ガイドライン（年平均値10μg／㎥、日平均値25μg／㎥）を設定している[7]。

PM2.5の健康影響を評価する際の留意点

$PM_{2.5}$の多くは燃焼によって発生するが、単一の物質ではなく、成分や粒径が異なる様々な物質の混合体である。$PM_{2.5}$とは粒径2・5μm以下の粒子を定義したものにすぎないため、使用される燃料や燃焼方法、気象条件の違いなどにより化学的組成や粒径分布には大きな差異があると考えられる。また、人への健康影響を評価する場合、国や地域による疾病構造やライフスタイルの相違との関連についても留意する必要があろう。

WHOは各国の状況を踏まえて対策を行うべきであるとし[7]、米国では人口および大気汚染の地域的な特性を考慮して、米国とカナダの疫学研究を重視している[6]。

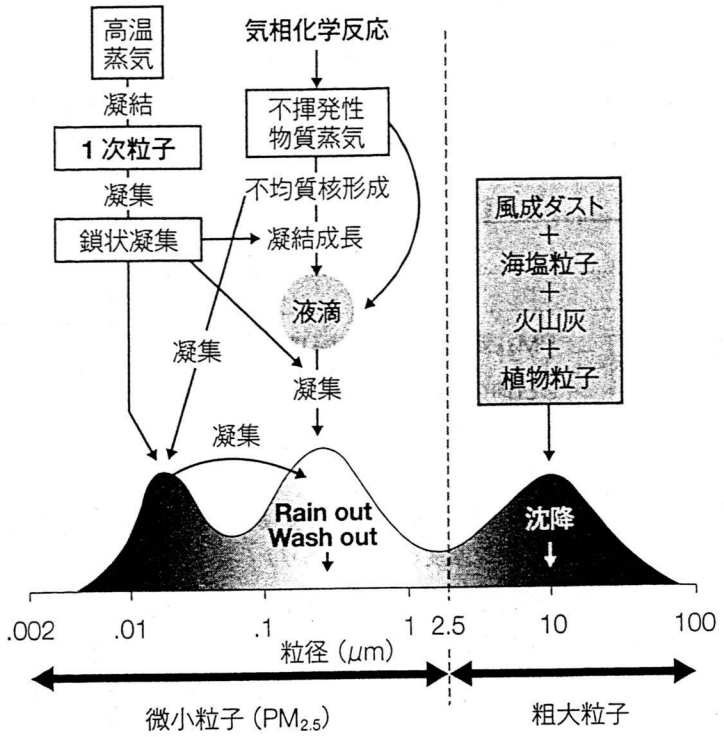

図1 大気粒子の粒径分布と組成

（文献[1]より一部改変）

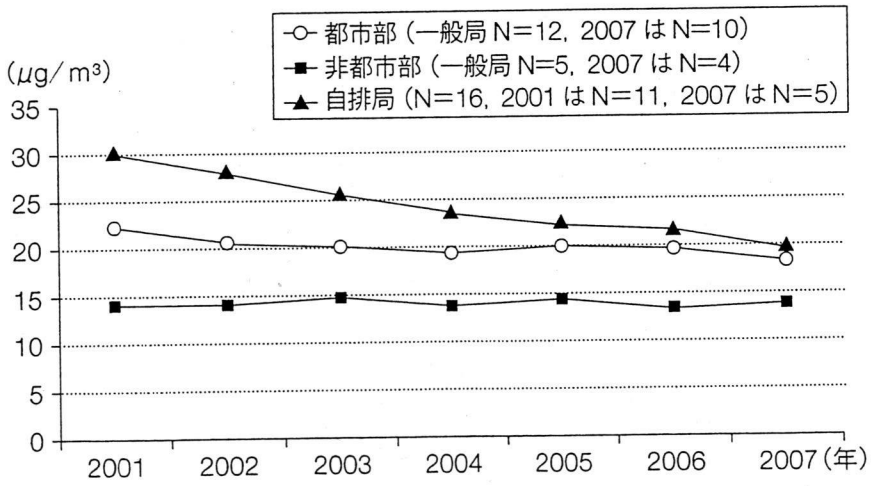

図2 $PM_{2.5}$濃度の年平均値の経年変化

一般局：一般環境大気測定局，自排局：自動車排出ガス測定局，N：調査地点数

（文献[8]より）

日本の大気環境中PM2.5濃度

我が国の大気環境中$PM_{2.5}$濃度に関するデータは少ないが、環境省により2001年から全国約30カ所で連続測定が行われている（図2）[8]。幹線道路近傍の自動車排出ガス測定局における$PM_{2.5}$濃度は年々低下しており、ディーゼル車の運行規制などの自動車排出ガス対策の効果が現れている。しかし、一般環境大気測定局では都市部、非都市部ともにほぼ横ばいである。

近年の$PM_{2.5}$濃度の年平均値は、自動車排出ガス測定局、

表1　環境省による健康影響調査結果の概要

調査項目		評価	主な結果
微小粒子状物質曝露影響調査[9]			
短期的影響			
死亡	全死亡	△	$PM_{2.5}$濃度の上昇により死亡リスクがわずかに増加
	呼吸器系	○	3日前の$PM_{2.5}$濃度の上昇により有意に増加
	循環器系	×	当日～5日前の$PM_{2.5}$濃度との関連なし
疾病	喘息による受診	×	喘息による急病診療所受診と$PM_{2.5}$濃度との関連なし（オゾン濃度とは関連あり）
	呼吸器系	○	$PM_{2.5}$濃度の上昇により喘息児，小学生のピークフロー値が有意に低下
	循環器系	×	SPM濃度と心室性不整脈との関連なし
長期的影響	呼吸器系	△	保護者の持続性の咳・痰は$PM_{2.5}$濃度が高い地域ほど高率であるが，小児の呼吸器症状とは関連なし
粒子状物質による長期曝露影響調査[11]			
長期的影響	全死亡	×	大気汚染との関連なし
	肺　癌	○	喫煙等のリスク因子を調整した後でSPM濃度と正の関連あり
	呼吸器系	△	女性では二酸化硫黄，二酸化窒素濃度と有意な関連あり（SPM濃度との関連は有意ではない）
	循環器系	×	SPM濃度と負の関連あり（ただし，血圧などの主要なリスク因子は未調整）

○：$PM_{2.5}$またはSPMとの関連あり．
△：関連は示唆されるが，明らかとは言えない．
×：$PM_{2.5}$またはSPMとの関連なし．

都市部の一般環境大気測定局ともにおよそ20μg／㎥程度であり、米国の環境基準（15μg／㎥）を超えている。非都市部における年平均値はおよそ14μg／㎥程度であり、都市部よりは低いが、WHOのガイドライン値（10μg／㎥）に比して高い。これらより、日本の大気環境中$PM_{2.5}$濃度は決して低くはなく、特に都市部においてはかなり高いと言わざるを得ない。

環境省による$PM_{2.5}$の健康影響調査

我が国の一般大気環境中の$PM_{2.5}$の健康影響に関する知見を得ることを目的として、1999年に環境庁（当時）は「微小粒子状物質曝露影響調査」を開始し、曝露評価、疫学、毒性の3分野で8年間にわたる調査研究が行われた[9]。この調査には筆者も当初より参画したが、米国で環境基準設定の根拠となった知見を参考に調査計画を立案し、我が国における$PM_{2.5}$への曝露と呼吸器系および循環器系の死亡や疾病等との関連性を評価した（一部の調査では$PM_{2.5}$濃度が

得られなかったため、SPM濃度との関連が評価されている）。疫学調査の結果の概要を表1に示した。

$PM_{2.5}$の短期的影響として、全国20市町における65歳以上の日別の死亡（2002～2004年）を検討したところ、$PM_{2.5}$の日平均濃度の増加により外因死を除く全死亡のリスクはわずかに増加し、呼吸器系疾患による死亡については有意な増加が観察された。これは諸外国での知見と同じ傾向であったが、死亡リスクは比較的小さく、特に米国で強い関連が示されている循環器系疾患による死亡については$PM_{2.5}$濃度との関連は見られなかった。

呼吸器系への短期的影響として、入院中の喘息児、水泳教室に通う喘息児、一般の小学生という三つの異なる集団を対象にピークフロー値を毎日測定した調査では、$PM_{2.5}$またはSPM濃度の上昇が数時間後のピークフロー値の低下と有意に関連しており、欧米諸国の研究と一致した結果が得られた。特に、入院中の喘息児では、午後4時以降の$PM_{2.5}$濃度（1時間平均値）が上昇すると、当日夜および翌日朝のピークフロー値の有意な低下が観察された（図3）。一方、喘息による受診については大気中オゾン濃度との関連が認められたが、$PM_{2.5}$濃度の影響は見られなかった[10]。

諸外国で報告の多い循環器系への影響については、埋込み型除細動器により心室性不整脈の治療を受けている患者の記録が検討されたが、SPM濃度との関連はなかった。

長期的影響については、全国7地域の小児とその保護者の呼吸器症状を5年間継続して調査したところ、$PM_{2.5}$濃度が高い地域ほど保護者の持続性の咳や痰の有症率は高かった。しかし、小児の呼吸器症状の有症および発症は、居住

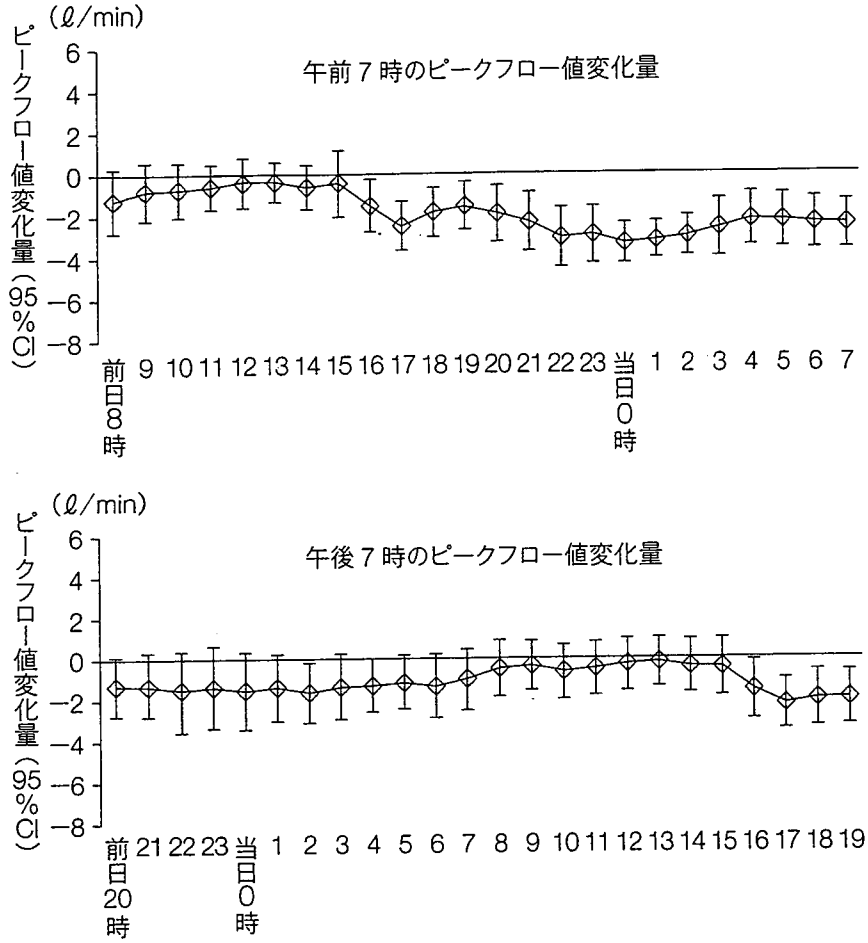

図3　喘息児のピークフロー値と$PM_{2.5}$濃度との関連

検査の24時間前から検査時までの1時間ごとの$PM_{2.5}$濃度が10 μg/m³増加した時のピークフロー値変化量とその95%信頼区間（性別，年齢，身長および気温の影響を調整）．

地域の$PM_{2.5}$濃度と関連がなかった。

粒子状物質への長期曝露と死亡との関連

最近、1982年から3府県で40歳以上の住民約10万人を15年間追跡したコホート調査の結果が環境省から報告された（表1）[11]。この調査では$PM_{2.5}$濃度は測定されておらず、大気汚染の指標として主に居住地域のSPM濃度が用いられている。肺癌による死亡は、喫煙等のリスク因子を調整した後でSPM濃度と正の関連が認められている。

