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Inhaled Multiwalled Carbon Nanotubes Modulate the Immune Response of Trimellitic Anhydride–induced Chemical Respiratory Allergy in Brown Norway Rats

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ABSTRACT

The interaction between exposure to nanomaterials and existing inflammatory conditions has not been fully established. Multiwalled carbon nanotubes (MWCNT; Nanocyl NC 7000 CAS no. 7782-42-5; count median diameter in atmosphere 61 ± 5 nm) were tested by inhalation in high Immunoglobulin E (IgE)-responding Brown Norway (BN) rats with trimellitic anhydride (TMA)-induced respiratory allergy. The rats were exposed 2 days/week over a 3.5-week period to a low (11 mg/m^3) or a high (22 mg/m^3) concentration of MWCNT. Nonallergic animals exposed to MWCNT and unexposed allergic and nonallergic rats served as controls. At the end of the exposure period, the allergic animals were rechallenged with TMA. Histopathological examination of the respiratory tract showed agglomerated/aggregated MWCNT in the lungs and in the lung-draining lymph nodes. Frustrated phagocytosis was observed as incomplete uptake of MWCNT by the alveolar macrophages and clustering of cells around MWCNT. Large MWCNT agglomerates/aggregates were found in granulomas in the allergic rats, suggesting decreased macrophage clearance in allergic rats. In allergic rats, MWCNT exposure decreased serum IgE levels and the number of lymphocytes in bronchoalveolar lavage. In conclusion, MWCNT did not aggravate the acute allergic reaction but modulated the allergy-associated immune response.

Keywords: MWCNT; nanomaterials; inhalation; asthma; TMA; Brown Norway rats; respiratory allergy.

INTRODUCTION

Carbon nanotubes are nanoparticles with specific health concerns (Holsapple et al. 2005; Ma-Hock et al. 2007; Liu et al. 2008; Shvedova et al. 2008; Yang et al. 2008; Savolainen et al. 2010; Aschberger et al. 2011; Donaldson et al. 2011; Sharifi et al. 2012). Nanotubes are manufactured but are also spontaneously formed in combustion processes and are present in the

outdoor air particulate matter (Murr et al. 2004; Lagally et al. 2012). Carbon nanotubes can cause pulmonary inflammation and can decrease pulmonary function upon inhalation. Most of the research on their toxicity has been done in healthy animals.

A number of studies used compromised animal models including asthma models, based on the observation that individuals with lung diseases such as asthma are hypersusceptible to exposure to environmental particles (McCreanor et al. 2007; Rueckerl et al. 2011; Knol et al. 2009; Iskander et al. 2012). Multiwalled and single-walled carbon nanotubes (MWCNT and SWCNT, respectively) aggravated airway inflammation induced by the respiratory allergen ovalbumin (OVA) in imprinting control region (ICR) mice, when instilled in the trachea together with OVA (Inoue 2011, 2009; Li et al. 2010). Aggravation of the allergic response against OVA was also shown in BALB/c mice upon intranasal application of MWCNT during the induction phase of the respiratory allergy (Nygaard et al. 2009). The causal relationship between the exposure and the interference with respiratory allergy is not clear; it may depend on induction of inflammatory cytokines, stimulation of mucus production, activation of bone marrow–derived inflammatory cell precursors, and enhanced oxidative stress.

The effects of nanotubes on OVA-induced respiratory allergy were studied in mice strains with a tendency to respond with the generation of high levels of serum IgE antibodies. This tendency is genetically determined and comparable with an atopic

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Abbreviations: AIM, aerosol instrument manager; ALP, alkaline phosphatase; ANOVA, analysis of variance; APS, aerodynamic particle sizer; BALF, bronchoalveolar lavage fluid; BN, Brown Norway; CMD, count median diameter; CPC, condensation particle counter; DMA, differential mobility analyzer; EDTA, Ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; GGT, γ -glutamyl transferase; GSD, geometric standard deviation; LDH, lactate dehydrogenase; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MMAD, mass median aerodynamic diameters; MWCNT, multiwalled carbon nanotubes; NC, nanocyl; OVA, ovalbumin; SEM, scanning electron microscopy; SLEW, super light element window; SMPS, scanning mobility particle sizer; SWCNT, single-walled carbon nanotubes; Th2, helper-2; TMA, trimellitic anhydride; WD, working distance.

TABLE 1.—Exposure schedule.

