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Letter

Effects of intranasal administration of multi-walled carbon nanotube (MWCNT) suspension on respiratory syncytial virus (RSV) infection in mice

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ABSTRACT — To evaluate the effects of multi-walled carbon nanotubes (MWCNT) on a host immune system, we assayed them using a murine model of respiratory syncytial virus (RSV) infection. MWCNT suspended in solution were intranasally administered to mice on days 1, 3, and 5 before RSV infection. On day 5 post-infection, the levels of representative inflammatory markers (interferon-γ, chemokines CCL3 and CCL5) in bronchoalveolar lavage fluid (BALF) were significantly increased in RSV-infected mice due to MWCNT exposure compared to the control. A histopathological analysis confirmed the exacerbation of the pneumonia. However, significant histopathological changes were not observed in mock-infected mice in this study. Some alveolar macrophages engulfing the MWCNT aggregates were localized in the inflammatory cells in the lung tissues, but RSV-positive cells immunohistopathologically stained with an anti-RSV antibody were observed apart from those cells. Thus, intranasal treatment with MWCNT should affect the pulmonary immune response against RSV, exacerbating RSV infection in mice.

Key words: MWCNT, RSV, Pneumonia

INTRODUCTION

Multi-walled carbon nanotubes (MWCNT) are nanomaterials used in various industries worldwide. MWCNT are utilized in some electronic products, because they are light, thermally stable, and good conductors. In contrast to these industrial merits of MWCNT, contamination of people in the environment have been suggested, and since the health disasters due to exposure to asbestos, various common chemical characters of MWCNT have been reported (Donaldson *et al*., 2011). Particularly, the induction of a malignant mesothelioma due to the exposure to MWNT-7, a representative MWCNT, was reported in a murine model (Sakamoto *et al*., 2009). Several carcinogenic research results of MWCNT were reported, but their safety for the immune system is poorly known.

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Respiratory syncytial virus (RSV) is an ss RNA virus and cause of the common cold in childhood. Although the symptoms of RSV-induced disease are generally not severe, pneumonia is induced in a small percentage of children with immune incompetence, because the severity of sickness due to RSV infection is reflected by the condition of the immune system (Holberg *et al*., 1991). We noticed this pathological property of RSV infection and established a novel assay system for evaluation of immunotoxicity of environmental chemical agents using a RSV infection murine model (Watanabe *et al*., 2008). Exposure to tetrabromobisphenol A, a brominated flame retardant, and titanium dioxide nanoparticles revealed the immune disorder and then resulted in the exacerbation of pneumonia in this model (Watanabe *et al*., 2010; Hashiguchi *et al*., 2015).

In this study, we evaluated the effects of intranasal exposure to MWCNT on a RSV infection in mouse model.

MATERIALS AND METHODS

Animals

Female (5 weeks old) BALB/c mice were purchased from Kyudo Animal Laboratory (Saga, Japan) and housed at 25 ± 2°C. The mice were allowed free access to the conventional solid diet CRF-1 (Oriental Yeast Co., Chiba, Japan) and water and used in this experiment after 7 days acclimation. The animal experimentation guidelines of the Kyushu University of Health and Welfare were followed in the animal studies.

Test substance

We used MWNT-7 (Mitsui Chemicals, Tokyo, Japan) supplied by Dr A. Hirose (one of the authors) in this study. The materials have an average fiber length of 3.3 μm and a fiber diameter of 40 to 50 nm. MWNT-7 readily aggregates to form microparticles in 0.02% Tween20/phosphate-buffered saline (PBS). To avoid aggregation, the particles in the suspension of MWNT-7 in 0.02% Tween20/PBS were dispersed using a portable ultrasonic disruptor just before treatment of mice.

Cell and virus

The A2 strain of RSV was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in HEp-2 cell (human epidermoid carcinoma, ATCC CCL-23) cultures. Viral titers of HEp-2 cells were measured by the plaque method and expressed as plaque-forming units per milliliter (PFU/mL).