一方、全死亡については、大気汚染との関連はなく、循環器系疾患による死亡は大気汚染と負の関連が見られたが、血圧などのリスク因子が調整できていないためであると考察されている。呼吸器系疾患による死亡は比較的少数であったが、女性においては大気中二酸化硫黄、二酸化窒素濃度との間に有意な関連が示された。しかし、SPM濃度との関連は有意ではなかった。

日本における$PM_{2.5}$の健康影響

これまでに示した通り、$PM_{2.5}$の呼吸器系への影響は、我が国でも喘息による受診を除いて諸外国とほぼ同様の結果が得られているが、循環器系に対しては短期曝露、長期曝露ともにまったく影響が見られていない。喘息による受診への影響が諸外国と異なるのは、医療制度の違いのほか、吸入ステロイド薬が普及して喘息発作が少なくなったことによるのかもしれない。我々が喘息児を対象に実施した調査では、大気中$PM_{2.5}$濃度の上昇により、ピークフロー値の低下だけでなく、喘鳴症状も発生しやすくなることを見出した[12]。

循環器系に対する$PM_{2.5}$の影響が見られなかった理由として、調査方法の限界だけでなく、日本は欧米諸国に比して循環器系疾患の有病率が低いという疾病構造の違いも関係していると考えられる。しかし、国内で採取された大気粒子を吸入曝露させた動物実験でも、肺の炎症性変化の増悪は観察されたが、循環器系への影響を示す知見は得られておらず[9]、大気中$PM_{2.5}$の成分や粒径分布が諸外国とは異なる可能性も否定できない。今後は、$PM_{2.5}$の成分や粒径と健康影響との関連性についての検討を進める必要があろう。

おわりに

$PM_{2.5}$が呼吸器系を中心に人の健康に影響を及ぼしていることは疑う余地がない。米国51地域を対象とした最近の研究では、1980年以降の大気中$PM_{2.5}$濃度が10μg／㎥低減することにより住民の平均余命が0・61年延長したと報告されている[13]。日本の$PM_{2.5}$濃度が米国に比して高いことからも、健康被害を未然に防ぎ、国民の生命を守るためには、1日も早く環境基準を設定して発生源に対する規制の強化等により大気中$PM_{2.5}$濃度の低減を図ることが求められる。

□■□文　献□■□

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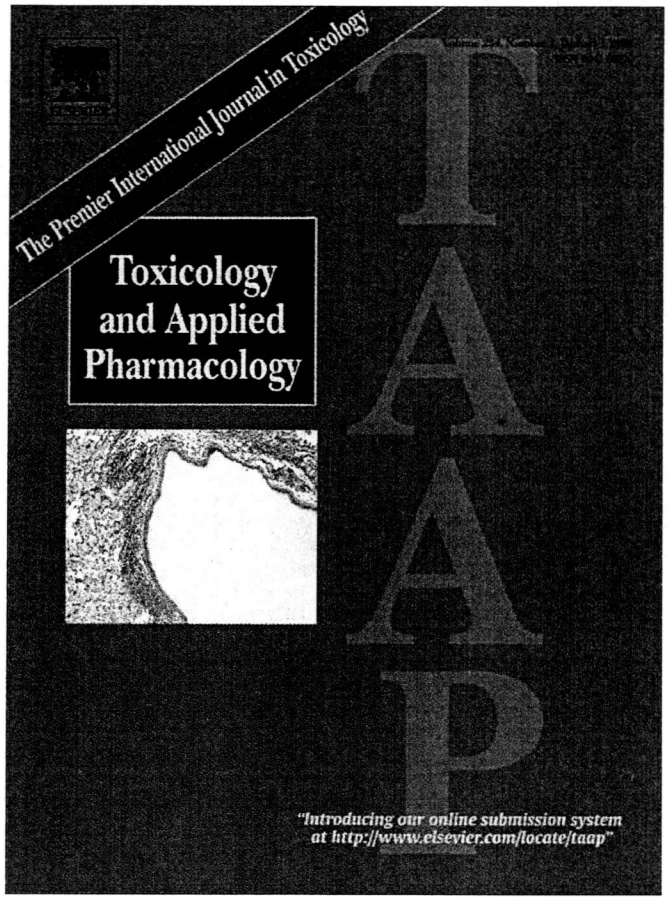

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Size effects of latex nanomaterials on lung inflammation in mice

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ABSTRACT

Effects of nano-sized materials (nanomaterials) on sensitive population have not been well elucidated. This study examined the effects of pulmonary exposure to (latex) nanomaterials on lung inflammation related to lipopolysaccharide (LPS) or allergen in mice, especially in terms of their size-dependency. In protocol 1, ICR male mice were divided into 8 experimental groups that intratracheally received a single exposure to vehicle, latex nanomaterials (250 μg/animal) with three sizes (25, 50, and 100 nm), LPS (75 μg/animal), or LPS plus latex nanomaterials. In protocol 2, ICR male mice were divided into 8 experimental groups that intratracheally received repeated exposure to vehicle, latex nanomaterials (100 μg/animal), allergen (ovalbumin: OVA; 1 μg/animal), or allergen plus latex nanomaterials. In protocol 1, latex nanomaterials with all sizes exacerbated lung inflammation elicited by LPS, showing an overall trend of amplified lung expressions of proinflammatory cytokines. Furthermore, LPS plus nanomaterials, especially with size less than 50 nm, significantly elevated circulatory levels of fibrinogen, macrophage chemoattractant protein-1, and keratinocyte-derived chemoattractant, and von Willebrand factor as compared with LPS alone. The enhancement tended overall to be greater with the smaller nanomaterials than with the larger ones. In protocol 2, latex nanomaterials with all sizes did not significantly enhance the pathophysiology of allergic asthma, characterized by eosinophilic lung inflammation and Igs production, although latex nanomaterials with less than 50 nm significantly induced/enhanced neutrophilic lung inflammation. These results suggest that latex nanomaterials differentially affect two types of (innate and adaptive immunity-dominant) lung inflammation.

Introduction

Environmental particulate matters (PM) are well recognized to be toxic for health, including respiratory systems (Dockery and Pope, 1994). One intriguing aspect of the epidemiologic data is that health effects of PM are primarily seen in people with predisposing factors, including bronchilitis, chronic obstructive pulmonary diseases, bronchial asthma, and pneumonia (Dockery et al., 1993). In particular, diesel exhaust particles (DEP), main constituents in urban PM, experimentally not only induce lung inflammation (Ichinose et al., 1995) but facilitate several respiratory diseases such as acute lung injury and bronchial asthma (Ichinose et al., 1995; Takano et al., 2002; Yanagisawa et al., 2003). Our previous in vivo studies have demonstrated that DEP and their components (organic chemicals extracted from DEP; referred to as "DEP-OC" residual particles after extraction of chemical components; referred to as "washed DEP") facilitate both lipopolysaccharide (LPS)-related lung inflammation (Takano et al., 2002; Yanagisawa et al., 2003) and allergic airway inflammation (Takano et al., 1997; Yanagisawa et al., 2006). Furthermore, washed DEP has been shown to augment systemic inflammation and coagulatory disturbance accompanied by lung inflammation related to LPS (Inoue et al., 2006a). More recently, we have demonstrated that carbon nanoparticles (less than 100 nm in their size) exacerbate LPS-related lung inflammation with systemic inflammation and coagulatory disturbance (Inoue et al., 2006b) and allergic airway inflammation in mice (Inoue et al., 2005). Taken together, these findings demonstrate that environment-existing particles have aggravating properties on complicated lung inflammation.

The development of nano-technology has increased the exposure risk to other types of particles in addition to combustion-derived particles in the environment, namely, engineered nanomaterials (Oberdorster et al., 2005). As these materials become more widespread, many questions arise as to the consequences that nanomaterials may have on the environment. In fact, previous reports have shown that the full impact, or even partial impact, of manufactured nanomaterials on health and the environment has yet to be explored in depth (Borm, 2002; Colvin, 2003; Guzman et al., 2006; Nel et al., 2006). In particular, nanomaterial exposure itself reportedly induces lung inflammation (Warheit et al., 2004; Shvedova et al., 2005; Chen

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et al., 2006; Warheit et al., 2006a; Warheit et al., 2006b). However, the facilitating effects of nanomaterials on the subjects with predisposing inflammatory disorders have not been fully examined. Furthermore, comparative studies of the size effects of nanomaterials via pulmonary exposure on these subjects have been less carried out.

Synthetic latex is used for variety of aims and contained in many materials such as paints, painting papers, carpet, wrapping papers, abradants, resins, gloves, etc. Besides, nano-level latex has been reported, for example, to be a useful tool for the investigation of pharmacological activity (Hasegawa et al., 2006). These facts suggest that the ambient and/or local concentrations of this type of nanomaterials mainly in manufacturing factories may increase in the future. Furthermore, its relative uniformed spherical form may allow us to estimate the size contribution, one of another important issue, to the effects. Therefore, to understand more directly the potential of latex nanomaterials to influence lung inflammation, the present study was designed to elucidate the effects of latex nanomaterials with three sizes on lung inflammation induced by intratracheal administration of LPS or allergen (ovalbumin: OVA), on which we have examined the synergistic/additive effects of environmental pollutants in vivo (Hiyoshi et al., 2005; Inoue et al., 2005; Inoue et al., 2006a; Inoue et al., 2006b; Inoue et al., 2006c; Takano et al., 1997; Takano et al., 2002).

Materials and methods

Animals. Male ICR mice 6 wk of age and weighing 29 to 33 g (Japan Clea Co., Tokyo, Japan) were used in all experiments. They were fed a commercial diet (Japan Clea Co.) and given water ad libitum. The mice were housed in an animal facility that was maintained at 24 to 26 °C with 55 to 75% humidity and a 12-h light/dark cycle.

Latex nanomaterials. All sizes (25, 50, and 100 nm) of latex nanomaterials were purchased as fluorescent latex particles (micromer®-blue F plain) from Micromod (Rostock, Germany). Their shape is round and they are monodisperse particles from polystyrene and, are designed with carboxylic acid groups on the particle surface for the covalent binding of proteins, antibodies or other molecules. Degree of agglomeration for each size of latex nanomaterial is not disclosed. They were used as suspension in the vehicle (phosphate-buffered saline [PBS] at pH 7.4 [Invitrogen Co., Carlsbad, CA] containing 0.05% Tween 80 [Nacalai Tesque, Kyoto, Japan]) by sonication for 3 min using an Ultrasonic disrupter (UD-201; Tomy Seiko, Tokyo, Japan).

Study protocol. In protocol 1, mice were divided into eight experimental groups. The vehicle group received above mentioned vehicle. The LPS group received 75 μg of LPS dissolved in PBS. The latex nanomaterial groups received (125 or) 250 μg of latex nanomaterial suspension in the vehicle. The LPS+latex nanomaterial groups received the combined treatment using the same protocols as the LPS and the nanomaterial groups (n=20–21 in each group; n=8 for BAL with ELISA for lung homogenates and plasma coagulation parameters, and n=12–13 for lung water contents [n=8], lung histology [n=4–5], and ELISA for serum proinflammatory cytokines [n=10]). Vehicle, latex nanomaterials, LPS, or LPS+latex nanomaterials were suspended in 0.1-ml aliquots, and inoculated once intratracheally through a polyethylene tube under anesthesia with 4% halothane (Takeda Chemical Industries, Tokyo, Japan). We have previously examined the effects of diesel exhaust-derived particles or carbon nanoparticles on LPS-related lung inflammation using 125 or 250 μg/animal in vivo (Yanagisawa et al., 2003; Inoue et al., 2006a; Inoue et al., 2006b). Based on and referred to the previous studies from our laboratory, we chose the dosage of 125 and 250 μg/body of the nanomaterials. The animals were euthanized and studied 24 h after the intratracheal administration with BAL, pulmonary vascular permeability of water and protein, protein levels of cytokines and chemokines in the lung tissue supernatants and the sera, and parameters related to coagulatory system. In protocol 2, mice were divided into 8 experimental groups. The vehicle group received above mentioned vehicle once a week for 6 wk. The OVA group received 1 μg of OVA (Grade IV: Sigma Chemical, St. Louis, MO) dissolved in the same vehicle every 2 wk for 6 wk (Fig. 1). The latex nanomaterial groups received (50 or) 100 μg of above mentioned three sizes of latex nanomaterials suspended in the same vehicle every week for 6 wk. The OVA+latex nanomaterial groups received the combined treatment in the same protocol as the OVA and the latex nanomaterial groups (n=12–13 in each group; n=8 for BAL with ELISA for lung homogenates, n=4–5 for lung histology, and n=12–13 for ELISA for serum Ig titers in experiments using 100 μg of latex nanomaterials). Vehicle, latex nanomaterials, OVA, or OVA+latex nanomaterials were suspended in 0.1-ml aliquots, and inoculated once by the intratracheal route through a polyethylene tube under anesthesia with 4% halothane (Takeda Chemical Industries). We have previously examined the effects of diesel exhaust-derived particles or carbon nanoparticles on allergic airway inflammation at a dose of 50 or 100 μg/animal in vivo (Yanagisawa et al., 2006; Inoue et al., 2005; Inoue et al., 2006c). Based on and referred to the previous studies from our laboratory, we chose the dosage of 50 and 100 μg/body of the nanomaterials. The animals were euthanized and studied 24 h after the final intratracheal administration, with BAL, lung histology, protein levels of cytokines and chemokines in the lung tissue supernatants and Ig production. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals according to Institutional Animal Care and Use Committee (IACUC: www.iacuc.org). All animal studies were approved by the Institutional Review Board.