Groups (number of animals)	Sensitization days 0 and 7	Challenge day 22	Exposure days (7 days in period day 23–42)	Rechallenge day 43	Necropsy day 44
TMA/low MWCNT (<i>n</i> = 6)	TMA	TMA	MWCNT low concentration	TMA	Bronchoalveolar lavage (BAL) right lung lobes Body weight
TMA/high MWCNT (<i>n</i> = 6)	TMA	TMA	MWCNT high concentration	TMA	Comet assay BAL and lung cells Breathing parameters during challenge
Air control (<i>n</i> = 9)	Vehicle	Air	Air	Air	Organ weight: left lung, liver, kidneys, spleen, heart, draining lymph nodes
TMA control (<i>n</i> = 12)	TMA	TMA	Air	TMA	Histopathology: draining lymph nodes, heart, larynx, liver, left lung, nasal tissues, trachea, Hematology
MWCNT control (<i>n</i> = 6)	Vehicle	Air	MWCNT high concentration	Air	

Note: TMA, trimellitic anhydride; MWCNT, multiwalled carbon nanotubes.

constitution in humans (*Dorlands Illustrated Medical Dictionary* 2012; Ober and Yao 2011; *Fireman Atlas of Allergies and Clinical Immunology* 2006). Atopic individuals are prone to develop asthma and other IgE-mediated inflammatory conditions. Atopic individuals and high IgE-responding animals may react differently to particle exposure in general and nanomaterials in particular than nonatopics, regardless of the presence of allergy. Some of the reported effects of MWCNT in the nonallergic control mice (Inoue et al. 2009) could be related to this particular constitution.

Nanotube-induced effects on chemically induced respiratory allergy have not been studied so far. Yet, co-exposure of nanotubes and chemical allergens may very well occur, especially at the workplace, for example, during the use of nanoparticle-filled coatings. The interaction between nanotube exposure and chemically induced respiratory allergy can be quite different from ovalbumin/protein-induced allergy, because chemical respiratory allergy is less IgE-mediated than protein-induced allergy (Dykewicz 2009). Moreover, chemical respiratory allergy is a complex mixture of both immune-related processes and irritation (Arts et al. 2003).

In the present study, the interaction between repeated (twice a week for 3.5 weeks) inhalation exposure to MWCNT and TMA-induced respiratory allergy (a TMA inhalation challenge before and after the MWCNT exposure period) was examined. Animals were exposed to MWCNT after the sensitization period to study effects on provocation of respiratory allergy. Effects on airways, lung parenchyma, and local lymph nodes were investigated in the Brown Norway (BN) rat, which is even more IgE-driven than the BALB/c and ICR mouse. The inhalation route was chosen over intranasal application or intratracheal instillation, because it is considered a far more realistic exposure route and does not require dispersion of the nanotubes in a medium. MWCNT exposure was expected to aggravate the TMA-induced allergy, and the allergic condition was expected to interfere with the clearance of MWCNT.

MATERIALS AND METHODS

Animals

BN rats were used, since this strain has a preference for Thelper-2 (Th2) type responses to respiratory allergens, including a high-IgE response (Arts et al. 2003). Female BN rats were obtained from a colony maintained under specific pathogen free (SPF) conditions at Charles River (Sulzfeld, Germany). Animals were housed in macrolon cages with a bedding of wood shavings. Food (RM3, from Special Diet Services, Witham, England) and water were supplied *ad libitum*. The temperature in the animal room was $22 \pm 2^\circ\text{C}$ and the relative humidity was 40 to 70%. This study was approved by the Institute's animal ethics committee.

Experimental Design

Animals were sensitized and challenged with TMA (1,2,4-benzenetricarboxylic anhydride; CAS no. 552-30-7; 97% purity, Aldrich, Brussels, Belgium) to induce respiratory allergy (Table 1). The animals were dermally sensitized with TMA on days 0 and 7 (150- μl 50% (w/v) TMA and 75- μl 25% (w/v) TMA, respectively, in a 4:1 acetone/olive oil mixture) on the flanks and the dorsum of the ears, respectively. This was followed by a respiratory challenge with 15-mg/m³ TMA for 15 min on day 22 (Arts and Kuper 2007). Animals were randomly divided into groups for exposure by inhalation to clean air (control group) or MWCNT (Nanocyl[®]-7000, CAS no. 7782-42-5, purity 90% [10% metal oxides, according to the supplier], Nanocyl S.A., Sambreville, Belgium). From day 23 onward, animals were exposed to a low concentration (surface area of about 500 $\mu\text{m}^2/\text{cm}^3$) or a high concentration (surface area of about 1,500 $\mu\text{m}^2/\text{cm}^3$) of MWCNT. The exposure was 5 hr/day, 2 days/week for 3.5 weeks, resulting in 7 exposure days. After a second respiratory challenge with 15 mg/m³ TMA for 15 min on day 43, the animals were necropsied on day 44.