Animal tests

The RSV infection test was performed as reported previously (Watanabe *et al*., 2008). Briefly, six-week-old mice were intranasally administered 0.10 mL of a suspension of MWNT-7 at 0.025 or 0.25 mg/kg of body weight on days 1, 3 and 5 before RSV infection under anesthesia. In the control group, mice were given 0.02% Tween20/PBS intranasally under anesthesia. These mice were infected intranasally with 3.0×10^5 PFU of the A2 strain of RSV under anesthesia. In a mock-infected group, mice were given PBS intranasally. On day 5 post-infection, bronchoalveolar lavage fluid (BALF) was obtained from the mice under anesthesia by instilling 0.8 mL of cold PBS into the lungs and aspirating it from the trachea using a tracheal cannula. Following the acquisition of BALF, the lungs were removed, immediately frozen in liquid N_2, and stored at −80°C until virus titration. Ice-cold BALF was centrifuged at 160 × g at 4°C for 10 min. After centrifugation, the supernatant was stored at −80°C until use. Virus titration was also performed as reported previously (Watanabe *et al*., 2008).

ELISA

Interferon (IFN) -γ levels in BALF were measured using a specific ELISA kit (Ready-set-go, eBioscience Inc., San Diego, CA, USA) according to the manufacturer's instructions. Levels of CCL3 (MIP-1α) and CCL5 (RANTES) in BALF were measured using specific ELISA kits (Quantikine, R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. The lower limits of detection of the kits are 15 (pg/mL) for IFN-γ, 1.5 (pg/mL) for CCL3 and 2.0 (pg/mL) for CCL5. The intra- and interassay coefficients of variation for the ELISA results were less than 10%.

Histological methods

For histological examination of mouse lungs, 3 to 6 mice per group of mock or RSV-infected mice were sacrificed by excess anesthesia on day 5 after infection, and the lungs were removed and placed in buffered formalin for a minimum of 24 hr. The tissue was then embedded in low-melting point paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin (HE).

Immunohistochemical evaluation

The lung tissue sections were deparaffinized and hydrated through xylenes and graded alcohols. After washing with water, they were incubated in unmasking solution (Vector Laboratories, Inc., Burlingame, CA, USA) at 90°C for 30 min. Then, the sections were incubated in the 0.3% H_2O_2 in PBS for 30 min to quench the

endogenous peroxidase activity and treated with blocking serum (Vector Laboratories, Inc.) for 30 min. The lung tissues were stained with a goat polyclonal antibody against RSV protein (1:250, Acris Antibodies GmbH, Inc., San Diego, CA, USA) for 90 min. Then, RSV proteins were detected using a VECTASTAIN ABC kit (Vector Laboratories, Inc.) according to the manufacturer's instructions. The sections were faintly counterstained with hematoxylin.

Statistical analysis

Comparisons between the levels of cytokines and chemokines of the control and MWCNT-exposed groups were carried out using a Mann-Whitney *U*-test. A *P* value of 0.05 or less was considered to be significant.

RESULTS AND DISCUSSION

Prior to this study, we evaluated the effects of one exposure to MWCNT on RSV infection using the murine model. Although the increase of the levels of several chemokines in BALF was detected by a protein array, significant exacerbation of pneumonia was not observed (data not shown). Therefore, we utilized three times exposure to MWCNT in this study. During intranasal exposure to MWCNT suspension on days 1, 3, and 5 before RSV infection, no toxicological sign, including decreases of body weight and food consumption, was observed (data not shown), and then the mice were intranasally infected with the A2 strain of RSV.