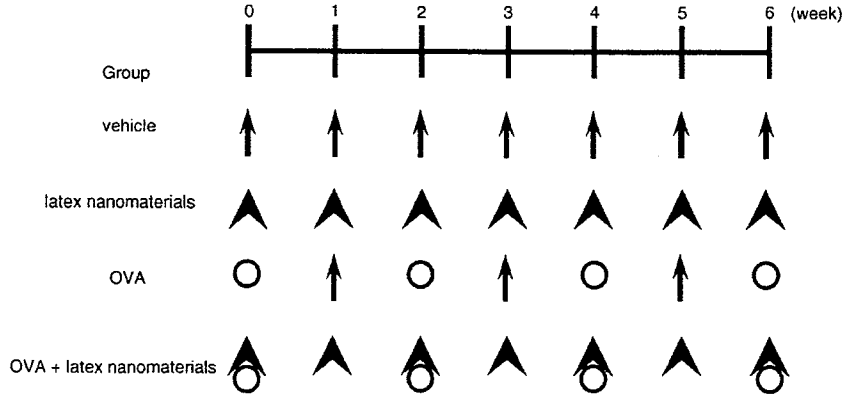

Fig. 1. Study design for protocol 2.

BAL process. BAL and cell counts were conducted as previously described ($n=8$ in each group at each protocol; Inoue et al., 2006a; Inoue et al., 2006b; Inoue et al., 2006c; Takano et al., 1997; Takano et al., 2002; Yanagisawa et al., 2003). After the BAL procedure, supernatants were analyzed for protein assay ($n=8$ in each group), and the lungs were removed, snap-frozen in liquid nitrogen, and stored at −80 °C until assay of cytokines and chemokines.

Pulmonary vascular permeability. In protocol 1, in a separate series of experiments, the lungs were weighed and dried. The wet lung weight−the dry lung weight/body weight was calculated (ref [Ichinose et al., 1995]: $n=8$ in each group). In another experiment, protein concentrations in BAL fluid were determined using the commercially available Bradford protein assay (Bio-Rad Laboratory Inc., Hercules, CA: $n=8$ in each group) with bovine serum albumin as the standard.

Lung histology. In another experiment, the lungs were fixed and stained with hematoxylin and eosin as previously described (Takano et al., 1997; Inoue et al., 2006a). For semi-quantitative assessment in protocol 1, neutrophil infiltration was assessed by averaging the number of neutrophils enumerated in 30 randomly selected high power fields (HPFs; ×600) in each slide. Histologic sections were evaluated in a blind fashion ($n=4$–5 in each group at each protocol).

Quantitation of cytokine and chemokine protein levels in the lung tissue supernatants. The frozen lungs after BAL were homogenized in each protocol as described previously (Takano et al., 1997; Inoue et al., 2004; Inoue et al., 2005). ELISA for interleukin (IL)-1β(R&D Systems, Minneapolis, MN), IL-5 (Endogen, Cambridge, MA), IL-13 (R&D Systems), IL-18 (MBL, Nagoya, Japan), macrophage chemoattractant protein (MCP)-1 (R&D Systems), and keratinocyte-derived chemoattractant (KC: R&D Systems), eotaxin (R&D systems), and macrophage inflammatory protein (MIP)-1α (R&D Systems) in the lung tissue homogenates were conducted according to the manufacturer's instructions and the values were expressed as pg or ng/ml total lung supernatants ($n=8$ in each group at each protocol).

Assays for circulatory levels of fibrinogen, von Willebrand factor (vWF), IL-1β, MCP-1, and KC, and activity of protein C (PC). In protocol 1, citrate plasma levels of fibrinogen and vWF, and the activity of PC ($n=8$ in each group) were determined using commercial kits (Diagnostica Stago, Roche, Tokyo, Japan) on STA Compact (Diagnostica Stago, Roche) as described previously (Inoue et al., 2004). Serum samples ($n=10$ in each group) were analyzed by ELISA for IL-1β, MCP-1, and KC (R&D Systems) according to the manufacturer's instructions as described above, and the values were expressed as pg or ng/ml.

Allergen-specific Ig determination. In protocol 2, blood was retrieved by cardiac puncture. Serum was prepared and frozen at −80 °C until assayed for allergen-specific IgG_1 and IgE. Allergen-specific IgG_1-antibody was measured by ELISA with solid-phase allergen as previously described (Inoue et al., 2005; Inoue et al., 2007: $n=12$–13 in each group). Allergen-specific IgE antibody was measured by commercial ELISA kit (Dainippon Sumitomo Pharmaceutical Co. Osaka, Japan) according to the manufacturer's instructions ($n=12$–13 in each group).

Statistical analysis. Data are reported as means±SE. Differences between groups were determined using analysis of variance (ANOVA: Stat view version 4.0; Abacus Concepts, Inc., Berkeley, CA). If differences between groups were significant ($P<0.05$) using one-way ANOVA, the Bonferroni correction was used for multiple comparison.

Results

Effects of latex nanomaterials on LPS-related lung inflammation and pulmonary vascular permeability

We examined the cellular profile of BAL fluid 24 h after the intratracheal instillation in protocol 1 and exhibited representative data (250 μg/animal of latex nanomaterials used: Table 1). Latex nanomaterials (25 or 50 nm) alone increased the number of neutrophils as compared with vehicle ($P<0.01$ for 25 or 50 nm latex nanomaterial). LPS exposure significantly increased the number as compared with vehicle exposure ($P<0.01$). The numbers in the LPS+25 or 50 nm latex nanomaterial group were identical with those in the LPS group, but greater than those in the corresponding latex nanomaterial groups ($P<0.01$). In contrast, the numbers were significantly smaller in the LPS+100 nm latex nanomaterial group than in the LPS group ($P<0.05$). Next, we examined the protein levels in BAL fluid and the lung water content 24 h after the intratracheal instillation (Table 1). Pulmonary exposure to latex nanomaterials elevated the protein level in BAL fluid ($P<0.05$ for 25 nm latex nanomaterial) and lung water content ($P<0.01$ for 25 or 50 nm latex nanomaterial, $P<0.05$ for 100 nm latex nanomaterial) as compared with exposure to vehicle. These values were significantly greater in the LPS group than in the vehicle group ($P<0.01$). These values were

Table 1
The number of neutrophils in bronchoalveolar lavage (BAL) fluid and lung histology, protein concentration in BAL fluid, and lung water content after intratracheal challenge (protocol 1)

Treatment	Neutrophils		Protein	Lung wet weight−dry weight [mg]/body weight [g]
	($\times 10^4$ /total BAL fluid)	(cell number/HPF)	(mg/ml of BAL fluid)	
Vehicle	22.1±4.0	1.6±0.4	0.42±0.03	5.63±0.11
25 nm latex nanomaterial	69.8±12.6**	5.6±1.2*	0.74±0.03*	6.54±0.09**
50 nm latex nanomaterial	65.4±12.5**	7.3±0.8*	0.58±0.03	6.48±0.11**
100 nm latex nanomaterial	26.2±4.8	4.1±0.5*	0.59±0.05	5.93±0.13*
LPS	326.5±88.2**	94.4±20.5**	1.05±0.13**	8.43±0.15**
LPS+25 nm latex nanomaterial	338.2±44.8**,$	166.1±21.7**,##,$	1.46±0.22**,##,$	10.28±0.16**,##,$
LPS+50 nm latex nanomaterial	323.2±16.8**,$	125.5±22.7**,$	1.36±0.13**,#,$	9.61±0.26**,##,$
LPS+100 nm latex nanomaterial	212.7±34.6**,#,$	101.2±12.4**,$	1.05±0.08**,$	8.93±0.27**,#,$

Results are expressed as mean±SE ($n=8$ in each group for neutrophil number and protein concentration in BAL and lung water content, $n=4$–5 in each group for neutrophil number in lung parenchyma).
* $P<0.05$ versus the vehicle group.
** $P<0.01$ versus the vehicle group.
\# $P<0.05$ versus the LPS group.
\## $P<0.01$ versus the LPS group.
$ $P<0.01$ versus the nanomaterial group.

Table 2
Protein levels of interleukin (IL)-1β and chemokines in the lung tissue supernatants after intratracheal challenge (protocol 1)

Treatment	IL-1β	MCP-1	KC
	(ng/total lung supernatants)	(pg/total lung supernatants)	
Vehicle	0.5±0.0	16.4±4.0	59.8±13.3
25 nm latex nanomaterial	1.2±0.1*	111.9±13.3**	34.8±8.2
50 nm latex nanomaterial	1.3±0.2*	83.2±14.0**	59.8±11.3
100 nm latex nanomaterial	0.8±0.1	48.7±7.4*	43.9±12.1
LPS	41.5±4.4**	1201.5±102.5**	445.4±116.1**
LPS+25 nm latex nanomaterial	77.2±10.8**,#,$	2714.1±346.6**,##,$	1391.2±231.5**,##,$
LPS+50 nm latex nanomaterial	55.7±4.8**,#,$	2305.6±292.9**,##,$	1475.3±231.1**,##,$
LPS+100 nm latex nanomaterial	37.4±4.3**,$	1438.1±268.0**,$	668.0±164.3**,$

Results are expressed as mean±SE (n=8 in each group).
* $P<0.05$ versus the vehicle group.
** $P<0.01$ versus the vehicle group.
\# $P<0.05$ versus the LPS group.
\#\# $P<0.01$ versus the LPS group.
$ $P<0.01$ versus the nanomaterial group.

further greater in the LPS+latex nanomaterial groups than in the LPS ($P<0.05$ for the LPS+50 nm latex nanomaterial group on the protein level of BAL fluid, and for the LPS+100 nm latex nanomaterial group on the lung water content; $P<0.01$ for the LPS+25 nm latex nanomaterial group on the protein level of BAL fluid and the lung water content, and for the LPS+50 nm latex nanomaterial group on the lung water content) or the corresponding latex nanomaterial ($P<0.01$) groups. Whereas in experiments using 125 μg/animal of latex nanomaterials, the data are similar as that using 250 μg/animal, although the significance did not show profoundly (data not shown).

Effects of latex nanomaterials on LPS-related histological changes in the lung

We evaluated lung specimens stained with hematoxylin and eosin 24 h after the intratracheal instillation in protocol 1. No pathological change was seen in the lung obtained from the vehicle group. Infiltration of neutrophils was moderately seen in the lungs from the latex nanomaterial groups and moderately seen in those from the LPS groups. Combined treatment with LPS and latex nanomaterials markedly enhanced leukocyte (mainly neutrophil) sequestration into the lung parenchyma as compared with LPS treatment alone, especially with smaller latex nanomaterials with overall trend. Furthermore, we determined the number of neutrophils in the lung (Table 1). Latex alone increased the number as compared with vehicle ($P<0.05$). LPS challenge significantly increased the number as compared with vehicle challenge ($P<0.01$). The number was further greater in the LPS+latex nanomaterial groups than in the LPS group ($P<0.01$ for LPS+25 nm latex nanomaterial) or the corresponding latex nanomaterial groups ($P<0.01$).

