinophils and neutrophils, and a late-phase increase in airway resistance by repeated antigen challenge. We have demonstrated that the depletion of neutrophils by anti-Gr-1 mAb greatly suppressed the late-phase increase in airway resistance in OVA+alum sensitized mice.³⁴⁾ Furthermore, in the present study, we observed the infiltration of neutrophils in BALF of mice sensitized with MWCNT and antigen, indicating that the late-phase increase in airway resistance may be mediated by neutrophilic airway inflammation. Regarding the mechanisms underlying the recruitment of neutrophils to inflamed sites, we have reported that complement C3a-mediated IL-17 plays an important role in the development of a late response *via* neutrophil accumulation in mice sensitized with anti-OVA IgE monoclonal antibody.²¹⁾ Therefore, in the present study, we found increased levels of C3a in BALF and production of IL-17 in lung tissue, suggesting that C3a-induced IL-17 contributes to the development of a late-phase increase in airway resistance through neutrophilic airway inflammation in mice sensitized with MWCNT and antigen; however, the mechanism of C3a production in this study is unclear. First, it is possible that in addition to activating the complement through the classical pathway, the antigen may directly activate C3 *via* an alternative pathway. Another possibility is that a neutral tryptase released from mast cells or pulmonary macrophages proteolytically cleaves C3 to generate C3a.^{35,36)} Thus, the antigen-triggered release of tryptase in mice sensitized with MWCNT and antigen could lead to C3a production without typical activation of the entire complement cascade. On the other hand, the important role of eosinophils in the immunopathogenesis of asthma was shown in studies using eosinophil-deficient mice that markedly attenuated the development of asthmatic responses.³⁷⁾ Thus, we showed that sensitization with MWCNT and antigen induced not only a late response but also the infiltration of eosinophils and neutrophils into the lungs, raising the possibility that eosinophilia and/or neutrophilia may contribute to the development of a late increase in

airway resistance.

In this sensitization with MWCNT and antigen, an early-phase increase in airway resistance was observed at the first and fourth challenge. It has been reported that mice sensitized with anti-OVA IgE monoclonal antibody showed an early response 10min after the antigen challenge, and the level of mouse mast cell protease-1 in serum 30min after the antigen challenge was increased.²⁰⁾ In mice sensitized with MWCNT and OVA, an increased level of IgE in serum was observed, suggesting that the early response may be IgE-dependent, probably through the activation of mast cells in the lung. Furthermore, the early response might have been influenced by not only IgE but also IgG1 and/or IgG2a because IgG1 and/or IgG2a production in serum was significantly observed in mice sensitized with MWCNT and antigen.

Goblet cell hyperplasia, a characteristic feature of allergic asthma,^{38–40)} was induced by co-sensitization with MWCNT and OVA. IL-4 and IL-13 are known to induce goblet cell hyperplasia *in vivo*.^{41,42)} In the present study, airway production of IL-4 and IL-13 was also induced by sensitization, suggesting that IL-4 and IL-13 are critical for the development of goblet cell hyperplasia in mice sensitized with MWCNT and OVA. In addition, it has been reported that multiple treatments with a C3a receptor antagonist during repeated antigen challenge also inhibited goblet cell hyperplasia in mice sensitized with OVA+alum,¹⁶⁾ and C3a induced goblet cell hyperplasia in the lung,⁴³⁾ suggesting that not only Th2-type cytokines but also C3a are important to goblet cell hyperplasia in mice sensitized with MWCNT and OVA.

Collectively, through these experiments, we demonstrated that sensitization with MWCNT and antigen can exacerbate the antigen-induced biphasic increase in airway resistance, airway inflammation, goblet cell hyperplasia, and the production of antigen-specific antibodies (IgE, IgG1, and IgG2a) in serum. This study highlights the risk of exposure to a combination of MWCNT and antigen from occupational and environmental exposure.

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