Because IFN-γ is known as a common marker of the severity of the pneumonia in humans and mice (Watanabe *et al*., 2008), we initially measured it in our model. On day 5 post-infection, the levels of IFN-γ in BALF were significantly increased due to MWCNT exposure at 0.25 mg/kg compared to the control (Fig. 1). These results suggested that the exposure to MWCNT exacerbated the pneumonia in RSV-infected mice. Subsequently, to define whether MWCNT suppressed the host immunity against RSV infection, we measured the pulmonary viral titer by a plaque method. However, there was no significant difference between MWCNT-exposed and control mice (data not shown). Therefore, to characterize the effects of MWCNT exposure on inflammation in the lung tissues, we measured the levels of chemokines CCL3 and CCL5 by the specific ELISA (Table 1). CCL5 and CCL3 are proinflammatory chemokines, regulated upon activation, normal T cell secreted (RANTES), and a macrophage inflammatory protein alpha (MIP-1α), respectively, and have been used to evaluate the severity of inflammation in lungs due to RSV infection (Hashiguchi *et al*., 2015). The levels of CCL5 and CCL3 were significantly elevated due to the MWCNT exposure at 0.25 mg/kg compared to the control (Table 1). We already report-

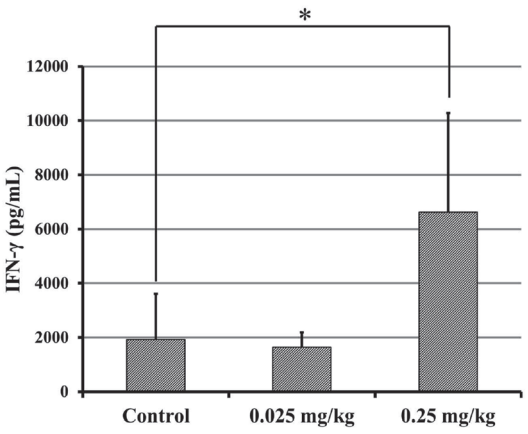

Fig. 1. Effects of MWCNT exposure on the levels of IFN-γ in BALF from RSV-infected mice on day 5 post-infection. The data represents mean ± standard deviations of values of six control or MWNCNT-exposed mice. *Significantly different from Control at $P < 0.05$ (Mann-Whitney *U*-test).

Table 1. Effects of MWCNT on the levels of chemokines in BALF from mock- or RSV-infected mice on day 5 post-infection.

MWCNT (mg/kg)	Concentration (pg/mL)			
	RSV-infected		Mock-infected	
	CCL3	CCL5	CCL3	CCL5
0	29 ± 9	86 ± 40	< 10	< 10
0.025	26 ± 4	86 ± 19	< 10	< 10
0.25	88 ± 23*	173 ± 35*	43 ± 32	< 10

Concentration (pg/mL) of each chemokine in BALF from mock- or RSV-infected mice with or without MWCNT (0.025 or 0.25 mg/kg) was measured by an ELISA for each specific chemokine. Data represents mean ± standard deviation values of 3–6 mice.
*Statistically different from control (0 mg/kg) at $P < 0.05$ (Mann-Whitney *U*-test).