Effects of latex nanomaterials on the expression of LPS-related proinflammatory cytokine and chemokines in the lung

We next measured the protein levels of IL-1β, MCP-1, and KC in the lung tissue supernatants 24 h after the intratracheal instillation in protocol 1 (Table 2). Pulmonary exposure to latex nanomaterials alone elevated the levels of IL-1β ($P<0.05$ for 25 or 50 nm latex nanomaterial) and MCP-1 ($P<0.05$ for 100 nm latex nanomaterial; $P<0.01$ for 25 or 50 nm latex nanomaterial) as compared to that to vehicle. LPS challenge significantly elevated the levels of all of these proteins as compared with vehicle challenge ($P<0.01$). The levels were further greater in the LPS+25 or 50 nm latex nanomaterial group than in the LPS ($P<0.05$ for the IL-1β level; $P<0.01$ for the MCP-1 or the KC) or the corresponding latex nanomaterial ($P<0.01$) groups.

Effects of latex nanomaterials on LPS-related coagulatory parameters

We next analyzed coagulatory parameters 24 h after the intratracheal challenge in protocol 1 (Table 3). LPS challenge significantly elevated the fibrinogen level as compared to vehicle challenge ($P<0.01$). The level was further greater in the LPS+25 or 50 nm latex nanomaterial group than in the LPS group ($P<0.05$ for the LPS+50 nm latex nanomaterial group; $P<0.01$ for the LPS+25 nm latex nanomaterial group) or the corresponding latex nanomaterial groups ($P<0.01$). As compared to vehicle challenge, LPS challenge caused a significant increase in the level of vWF ($P<0.05$). The level was further greater in the LPS+25 nm ($P<0.01$) or the LPS+50 nm ($P<0.05$) latex nanomaterial group than in the LPS group or the corresponding latex nanomaterial groups ($P<0.01$). LPS significantly decreased the activity of PC as compared with vehicle ($P<0.01$). The activity was significantly lower in the LPS+latex nanomaterial groups than in the corresponding latex nanomaterial groups ($P<0.05$).

Effects of latex nanomaterials on circulatory levels of LPS-related proinflammatory cytokine and chemokines

We measured the protein levels of IL-1β, MCP-1, and KC in the sera 24 h after the intratracheal instillation in protocol 1 (Table 4). LPS challenge significantly elevated the level of IL-1β as compared with

Table 3
Plasma coagulatory parameters after intratracheal instillation (protocol 1)

Treatment	Fibrinogen	vWF	Activity of PC
	(mg/dl)	(%)	
Vehicle	336.3±6.2	73.9±6.7	6.1±0.4
25 nm latex nanomaterial	366.0±6.5	72.1±3.0	5.0±0.2
50 nm latex nanomaterial	350.3±18.2	72.8±1.1	5.1±0.1
100 nm latex nanomaterial	345.7±10.4	64.8±9.5	5.6±0.3
LPS	592.3±26.4**	87.9±6.2*	3.5±0.3**
LPS+25 nm latex nanomaterial	797.8±40.6**,##,$$	97.5±5.9**,##,$$	3.3±0.3**,$
LPS+50 nm latex nanomaterial	687.3±54.1**,#,$$	92.3±5.0**,#,$$	3.3±0.3**,$
LPS+100 nm latex nanomaterial	585.3±65.5**,$$	84.9±4.5*,$$	3.6±0.3**,$

Results are expressed as mean±SE (n=8 in each group).
* $P<0.05$ versus the vehicle group.
** $P<0.01$ versus the vehicle group.
\# $P<0.05$ versus the LPS group.
\#\# $P<0.01$ versus the LPS group.
$ $P<0.05$ versus the nanomaterial group.
$$ $P<0.01$ versus the nanomaterial group.

Table 4
Circulatory levels of interleukin (IL)-1β and chemokines after intratracheal instillation (protocol 1)

Treatment	IL-1β	MCP-1	KC
	(ng/ml)	(pg/ml)	
Vehicle	5.7±1.2	12.9±1.5	59.8±13.3
25 nm latex nanomaterial	4.7±0.9	21.4±4.3	34.8±8.2
50 nm latex nanomaterial	4.5±0.9	24.2±2.6*	59.8±11.3
100 nm latex nanomaterial	2.8±0.9	23.6±2.5*	43.9±12.1
LPS	9.2±1.8*	191.1±29.9**	445.4±116.1**
LPS+25 nm latex nanomaterial	11.7±1.9**,$$	504.9±74.3**,##,$$	1391.2±231.5**,##,$$
LPS+50 nm latex nanomaterial	13.5±2.0**,#,$$	687.1±71.7**,##,$$	1475.3±231.1**,##,$$
LPS+100 nm latex nanomaterial	4.5±1.2$	313.1±73.2**,#,$$	668.0±164.3**,$$

Results are expressed as mean±SE ($n=10$ in each group).
* $P<0.05$ versus the vehicle group.
** $P<0.01$ versus the vehicle group.
\# $P<0.05$ versus the LPS group.
\#\# $P<0.01$ versus the LPS group.
$ $P<0.05$ versus the nanomaterial group.
$$ $P<0.01$ versus the nanomaterial group.

vehicle challenge ($P<0.05$). The level was further greater in the LPS+25 nm or the LPS+50 nm latex nanomaterial group than in the LPS ($P<0.05$ for LPS+50 nm latex nanomaterial) or the corresponding latex nanomaterial groups ($P<0.01$). Latex nanomaterial (50 and 100 nm) challenge significantly elevated MCP-1 level ($P<0.05$ vs. vehicle). LPS challenge also significantly elevated the levels of MCP-1 and KC as compared with vehicle challenge ($P<0.01$). The levels were further greater in the LPS+latex nanomaterial groups than in the LPS ($P<0.01$ for LPS+25 or 50 nm latex nanomaterial group) or the corresponding latex nanomaterial groups ($P<0.01$).

Effects of latex nanomaterials on allergic airway inflammation

We investigated the cellular profile of BAL fluid in protocol 2 and exhibited representative data (100 μg/animal of latex nanomaterials used: Fig. 2). The number of total cells was significantly greater in the latex nanomaterial or the OVA+latex nanomaterial groups than in the vehicle group ($P<0.05$ for 50 nm latex nanomaterial, $P<0.01$ for 25 nm latex nanomaterial and OVA+25 or 50 nm latex nanomaterial). The number was greater in the OVA+latex nanomaterial groups than in the OVA group ($P<0.01$ for OVA+25 or 50 nm latex nanomaterial). The number of macrophages was greater in the latex nanomaterial or the OVA+latex nanomaterial groups than in the vehicle group ($P<0.05$ for 25 nm latex nanomaterial and OVA+25 or 50 nm latex nanomaterial). The number was greater in the OVA+latex nanomaterial groups than in the OVA group ($P<0.05$ for OVA+25 nm latex nanomaterial; $P<0.01$ for OVA+50 nm latex nanomaterial). Exposure to OVA or OVA+latex nanomaterials significantly increased the number of eosinophils as compared with vehicle exposure ($P<0.05$ for OVA or OVA+50 nm latex nanomaterial; $P<0.01$ for OVA+25 or 100 nm latex nanomaterial). However, the numbers were comparable among the OVA and OVA +latex nanomaterial groups. The number of lymphocytes was greater in the 25 nm latex nanomaterial group or the OVA+25 or 50 nm latex nanomaterial group than in the vehicle group ($P<0.01$ for 25 nm latex nanomaterial or OVA+25 or 50 nm latex nanomaterial). The number was greater in the OVA+latex nanomaterial groups than in the OVA ($P<0.01$) or the corresponding latex nanomaterial groups ($P<0.05$ for OVA+50 nm latex nanomaterial). The number of neutrophils was greater in the 25 and 50 nm latex nanomaterial groups and the OVA +25 or 50 nm latex nanomaterial group than in the vehicle group ($P<0.01$). The number was greater in the OVA+latex nanomaterial groups than in the OVA or the corresponding latex nanomaterial groups ($P<0.01$ for OVA+25 or 50 nm latex nanomaterial). Whereas in experiments using 50 μg/animal of latex nanomaterials, the data are similar as that using 100 μg/animal, although the significance did not show profoundly (data not shown).

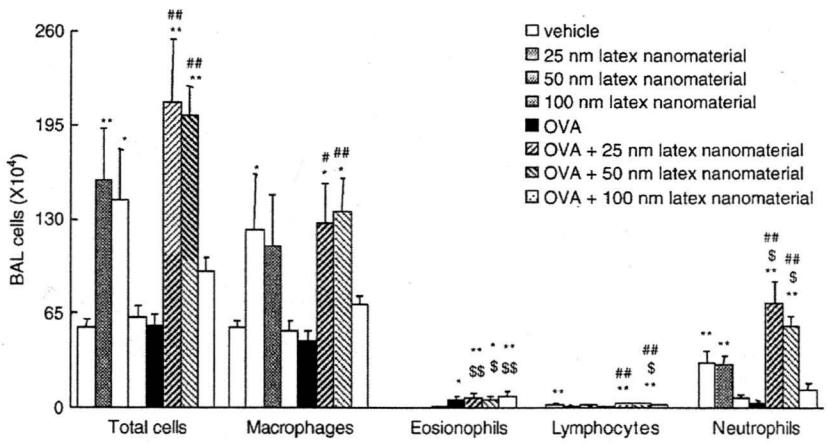

Fig. 2. Cellularity in BAL fluid after intratracheal challenge (protocol 2). Twenty four hours after the final intratracheal administration, lungs were lavaged for the analysis of BAL fluid. Count for total cells was determined on a fresh fluid specimen using a hemocytometer. Differential cell counts were assessed on cytologic preparations stained with Diff-Quik. Results are means±SE ($n=8$ in each group). *$P<0.05$ versus the vehicle group, **$P<0.01$ versus the vehicle group, #$P<0.05$ versus the OVA group, ##$P<0.01$ versus the OVA group, $$P<0.05$ versus the nanomaterial group, $$$P<0.01$ versus the nanomaterial group.

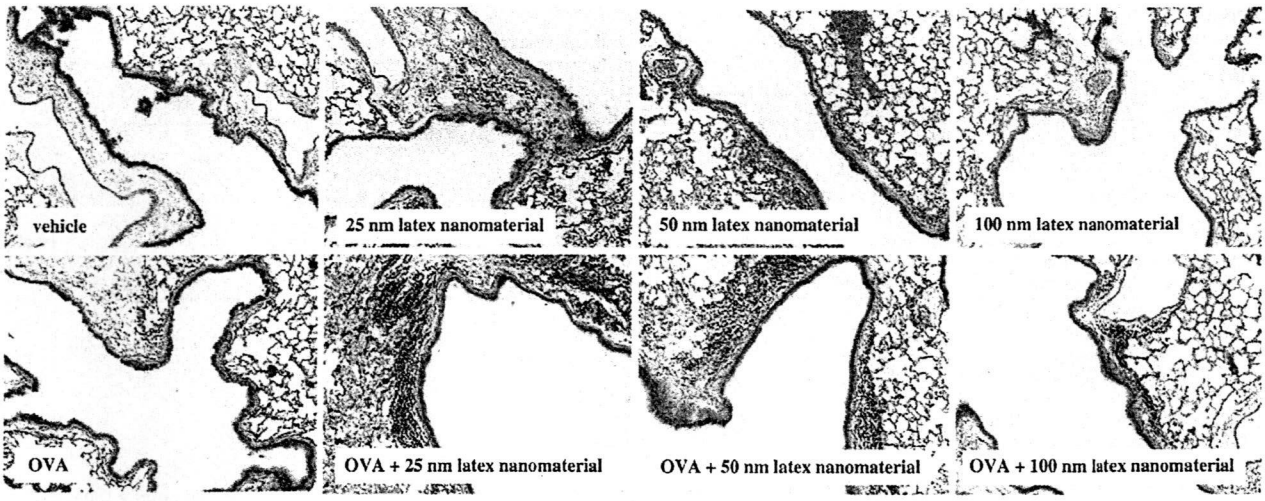

Fig. 3. Representative histological findings of the hematoxylin and eosin-stained lung obtained from the vehicle, latex nanomaterial, OVA, or OVA+latex nanomaterial groups 24 h after the final intratracheal administration. Animals received intratracheal instillation of vehicle, latex nanomaterials, OVA, or OVA+latex nanomaterials for 6 wk. Lungs were removed and fixed 24 h after the last intratracheal administration (n=4–5 in each group). Original magnification ×25.

Effects of latex nanomaterials on allergen — related histological changes in the lung

We evaluated lung specimens stained with hematoxylin and eosin 24 h after the final intratracheal instillation in protocol 2 (Fig. 3). No pathological change was seen in the lung obtained from the vehicle group. Infiltration of neutrophils was slightly seen in the lungs from the latex nanomaterial groups. On the other hand, infiltration of eosinophils was moderately observed in the lung from the OVA group. Combined treatment with OVA and latex nanomaterials apparently worsened polymorphonuclear leukocyte (mainly neutrophil) sequestration into the lung parenchyma as compared with OVA treatment alone, especially with smaller latex nanomaterials with overall trend.

Effects of latex nanomaterials on local expression of cytokines and chemokines in the presence of allergen

We quantitated protein levels of IL-5, IL-13, IL-18 (Table 5), eotaxin, MIP-1α, MCP-1, and KC (Table 6) in the lung tissue supernatants. OVA challenge increased the level of IL-5 and IL-13 as compared with vehicle challenge (P<0.01). The levels were not significantly different between the OVA and the OVA+latex nanomaterial groups. The level of IL-18 was significantly greater in the latex nanomaterial groups and the OVA+latex nanomaterial groups than in the vehicle group (P<0.01). The level was further greater in the OVA+latex nanomaterial groups than in the OVA group (P<0.01). The level of eotaxin was significantly greater in the OVA and the OVA+latex nanomaterial groups than in the vehicle group (P<0.01). The level was significantly greater in the OVA+latex nanomaterial groups than in the corresponding latex nanomaterial groups (P<0.01). The level was not significantly different among the OVA and the OVA+latex nanomaterial groups. The level of MIP-1α was greater in the latex nanomaterial groups (P<0.01) or the OVA group (P<0.05) than in the vehicle group. The level was further greater in the OVA+latex nanomaterial groups than in the vehicle (P<0.01), the OVA (P<0.01 for OVA+25 or 50 nm latex nanomaterial; P<0.05 for OVA+100 nm latex nanomaterial), or the corresponding latex nanomaterial groups (P<0.05 for OVA+25 nm latex nanomaterial). The level of MCP-1 was also greater in the latex nanomaterial groups (P<0.01) or the OVA group (P<0.05) than in the vehicle group. The level was further greater in the OVA+latex nanomaterial groups than in the vehicle, the OVA, or the corresponding latex nanomaterial groups (P<0.01). The level of KC was greater in the latex nanomaterial groups than in the vehicle group (P<0.05 for 25 or 50 nm latex nanomaterial). The level was further greater in the OVA+latex nanomaterial groups than in the vehicle (P<0.01 for OVA+25 or 50 nm latex nanomaterial; P<0.05 for OVA+100 nm latex

Table 5
Protein levels of cytokines in the lung tissue supernatants after the final intratracheal challenge (protocol 2)

Treatment	IL-5	IL-13	IL-18
	(pg/ml)		
Vehicle	13.0±1.1	3.5±2.7	828.0±31.9
25 nm latex nanomaterial	12.6±0.6	3.7±1.1	2227.5±178.3**
50 nm latex nanomaterial	17.3±2.1	2.2±1.5	2196.0±201.1**
100 nm latex nanomaterial	12.6±1.0	4.1±2.2	1391.5±116.0*
OVA	120.7±32.2**	143.9±37.5**	749.1±73.4
OVA+25 nm latex nanomaterial	118.9±35.2**,§§	89.1±31.4**,§§	2786.1±283.9**,$^{\#\#}$
OVA+50 nm latex nanomaterial	150.3±19.8**,§§	139.8±41.2**,§§	2068.9±182.8**,$^{\#\#}$
OVA+100 nm latex nanomaterial	106.1±29.2**,§§	92.9±26.2**,§§	1719.4±136.8**,$^{\#\#}$

Results are expressed as mean±SE (n=8 in each group).
$^{\#}P$<0.05 versus the OVA group.
$^{\S}P$<0.05 versus the nanomaterial group.
* P<0.05 versus the vehicle group.
** P<0.01 versus the vehicle group.
$^{\#\#}$ P<0.01 versus the OVA group.
§§ P<0.01 versus the nanomaterial group.

Table 6
Protein levels of chemokines in the lung tissue supernatants after the final intratracheal challenge (protocol 2)

Treatment	Eotaxin	MIP-1α	MCP-1	KC
	(pg/ml)			
Vehicle	74.7±5.0	2.0±1.4	25.2±9.5	156.7±113.9
25 nm latex nanomaterial	111.2±7.4	237.9±16.6**	363.1±46.2**	505.2±73.0*
50 nm latex nanomaterial	126.9±12.3	188.0±21.7**	314.9±44.2**	510.1±44.8*
100 nm latex nanomaterial	108.4±10.6	73.3±11.1**	126.7±13.9**	237.5±28.0
OVA	513.2±116.4**	16.3±4.5*	94.7±15.0*	142.4±40.4
OVA+25 nm latex nanomaterial	522.7±177.2**,$$	496.0±71.9**,##,$	830.4±143.6**,##,$$	934.5±180.4**,##,$$
OVA+50 nm latex nanomaterial	828.1±175.0**,$$	206.2±24.6**,##	855.1±150.6**,##,$$	802.5±149.8**,##
OVA+100 nm latex nanomaterial	877.0±357.5**,$$	66.7±17.0**,#	364.6±139.8**,##,$$	342.0±110.5#

Results are expressed as mean±SE (n=8 in each group).
* P<0.05 versus the vehicle group.
** P<0.01 versus the vehicle group.
\# P<0.05 versus the OVA group.
\#\# P<0.01 versus the OVA group.
$ P<0.05 versus the nanomaterial group.
$$ P<0.01 versus the nanomaterial group.

nanomaterial), the OVA (P<0.01 for OVA+25 or 50 nm latex nanomaterial), or the corresponding latex nanomaterial groups (P<0.01 for OVA+25 nm latex nanomaterial).

Effects of latex nanomaterials on allergen-specific production of IgG_1 and IgE

We measured allergen-specific IgG_1 and IgE (Fig. 4). The allergen-specific IgG_1 and IgE were significantly greater in the OVA or the OVA+latex nanomaterial groups than in the vehicle group (P<0.01). The titers of both IgG_1 and IgE were comparable among the OVA and the OVA+latex nanomaterial groups.

Discussion

The present study has demonstrated that latex nanomaterials instilled intratracheally enhance neutrophilic lung inflammation with pulmonary vascular permeability related to LPS resulted from activated innate immunity. The enhancement is concomitant with the increased local (lung) expression of proinflammatory cytokine

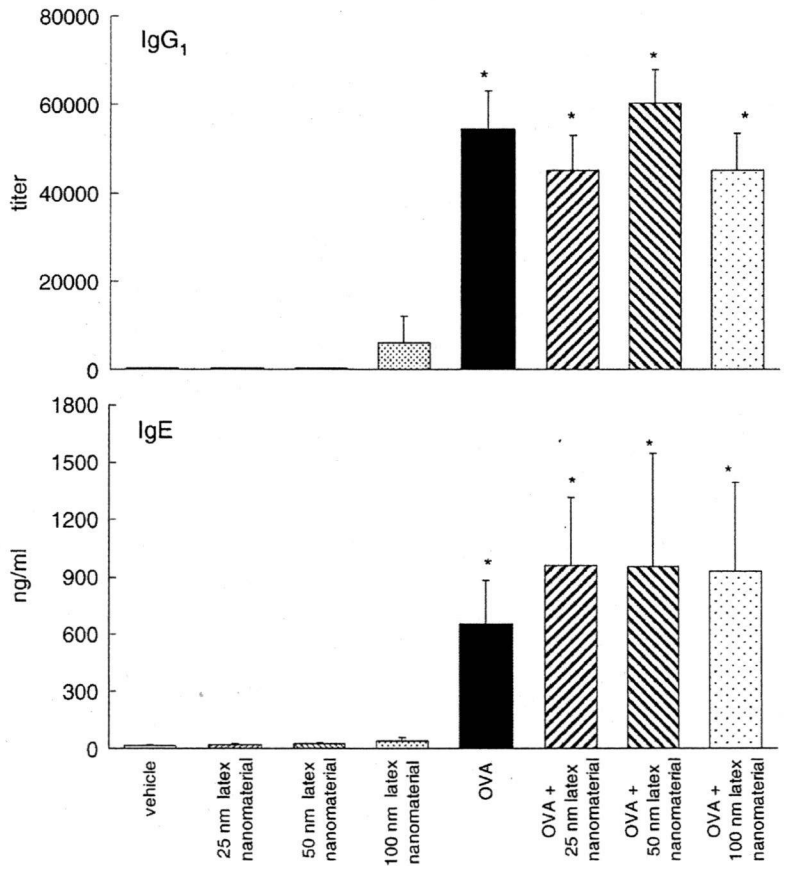

Fig. 4. Allergen-specific IgG_1 and IgE titers. Eight groups of mice were intratracheally administered vehicle, latex nanomaterials, OVA, or the combination of OVA+latex nanomaterials for 6 wk. Plasma samples were retrieved 24 h after the last intratracheal instillation. Antigen-specific IgG_1 and IgE were analyzed using ELISA. Results are expressed as mean±SE (n=12–13 in each group). *P<0.01 versus vehicle.

such as IL-1β, and chemokines such as MCP-1 and KC. In addition, combined challenge with LPS and nanomaterials further increases circulatory levels of fibrinogen, vWF, IL-1β, MCP-1, and KC, as compared with challenge with LPS alone. The enhancing effects of nanomaterials on the pathology tend to be greater with the smaller nanomaterials than with larger ones in overall trend. In contrast, repetitive exposure to the latex nanomaterials does not significantly exacerbate the hallmarks of allergic airway inflammation characterized by eosinophilic lung inflammation with Ig productions predominant consequence of activated adaptive immunity.

Regarding the effects of PM including nanoparticles on LPS-related lung inflammation, we have previously demonstrated that DEP, important constituents in PM, enhance the lung inflammation (Takano et al., 2002; Yanagisawa et al., 2003) and the accompanying coagulatory disturbance (Inoue et al., 2006a) using a similar protocol to that in the present study. Furthermore, we have demonstrated that carbon nanoparticles, used as a relevant type of ambient PM, also deteriorate the pathophysiology (Inoue et al., 2006b). In that study, smaller (14 nm) nanoparticles have more markedly aggravated the features than larger (56 nm) ones (Inoue et al., 2006b). On the other hand, previous reports have shown the full impact, or even partial impact, of manufactured nanomaterials on health and the environment (Borm, 2002; Colvin, 2003; Guzman et al., 2006; Hardman, 2006; Nel et al., 2006). For example, a single pulmonary exposure to nano TiO_2 particles (19–21 nm) reportedly induces emphysema-like lung injury (Chen et al., 2006). Also, single-walled carbon nanotubes (1–4 nm in a diameter) instilled through the airways (pharynges) induce lung inflammation, which is characterized by fibrogenic changes with granuloma formation (Shvedova et al., 2005). However, all these studies have examined the effects of (nano) materials on physical (normal) conditions. The present results have, for the first time to our knowledge, shown that exposure to latex nanomaterials can additively/synergistically augment both local (lung) and systemic inflammation with coagulatory disturbance related to LPS, and that the effects are greater with the smaller materials than with the larger ones with overall trend, which is similar to the effects of carbonaceous nanoparticles found in our previous study (Inoue et al., 2006b).

Mechanistically, the pathogenesis of acute lung inflammation reportedly involves amplified lung expression of proinflammatory cytokines including IL-1β and chemokines such as IL-8 and MCP-1 (Standiford et al., 1995; Martin, 1999; Puneet et al., 2005). In our previous studies, indeed, we have confirmed the lung expression of proinflammatory cytokine and chemokines including IL-1β, MCP-1, and KC in the lung 24 h after the intratracheal administration of LPS, DEP, washed DEP, or carbon nanoparticles is concomitant with the aggravated lung injury (Takano et al., 2002; Sanbongi et al., 2003; Yanagisawa et al., 2003; Inoue et al., 2006b). In the present study, likewise, the lung expression of these molecules paralleled the lung inflammation with overall trend (Tables 2 and 4). Thus, the exaggerating effects of latex nanomaterials on this lung inflammation might be mediated, at least in part, through the enhanced lung expression of IL-1β, MCP-1, and KC (a murine homologue of IL-8).