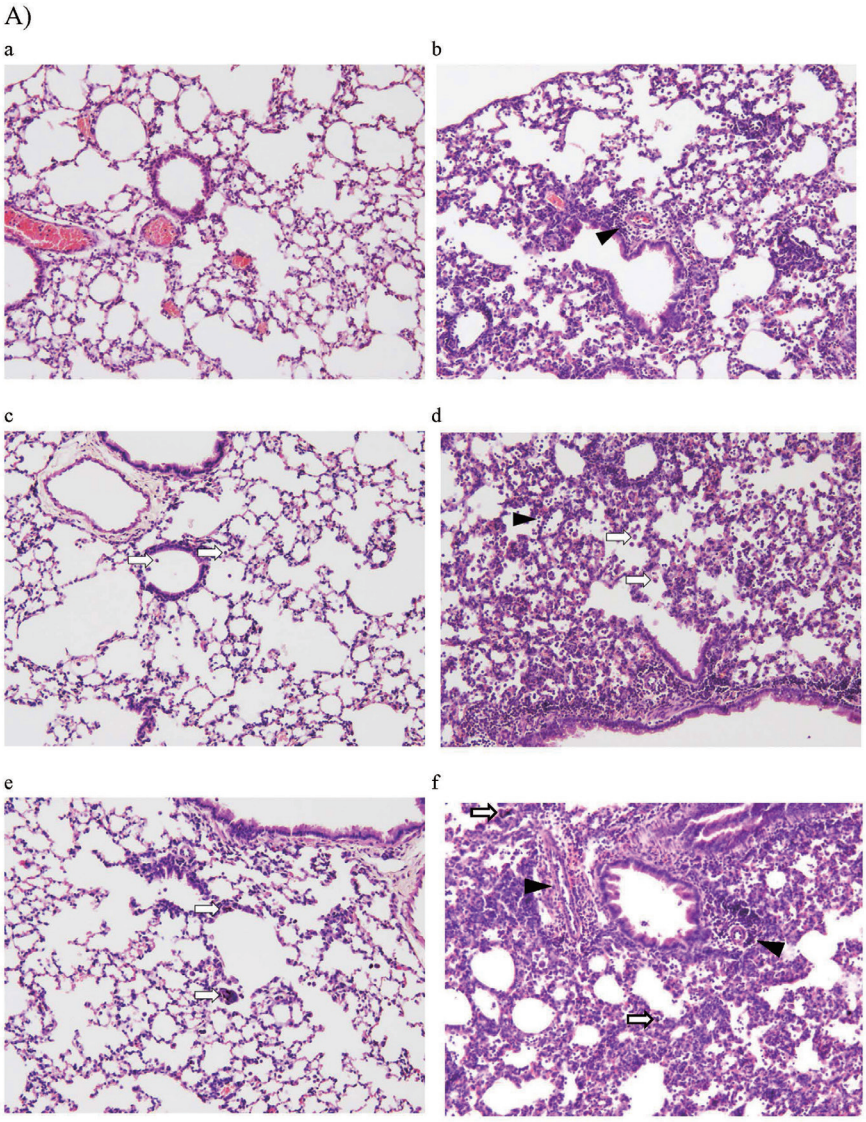

Fig. 2. Lungs of mice 5 days after RSV infection. A) Hematoxylin and eosin staining (× 100). (a) Control mouse with mock infection. (b) Control mouse with RSV infection. (c) MWCNT-exposed (0.025 mg/kg) mouse with mock infection. (d) MWCNT-exposed (0.025 mg/kg) mouse with RSV infection. (e) MWCNT-exposed (0.25 mg/kg) mouse with mock infection. (f) MWCNT-exposed (0.25 mg/kg) mouse with RSV infection. Open arrows indicate MWCNT fibers in macrophages. Closed arrowheads indicate infiltration of lymphocytes, neutrophils and macrophages around the pulmonary artery. B) Immunostained with anti-RSV antibodies (1:250) and counterstained with hematoxylin (× 100). (a) Control mouse with mock infection. (b) Control mouse with RSV infection. (c) MWCNT-exposed (0.25 mg/kg) mouse with RSV infection (× 200). Closed arrowheads indicate RSV-positive cells. Open arrows indicate MWCNT fibers in macrophages.

ed that titanium dioxide (TiO_2) nanoparticles affected the immune system and exacerbated pneumonia in RSV-infected mice (Hashiguchi *et al*., 2015). Although CCL5 was increased due to TiO_2 exposure at 0.5 mg/kg, the levels of CCL3 were not increased in the study. We speculated that MWCNT would be more effective on the function of macrophages than TiO_2.