Nano-sized particles and materials are reportedly able to penetrate deeply into the respiratory tract and may even pass the lung to reach the systemic circulation (MacNee and Donaldson, 2000; Nemmar et al., 2001), implying that they can affect the circulatory system in the context of systemic inflammation with coagulopathy. In accord with these studies, we have previously demonstrated that pulmonary exposure to carbon nanoparticles enhance systemic inflammation with coagulatory disturbance in the same model in the present study (Inoue et al., 2006b). In the present study, LPS combined with nanomaterials, specifically with those with a diameter of less than 50 nm, significantly elevated the levels of fibrinogen, MCP-1, and KC compared to LPS alone. Additionally, the elevated level of vWF induced by LPS was further increased by the combination of LPS with nanomaterials, especially with latex with a diameter of less than 50 nm. These findings suggest that pulmonary exposure to nanomaterials, in particular to smaller ones, can directly/indirectly facilitate systemic inflammation with coagulatory disturbance accompanied by lung inflammation, like combustion-derived nanoparticles (Inoue et al., 2006b), although evidence of translocation remains unaddressed.

Overall, the (above exhibited) response to 25 or 50 nm (and partial one to 100 nm) latex nanomaterials plus LPS was almost greater than the sum of the individual responses. Thus, it is noted that these observations could be considered as synergistic effects of two inflammation-inducing agents such as latex nanomaterials and LPS.

Independently, we have previously reported that PM including DEP and carbon nanoparticles experimentally enhance allergic asthma, another lung inflammation, characterized by eosinophilic inflammation and airway hyperresponsiveness (Takano et al., 1997; Ichinose et al., 1998; Takano et al., 1998; Inoue et al., 2005; Inoue et al., 2006c), rising the possibility that nano-leveled materials can act on the pathophysiology with similar fashion to PM. In fact, TiO_2 nanomaterials (14–29 nm in size) have more prominent adjuvant effects on allergen-specific response with Ig production than fine (250–260 nm in size) ones in vivo (de Haar et al., 2006). In the present study, however, repetitive exposure to latex nanomaterials did not exacerbate the murine model of allergic asthma in the context of eosinophilic lung inflammation and Ig production in the same protocol as our previous studies (Takano et al., 1997; Inoue et al., 2005; Yanagisawa et al., 2006). The reason of this different observation from our previous ones remains puzzled. It is possible that the differences in facilitating effects on this condition between PM and latex nanomaterials might be due to those in characteristics of the materials (particles), in particular, on their surface. In support of this concept, we have shown that chemical components extracted from DEP augment this model in the same protocol as the present study (Hiyoshi et al., 2005; Yanagisawa et al., 2006; Inoue et al., 2007). Alternate, the high absorption activity of PM, which is a characteristic feature of carboneous materials might be important for the deterioration of allergic lung inflammation.

On the other hand, in the present study, the latex nanomaterials apparently exacerbated neutrophilic lung inflammation also related to allergen in a size dependent-manner. Besides, they clearly amplified lung expression of KC, a potent chemotactic factor for neutrophils, and MIP-1α, also chemotactic factors for various leukocytes, in paralleled with the neutrophilic lung inflammation. These results may suggest that latex nanomaterials mainly induce/enhance neutrophilic inflammation in vivo. To date, neutrophilic asthma has been recognized to be a distinct phenotype of asthma (Gibson et al., 2001; Douwes et al., 2002; Simpson et al., 2006; Macdowell and Peters, 2007; Simpson et al., 2008). Thus, latex nanomaterials may relate to development/exacerbation of this type of asthma. The observation that lung expression level of IL-18 was paralleled to neutrophilic lung inflammation in the present study should support the concept, since IL-18 reportedly activates neutrophils in vitro (Leung et al., 2001) and induces neutrophil-dominant allergic airway inflammation in vivo (Sugimoto et al., 2004).

In conclusion, this study demonstrated that pulmonary exposure to latex nanomaterials enhanced lung inflammation induced by LPS. The enhancement was mediated through the increased local expression of proinflammatory cytokines and chemokines. The enhancing effects were more prominent with smaller nanomaterials than with larger ones in overall trend. Smaller nanomaterials also enhanced systemic inflammation and coagulatory disturbance accompanying the lung inflammation. Conversely, the nanomaterials did not exacerbate eosinophilic lung inflammation but did neutrophilic lung inflammation related to allergen. These results suggest that nanomaterials can differentially facilitate two types of lung inflammation (innate- or adaptive immunity-dominant). These effects of nanomaterials should

shed light on the understanding of nanotoxicity, especially for sensitive populations with lung inflammation.

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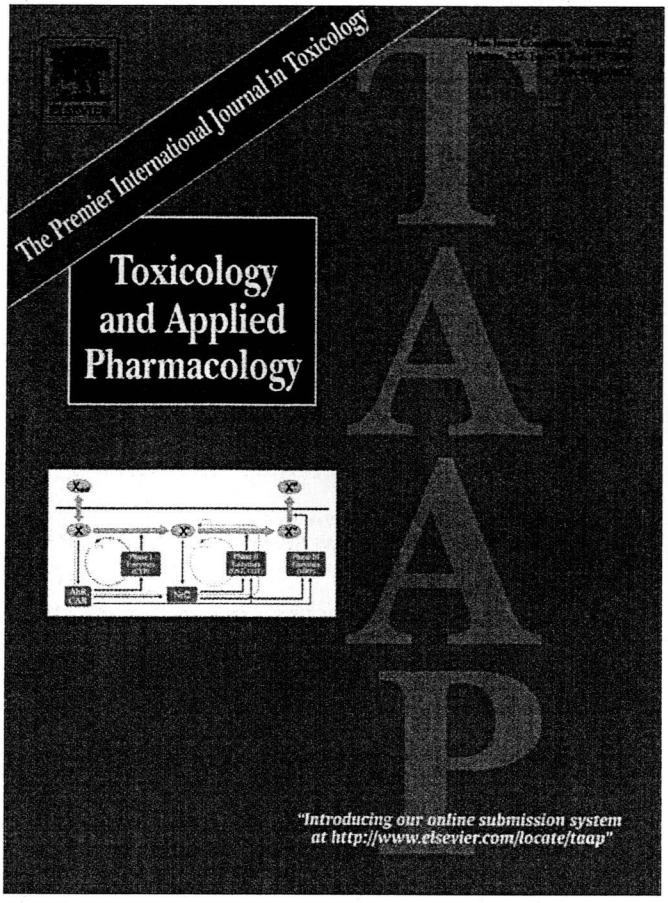

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Effects of multi-walled carbon nanotubes on a murine allergic airway inflammation model

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ABSTRACT

The development of nanotechnology has increased the risk of exposure to types of particles other than combustion-derived particles in the environment, namely, industrial nanomaterials. On the other hand, patients with bronchial asthma are sensitive to inhaled substances including particulate matters. This study examined the effects of pulmonary exposure to a type of nano-sized carbon nanotube (multi-walled nanotubes: MWCNT) on allergic airway inflammation *in vivo* and their cellular mechanisms *in vitro*. *In vivo*, ICR mice were divided into 4 experimental groups. Vehicle, MWCNT (50 μg/animal), ovalbumin (OVA), and OVA + MWCNT were repeatedly administered intratracheally. Bronchoalveolar lavage (BAL) cellularity, lung histology, levels of cytokines related to allergic inflammation in lung homogenates/BAL fluids (BALFs), and serum immunoglobulin levels were studied. Also, we evaluated the impact of MWCNT (0.1–1 μg/ml) on the phenotype and function of bone marrow-derived dendritic cells (DC) *in vitro*. MWCNT aggravated allergen-induced airway inflammation characterized by the infiltration of eosinophils, neutrophils, and mononuclear cells in the lung, and an increase in the number of goblet cells in the bronchial epithelium. MWCNT with allergen amplified lung protein levels of Th cytokines and chemokines compared with allergen alone. MWCNT exhibited adjuvant activity for allergen-specific IgG_1 and IgE. MWCNT significantly increased allergen (OVA)-specific syngeneic T-cell proliferation, particularly at a lower concentration *in vitro*. Taken together, MWCNT can exacerbate murine allergic airway inflammation, at least partly, via the promotion of a Th-dominant milieu. In addition, the exacerbation may be partly through the inappropriate activation of antigen-presenting cells including DC.

Introduction

Bronchial asthma is recognized as a chronic type of airway inflammation with an associated hyperresponsiveness. Intensive research has accumulated evidence regarding the molecular mechanisms for therapeutic targets against the pathology; in contrast, relatively fewer investigations on the (environmental) exacerbating factors have been conducted. However, the latter type of research should be focused on, since morbidity due to bronchial asthma has markedly increased over the past few decades and the large environmental changes occurring throughout the world may be a causal factor (Umetsu et al., 2002; Matsui et al., 2008).

Atmospheric inhalable particulate matter (PM) is one of the important environmental factors leading to the aggravation of asthma and an increase in patient numbers (Leikauf, 2002). In particular, during PM-polluting events, asthmatic subjects reportedly exhibit increases in respiratory symptoms, bronchoconstriction, medication use, bronchial hyperreactivity, and emergency care visits (Schwartz et al., 1993; Boezen et al., 1998). Compatible with epidemiological studies, we and others have experimentally demonstrated that exposure to diesel exhaust or diesel exhaust particles (DEP; representative constituents in $PM_{2.5}$) exacerbate allergic asthma *in vivo* (Takano et al., 1997, 1998; Hao et al., 2003; Dong et al., 2005; Matsumoto et al., 2006). Extensive efforts are being made by several governments to reduce atmospheric PM in the environment throughout the world.

To date, in contrast, the development of nanotechnology has increased the exposure risk to other types of particles in addition to combustion-derived ones, namely, engineered nanomaterials (Oberdorster et al., 2005). As these materials have become more widespread, many questions have arisen regarding the effects they may have on the environment. In fact, previous reports have shown that the full, or even partial, impact of manufactured nanomaterials on health and the environment has yet to be explored in depth (Borm, 2002; Colvin, 2003; Guzman et al., 2006; Nel et al., 2006). It has been reported that nanomaterial exposure itself induces lung inflammation

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(Warheit et al., 2004, 2006; Shvedova et al., 2005; Chen et al., 2006). We previously demonstrated that industrial carboneous particles (carbon black [CB] nanoparticles) enhance allergic asthma hallmarks (Inoue et al., 2005, 2006c, 2007b), suggesting that these inhalable nano-level materials/particles may also promote the pathophysiology. However, the facilitating effects of nanomaterials on subjects with predisposing inflammatory disorders including asthma have not been sufficiently examined.

Among these nanomaterials, carbon nanotubes (CNT) are unique. CNT possess significant characteristics in size (1–20 nm width, and many microns in length), strength, and surface chemistry (Mitchell et al., 2007). Notably, their length/width (aspect) ratios of >1000, reactive surface chemistry, and/or poor solubility raise concerns linked to previous experiences involving hazardous fibers, including asbestos. CNT are being applied in wide-ranging areas from the semiconductor industry to drug delivery and super-light and strong composite materials (de Jonge and Bonard, 2004), and, simultaneously, levels are implicated to be increasing, in particular in the indoor environment (Murr et al., 2005). Furthermore, there is *in vivo* data implicating CNT in the induction of inflammatory lung disorders (Muller et al., 2005; Shvedova et al., 2005).

In this study, we examined the effects of multi-walled CNT (MWCNT), a type of CNT, on allergic airway inflammation *in vivo* and the underlying cellular mechanisms in the context of the maturation/activation/function of antigen-presenting cells (APC) and the allergen-specific T-cell response (using *ex vivo*-generated murine dendritic cells [DC] *in vitro*).

Materials and methods

Animals. Male ICR mice 6–7-wk-of-age (weighing 29 to 33 g: Japan Clea Co., Tokyo, Japan) were used for the *in vivo* studies; mice 11–15-wk-of-age (38–42 g) were used for *in vitro* studies. Mice were housed in an animal facility maintained at 24 to 26 °C with 55 to 75% humidity and a 12-h light/dark cycle, and fed a commercial diet (Japan Clea Co.) and given water ad libitum.