The effects of MWCNT exposure on lung tissues of RSV-infected mice were analyzed histopathologically, and representative results are shown in Fig. 2. The MWCNT fibers and their aggregates engulfed by alveolar macrophages were found in all lung lobes of mock-

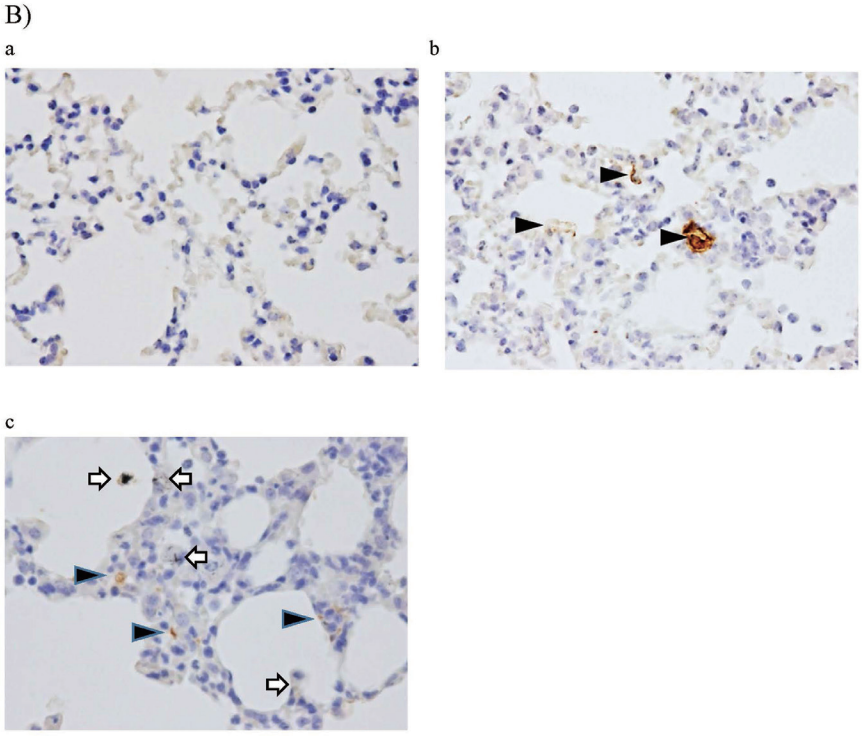

Fig. 2. (Continued).

or RSV-infected mice exposed to MWCNT (Fig. 2A c–f). These results suggested that the intranasal administration of MWCNT suspension was successful, like the TiO2 experiments reported previously (Hashiguchi *et al*., 2015). Although MWCNT in macrophages were observed dose-dependently in mock-infected mice, typical features of inflammation were slightly detected due to the high-dose exposure (Fig. 2A a, c, e). In RSV-infected mice, thickening of the alveoli was broadly observed and infiltrations of lymphocytes, neutrophils, and macrophages were enhanced dose-dependently due to the exposure to MWCNT (Fig. 2A b, d, f). We confirmed that MWCNT exposure induced disorder of the inflammatory response of immune cells and exacerbated pneumonia. Therefore, to clarify whether MWCNT-engulfed macrophages were involved in progression of RSV infection, immune histochemical analysis using a goat anti-RSV protein antibody was carried out (Fig. 2B). While RSV-positive cells were observed in the lung tissues of mice with or without exposure to MWCNT, the localization of RSV-positive cells was apart from that of MWCNT-engulfed macrophages (Fig. 2B b, c). These results suggested that MWCNT exposure enhances the immune response against RSV from an early phase of RSV infection, rather than dysfunction of the immune cells, including MWCNT-engulfed macrophages, resulting in exacerbation of pneumonia.

In this study, we demonstrated that intranasal exposure to MWCNT exacerbated pneumonia in RSV-infected mice. However, although MWCNT were found in all lobes of the lung in the exposed mice, they predominantly formed aggregations in the lung tissues and presented in terminal bronchioles. Dr Y. Taqahashi's group at the National Institute of Health Sciences in Japan developed an innovative system (Taquann system) to efficiently disperse a multi-wall carbon nanotube applicable for inhalation tests of mice (Taquahashi *et al*., 2013). We started the collaboration project with them and adopted a Taquann system for our assay system and proceeded to evaluate MWCNT. Efficient delivery of MWCNT to lung tissue should be useful to clarify the mechanism of interaction between the expansion of RSV infection and the immune cells, including macrophages, responding to MWCNT fibers.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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