MWCNT. Two types of MWCNT, purchased from Bussan Nanotech Research (Ibaraki, Japan) and SES Research (TX), respectively, were used in this study. We previously reported the characterization of MWCNT (XNRIWMVT-7, Lot# 05072001K28) prepared by a catalytic chemical vapor deposition method (Kim et al., 2005) from Bussan Nanotech Research (Hirano et al., 2008). In brief, the nominal characteristics of the MWCNT were as follows: average diameter, 67 nm; surface area, 26 m^2/g; carbon purity, 99.79 wt.%; fiber length, not specified. The precise length distribution of the MWCNT varies from 3 to approximately 30 μm when the fiber suspension was filtered through a Nuclepore® filter with a pore size of 3 μm. According to the manufacturer's disclosing information (http://www.sesres.com/index.asp), MWCNT from SES Research were formed in the arc process and ranged from 2–20 nm in diameter, and 100 nm to several microns long, with 5–20 graphitic layers. The MWCNT contain 40–50% nanotubes with the remaining material consisting of Amorphous carbon and other carbon nano-particles. The catalytic iron is entrapped within the CNT structure and does not leach out under neutral pH conditions. The amount of metallic impurities of both types of MWCNT was analyzed using nitric acid dissolution and inductively coupled plasma-atomic emission spectrometry (ICP-AES), performed according to the method described previously (Okamoto, 1987) and shown in Table 1. Both MWCNT were autoclaved at 250 °C for 2 h before use, and the suspension was sonicated for 3 min using an Ultrasonic Disrupter (UD-201; Tomy Seiko, Tokyo, Japan) immediately before exposure. LPS activity in the autoclaved MWCNT, determined by the Limulus Amebocyte Lysate assay (Seikagaku-kogyo, Tokyo, Japan), was lower than the assay's detection limit (0.001 EU/mg CNT). It has been demonstrated that well-dispersed CNT are less toxic than agglomerated or pelleted CNT in a mesothelioma cell line (Wick et al., 2007); thus, the degree of agglomeration is critical in evaluation of the adverse effects of CNT. Serum (Lam et al., 2004) or Tween 80 (Warheit et al., 2004) has been used to disperse CNT for *in vivo* studies, and polyoxyethlene sorbitan monooleate (PS80 or Tween 80) (Wick et al., 2007) or Pluronic surfactant (Cherukuri et al., 2004) may be proper dispersing media for *in vitro* studies. Therefore, we used Tween 80 for dispersing these MWCNT. Nonetheless, CNT occur, at least in part, in agglomerated forms due to van der Waals forces, no matter what concentration of detergent is used.

Table 1
Multi-walled nanotubes (MWCNT) characterization.

Element	ng/mg CNT	
	Bussan Nanotech Research	SES Research
Al	<300	<400
As	(20)	ND
Cd	(10)	ND
Co	ND	(10)
Cr	19.5	ND
Cu	(10)	19.2
Fe	3633	146
K	ND	ND
Mg	20.1	26.2
Mn	(10)	ND
Mo	ND	ND
Ni	(20)	(20)
Pb	65.7	(100)
Ti	12.5	27.0
V	(10)	ND
Y	ND	ND
Zn	32.3	284

ND: Undetectable value.
( ): Semi quantitative value.

In vivo studies

Study protocol. Mice were divided into 4 experimental groups (Fig. 1). The vehicle group received phosphate-buffered saline (PBS) at pH 7.4 (Invitrogen Co., Carlsbad, CA) containing 0.05% Tween 80 (Nacalai Tesque, Kyoto, Japan) once a week for 6 wk. The OVA group received 1 μg of OVA (Grade IV: Sigma Chemical, St. Louis, MO) dissolved in the same vehicle every 2 wk and only PBS containing 0.05% Tween 80 every another 2 wk for totally 6 wk (Fig. 1). The MWCNT group received (25 or) 50 μg of MWCNT suspended in the same vehicle every week for 6 wk. The OVA + MWCNT group received combined treatment using the same protocol as for the OVA and MWCNT groups ($n = 12$–13 in each group).

Vehicle, MWCNT, OVA, or OVA + MWCNT were suspended in 0.1-ml aliquots, and inoculations were delivered by the intratracheal route through a polyethylene tube, under anesthesia with 4% halothane (Takeda Chemical Industries, Osaka, Japan). We previously examined the effects of diesel exhaust-derived particles or carbon nanoparticles on allergic airway inflammation at a dose of 50 or 100 μg/animal *in vivo* (Inoue et al., 2005, 2006c; Yanagisawa et al., 2006). Based on (and referred to) the previous studies from our laboratory, we chose the dosage of 25 and 50 μg/body of MWCNT.

The animals were euthanized and studied 24 h after the final intratracheal administration, with bronchoalveolar lavage (BAL) cellularity, lung histology, protein levels of cytokines and chemokines in the lung tissue supernatants, and systemic Ig production then being analyzed. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals according to the Institutional Animal Care and Use Committee (IACUC: www.iacuc.org). All animal studies were approved by the Institutional Review Board.

BAL process. BAL and cell counts were conducted as previously described ($n = 8$ for each group for each experiment) (Takano et al.,

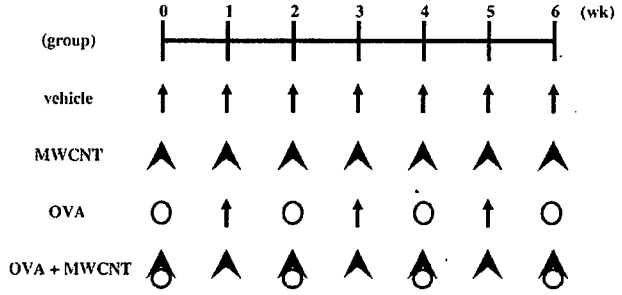

Fig. 1. Protocol for *in vivo* study. MWCNT: multi-walled carbon nanotubes, OVA: ovalbumin.

2002; Inoue et al., 2006a, 2006b, 2006c; Yanagisawa et al., 2006). After the BAL procedure, the lungs were removed, snap-frozen in liquid nitrogen, and stored at −80 °C until assays of cytokines and chemokines. On the other hand, all the BAL fluid (BALF) supernatants were then analyzed for the (cytokine) protein assay ($n=8$ in each group).

Histologic evaluation. After exsanguinations, the lungs were fixed and all the sections (at 4 μm) were prepared. For histologic studies, all the sections were then stained with hematoxylin and eosin or periodic acid-Schiff (PAS), as previously described (Takano et al., 1997, 2002; Inoue et al., 2009a).

Morphometric analysis of the numbers of polymorphonuclear leukocytes (PMNs), mononuclear cells (MONs), and goblet cells around the airways. All the sections of the lungs were stained with Diff-Quik to quantify the numbers of infiltrated PMNs. The length of the basement membrane of airways was measured using a videomicrometer (Olympus, Tokyo, Japan) in each sample slide. The number of PMNs and MONs around the airways was counted with a micrometer under oil immersion. The results are expressed as the number of inflammatory cells per millimeter of basement membrane, as described previously (Takano et al., 1997; Inoue et al., 2005).

To quantify goblet cells, all the sections of the lungs were stained with PAS. The number of goblet cells in the bronchial epithelium was counted by employing a micrometer. The results are expressed as the number of goblet cells per millimeter of basement membrane, as described previously (Inoue et al., 2005).

Quantitation of cytokine and chemokine protein levels in the lung tissue and BALF supernatants. The frozen lungs (after lavaging) were homogenized as described previously (Inoue et al., 2006a and 2006b). ELISAs for tumor necrosis factor (TNF)-α (Endogen, Cambridge, MA), interleukin (IL)-1β (R&D Systems, Minneapolis, MN), IL-4 (Amersham, Buckinghamshire, UK), IL-5 (Endogen), IL-6 (Invitrogen Co.), IL-13 (R&D Systems), IFN-γ (Endogen), IL-18 (MBL, Nagoya, Japan), IL-33 (R&D Systems), eotaxin (R&D Systems), thymus and activation-regulated chemokine (TARC: R&D Systems), macrophage-derived chemokine (MDC: R&D Systems), macrophage chemoattractant protein (MCP)-1 (R&D Systems), and keratinocyte-derived chemoattractant (KC: R&D Systems) in the lung tissue homogenates/BALFs were conducted according to the manufacturer's instructions. All values are expressed in pg/total lung supernatants ($n=8$ in each group for each protocol).

Allergen-specific Ig determination. Allergen (OVA)-specific IgG_1 or IgE antibodies were measured by ELISA using sera obtained from each mouse. OVA-specific IgG_1 were measured according to the methods employed in our previous reports (Inoue et al., 2005; Yanagisawa et al., 2006) ($n=12$–13 in each group). OVA-specific IgE were measured using an ELISA kit (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) according to the manufacturer's instructions ($n=12$–13 in each group).

In vitro studies

Preparation and generation of bone marrow-derived dendritic cells (BMDC). The preparation of bone marrow cells from naïve ICR mice was conducted as previously described (Koike et al., 2008a and 2008c). BMDC were differentiated using a modified protocol of Lutz et al. (1999), as previously conducted (Koike et al., 2008c).

Exposure to MWCNT. On Day 8 of their culturing, the immature BMDC (as stimulators of the lymphocyte reaction) were treated with 50 μg/ml mitomycin C (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) for 20 min in a water bath at 37 °C before exposure to MWCNT, as previously described (Koike et al., 2008a and 2008c). The cells were then washed three times with R10 (RPMI 1640 medium [GIBCO BRL, Eggenstein, Germany] supplemented with 10% heat-inactivated fetal bovine serum [FBS; Dainippon Sumitomo Pharma Co., Ltd.], 100 U/ml penicillin, 100 μg/ml streptomycin [Sigma Chemical], and 50 μM 2-mercaptoethanol [GIBCO BRL]). Cells (2.4×10^6) were then placed in 6-well plates, and exposed to different concentrations of MWCNT (0.1–1 μg) in vehicle (2.4 ml of R10 medium, containing 10 ng/ml recombinant mouse granulocyte macrophage-colony stimulating factor [GM-CSF; Sigma Chemical] plus 0.05% Tween 80) or vehicle alone (control) for 24 h at 37 °C in an atmosphere of 5% CO_2/95% air.

FACS analysis. For FACS analysis, the following monoclonal antibodies were used: MHC class II molecules: I-A/I-E (2G9, FITC-conjugated, BD Biosciences Pharmingen, CA); co-stimulatory molecules: CD80 (16-10A1, PE-conjugated, BD Biosciences Pharmingen), CD86 (GL1, PE-conjugated, BD Biosciences Pharmingen), DC markers: CD11c (HL3, PE-conjugated, BD Biosciences Pharmingen), DEC-205 (NLDC-145, PE-conjugated, Cedarlane Labs, Ontario, Canada). Immunostaining was performed as previously described (Koike et al., 2008a and 2008b), and flow cytometry was performed using a FACSCalibur (Becton, Dickinson and Company, NJ). Fluorescence data are expressed as the percentage of positive cells. This experiment was repeated three times using three animals in each experiment.

Preparation of allergen-sensitized T-cells and antigen-specific lymphocyte reaction. OVA-sensitized T-cells were derived from a pool of spleens from OVA-sensitized syngeneic (ICR) mice, as previously described (Koike et al., 2008a and 2008c). Thereafter, OVA-specific T-cells (2×10^5) were co-cultured with BMDC (5×10^3) in the presence of OVA (40 μg) in 96-well flat-bottom plates for 4 days, and T-cell proliferation was then measured using a Cell-Proliferation-ELISA Kit (Roche Molecular Biochemicals, Mannheim, Germany), as previously described (Koike et al., 2008c). This experiment was repeated twice using three animals in each experiment.

Statistical analysis. Data are reported as the mean ± SE. Differences between experimental groups were determined using analysis of variance (Stat view version 4.0; Abacus Concepts, Inc., Berkeley, CA) followed by the post-hoc test (Fisher's protected least significant difference test). Significance was assigned to *P*-values smaller than 0.05.

Results

Effects of MWCNT (from Bussan Nanotech Research) on lung immune cell profiles

We investigated the cellular profile of BALF and showed representative data (50 μg/animal of MWCNT; Fig. 2). The number of total cells was significantly greater in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.05$

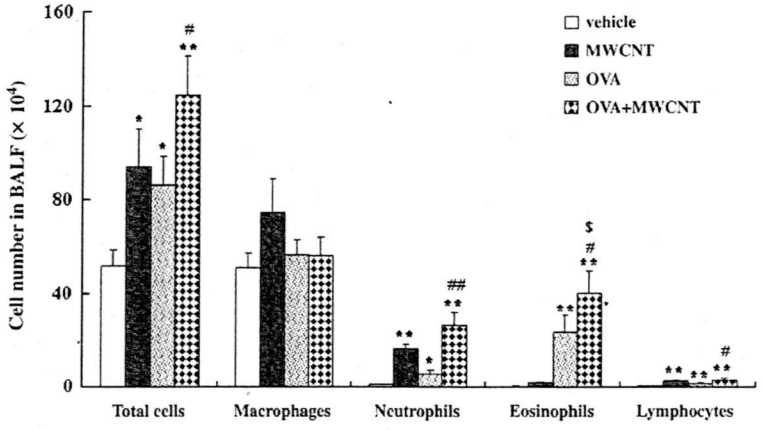

Fig. 2. Cellularity in bronchoalveolar lavage fluid (BALF) after intratracheal challenge. Twenty-four hours after the final intratracheal administration of the vehicle, multi-walled carbon nanotubes (MWCNT [50 μg/treatment]), ovalbumin (OVA), or OVA + MWCNT over a period of 6 wk, lungs were lavaged for the analysis of BALF. Total cell counts were determined in fresh fluid specimens using a hemocytometer. Differential cell counts were assessed in cytologic preparations stained with Diff-Quik. Results are means ± SE ($n = 8$ in each group). $^{*}P<0.05$ vs. vehicle group, $^{**}P<0.01$ vs. vehicle group, $^{\#}P<0.05$ vs. OVA group, $^{\#\#}P<0.01$ vs. OVA group, $^{\$}P<0.01$ vs. MWCNT group.

for MWCNT and OVA, $P<0.01$ for OVA + MWCNT). The number was greater in the OVA + MWCNT group than in the MWCNT group (N. S.) or OVA group ($P<0.05$).

Exposure to OVA or OVA + MWCNT significantly increased the number of eosinophils as compared with vehicle exposure ($P<0.01$). The number was significantly greater in the OVA + MWCNT group than in the MWCNT group ($P<0.01$) or OVA group ($P<0.05$). The number of lymphocytes was greater in the MWCNT group, OVA group or OVA + MWCNT group than in the vehicle group ($P<0.01$). The number was greater in the OVA + MWCNT group than in the OVA group ($P<0.05$) or the MWCNT group (N. S.). The number of neutrophils was greater in the MWCNT group ($P<0.01$), OVA group ($P<0.05$), or OVA + MWCNT group ($P<0.01$) than in the vehicle group. The number was higher in the OVA + MWCNT group than in the OVA group ($P<0.01$) or MWCNT group (N. S.). The number of macrophages did not significantly differ among the experimental groups. In experiments using 25 μg/animal of MWCNT, the data are similar to those using 50 μg/animal, although the level of significance was not marked except for eosinophil number ($P<0.01$, OVA + MWCNT vs. OVA or MWCNT) (data not shown).

Effects of MWCNT on allergen-related histological changes in the lung

We evaluated lung specimens stained with hematoxylin and eosin 24 h after the final intratracheal instillation (Fig. 3). No pathological change was seen in lungs obtained from the vehicle group. The infiltration of neutrophils was moderately increased in the lungs of hosts in the MWCNT group or OVA group. On the other hand, the infiltration of eosinophils was moderate in lungs from the OVA group. Combined treatment with OVA + MWCNT appeared to worsen PMN (mainly neutrophils and eosinophils) and MON (mainly lymphocytes) sequestration into the lung parenchyma, as compared with OVA treatment alone.

To quantify the infiltration of inflammatory leukocytes around the airways, we expressed the number of these cells per length of basement membrane of the airways. The number of PMNs was greater in the MWCNT group (N. S.), OVA group ($P<0.05$), or OVA + MWCNT group ($P<0.01$) than in the vehicle group. The number was greater also in the OVA + MWCNT group than in the OVA group or MWCNT group ($P<0.01$). The number of MONs was larger in the MWCNT group, OVA group ($P<0.05$), or OVA + MWCNT group ($P<0.01$) than in the vehicle group. The number was also higher in the OVA + MWCNT group than in the MWCNT group ($P<0.05$) or OVA group (N. S.).

MWCNT potentiates goblet cell hyperplasia

To evaluate airway epithelial injury and the hypersecretion of mucus, lung sections were stained with PAS (Fig. 4). MWCNT or OVA alone significantly induced goblet cell hyperplasia in the airway as compared with the vehicle. However, the phenomenon had markedly progressed in the OVA + MWCNT group compared to the other groups. Semi-quantitative analyses also showed that MWCNT or OVA alone significantly increased the number of goblet cells as compared with the vehicle ($P<0.01$). The number was significantly greater in the OVA + MWCNT group than in the vehicle group ($P<0.01$), OVA group ($P<0.05$), or MWCNT group (N. S.).

Impact of MWCNT on local expressions of cytokines and chemokines in the presence of allergen

We quantified protein levels of cytokines such as IL-4, IL-5, IL-13, IFN-γ, IL-18, and IL-33 (Table 2), and chemokines such as eotaxin, TARC, MDC, MCP-1, and KC (Table 3) related to allergic inflammation in the lung tissue homogenates and proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 in the BALFs (Table 4). As for cytokine and chemokine profiles in the lung tissue homogenates, OVA ($P<0.05$) or OVA + MWCNT ($P<0.01$) exposure increased the level of IL-4 as compared with vehicle challenge. The level was greater in the OVA + MWCNT group than in the OVA group or MWCNT group ($P<0.01$). The levels of IL-5 and IL-13 were significantly greater in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.01$). The levels were further higher in the OVA + MWCNT group than in the OVA group (N. S.) or MWCNT group ($P<0.01$). The level of IFN-γ was significantly greater in the MWCNT group or OVA + MWCNT group than in the vehicle group ($P<0.01$). Although the level was significantly larger in the OVA + MWCNT group than in the OVA group ($P<0.01$), it tended to be lower in the OVA + MWCNT group than in the MWCNT group (N. S.). The level of IL-18 was significantly higher in the MWCNT group or OVA + MWCNT group than in the vehicle group ($P<0.01$). The level was greater in the OVA + MWCNT group than in the OVA group ($P<0.01$). The level of IL-33 was significantly greater in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.01$). The level was greater in the OVA + MWCNT group than in the OVA group ($P<0.01$). Eotaxin level was greater in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.01$).

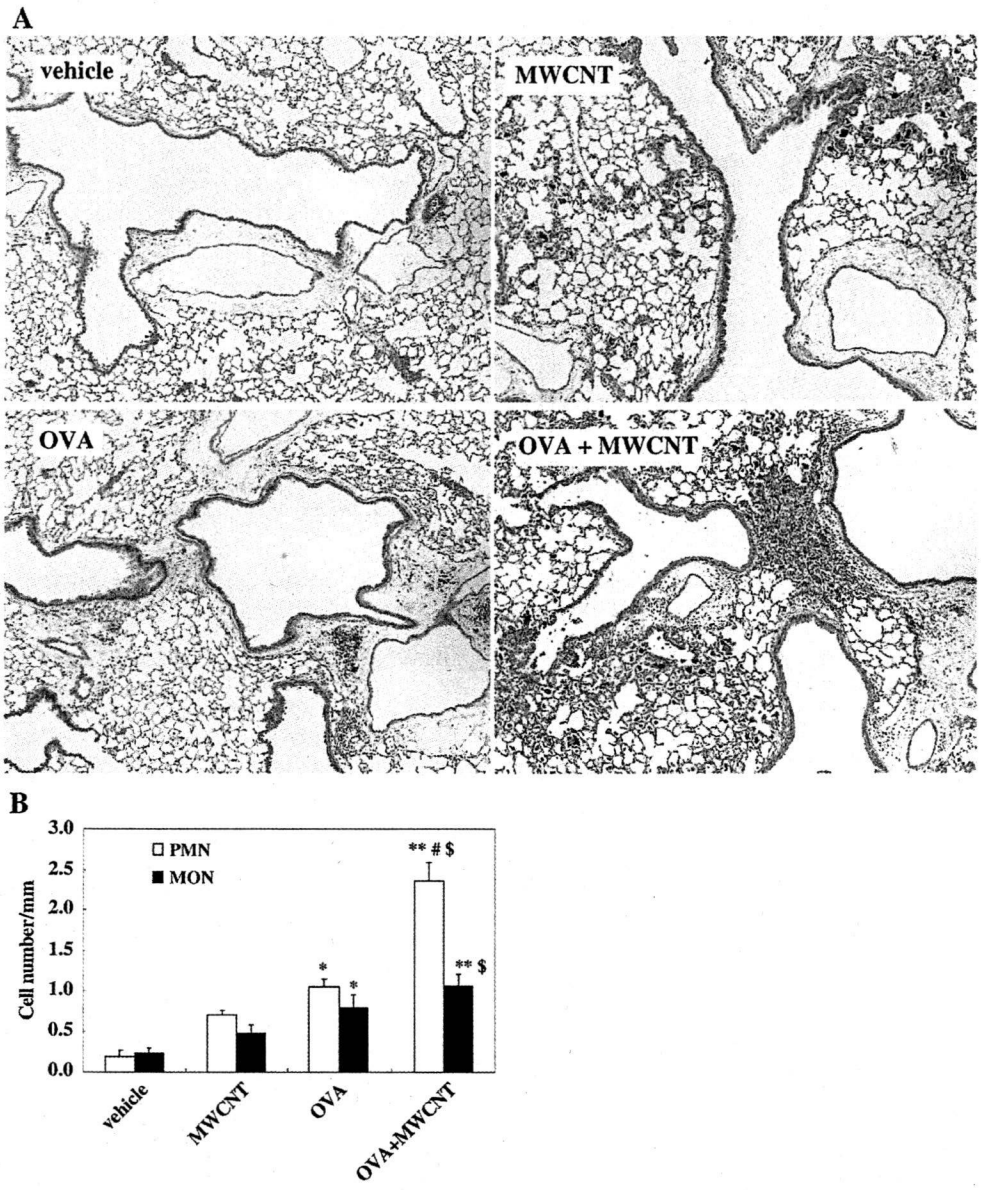

Fig. 3. Representative histological findings of hematoxylin and eosin-stained lungs obtained from the vehicle, MWCNT (50 μg/treatment), OVA, or OVA + MWCNT groups 24 h after the final intratracheal administration (A), and semi-quantitative analysis of polymorphonuclear leukocyte (PMN) and mononuclear cell (MON) sequestration into the lung (B; $n=4$–5 in each group). Animals received intratracheal instillation of vehicle, MWCNT, OVA, or OVA + MWCNT over a period of 6 wk. Lungs were removed and fixed 24 h after the last intratracheal administration. Original magnification × 100. PMN and MON infiltration was assessed by averaging the number of PMNs and MONs per millimeter of basement membrane. Values are the mean ± SE in each group. $^{*}P<0.05$ vs. vehicle group, $^{**}P<0.01$ vs. vehicle group, $^{\#}P<0.05$ vs. OVA group, $^{\$}P<0.05$ vs. MWCNT group.

The level was further greater in the OVA + MWCNT group than in the OVA group (N. S.) or MWCNT group ($P<0.01$). The levels of TARC, MDC, MCP-1, and KC were higher in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.01$ except for KC vs. OVA [$P<0.05$]). The level was greater in the OVA + MWCNT group than in the OVA group or MWCNT group ($P<0.01$ except for MDC vs. MWCNT [$P<0.05$]).

IL-1β and IL-6 levels (Table 4) in the BALFs were greater in the MWCNT group ($P<0.05$) or OVA + MWCNT group ($P<0.01$) than in the vehicle group. The levels were further significantly greater in the OVA + MWCNT group than in the MWCNT group ($P<0.05$) or OVA group ($P<0.01$). TNF-α level was not different among the experimental groups (data not shown).

Effects of MWCNT on allergen-specific production of Igs

We measured allergen-specific IgG_1 and IgE levels (Fig. 5). These levels were significantly greater in the OVA group or OVA + MWCNT group than in the vehicle group ($P<0.01$). The titers were further greater in the OVA + MWCNT group than in the OVA group ($P<0.01$ for IgG_1 and $P=0.07$ for IgE) or MWCNT group ($P<0.01$).

Action of MWCNT on the expression of cell surface molecules in BMDC

Immature BMDC were exposed to MWCNT for 24 h. The expression of MHC Class II, CD86, CD80, CD11c, and DEC205 (Fig.