Exposure Procedure

The respiratory challenge to TMA was performed in a four-armed plethysmograph connected to a central exposure chamber for head-nose-only exposure (Institute's design) as

described previously (Arts et al. 1998). Four rats at a time were first exposed to fresh air for about 7 min (prechallenge period for acclimatization) and then to the TMA atmosphere for 15 min (challenge period), followed by a recovery period (exposure to fresh air) of 15 min. Exposure to the MWCNT aerosols was performed in a different head-nose-only exposure (ADG Developments Ltd., Codicote Herts, UK) in which the allergic and nonallergic animals were simultaneously exposed to either the low or the high concentration test atmosphere of MWCNT. The animals were secured in plastic animal holders (Battelle) during exposure.

Generation and Monitoring of the Test Atmosphere

TMA. For the TMA challenges, TMA (dissolved in acetone in a concentration of 9–18 g/L) was nebulized using an ultrasonic nebulizer (Sono-Tek Corporation, Poughkeepsie, NY) and a motor-driven syringe pump (WPI type SP220i, World Precision Instruments, Sarasota, FL). The flow of humidified air to the exposure chamber was monitored by a mass stream meter (Bronkhorst Hi Tec, Ruurlo, The Netherlands) and was between 9 and 16 L/min. The acetone concentration was kept below 3 g/m³, which is considered to be far below a level inducing sensory irritation (Alarie 1973; de Ceaurriz et al. 1981; Schaper and Brost 1991). Due to the short exposure period, TMA concentrations were determined gravimetrically before and/or after challenge using the same exposure conditions and settings but using longer time periods of TMA generation. In addition, the stability of the test atmosphere was monitored by measuring the acetone concentration using a total carbon analyzer with a flame ionization detector (RS55-T, Ratfisch, Poing, Germany). Particle size distribution measurements were also carried out before and after challenge using a 10-stage cascade impactor (Sierra Instruments, Carmel Valley, CA); the mass median aerodynamic diameters (MMAD) and geometric standard deviations (GSDs) were determined (Lee 1972).

MWCNT

Target concentrations were based on surface area and were 500 $\mu\text{m}^2/\text{cm}^3$ for the low concentration and 1,500 $\mu\text{m}^2/\text{cm}^3$ for the high concentration. The test material was delivered as a dry powder to a Fox eductor (Fox Valve Development Corp., Dover, NJ) using a Gericke feeder (Gericke AG, Regensdorf, Switzerland). The output of the eductor was fed to a jet mill (Institute's design) to deagglomerate the MWCNT, after which the test atmosphere entered the exposure chamber. Compressed dry air was supplied to both the eductor and the jet mill. The number of particles in the low concentration test atmosphere (measurement of particle number in the high concentration test atmosphere was not feasible) was recorded by a butanol-based Condensation Particle Counter (CPC, model 3022A, TSI Incorporated, Shoreview, MN) or a water-based Ultrafine Condensation Particle Counter (uCPC, Model 3786, TSI Inc.). Control animals received clean air but were otherwise treated identically.

The temperature and relative humidity of the test atmospheres were measured 3 times a day during particle exposure

and once every session during TMA challenge, using an RH/T device (TESTO 635, TESTO GmbH & Co, Lenzkirch, Germany). The test atmospheres were generated in filtered compressed dry air.

Particle size distribution analysis during MWCNT generation was carried out using a Scanning Mobility Particle Sizer (SMPS) and an Aerodynamic Particle Sizer (APS Model 3321). The SMPS consisted of an Electrostatic Classifier (Model 3080) with a Differential Mobility Analyzer (DMA, Model 3081). A CPC (Model 3022A) was used to count the particle output of the SMPS. To prevent overload of the APS, an aerosol diluter (Model 3302A) was used to dilute the test atmospheres 20 times. Both the SMPS and APS were controlled by the Aerosol Instrument Manager software package (AIM, release version 8.0.0.0). Although the particles are non-spherical, the equivalent CMD of spherical particles with the same air resistance was used to characterize the particle size. All appliances and software were obtained from TSI Incorporated (Shoreview, MN). The surface area of the test material in the atmosphere was measured using a LQ1 Diffusion Charger (LQ1-DC, Matter Engineering AG, Wohlen, Switzerland). The mass was determined gravimetrically. Test atmosphere samples were obtained by passing the atmosphere through fiber glass filters (Schleicher and Schuell, GF10, Ø 47 mm) at a sample flow of approximately 5 L/min.

After weighing, the gravimetric filters, collected during exposure of the high concentration, were used for analysis of elementary and organic carbon levels. To eliminate the presence of trace amounts of carbon, all fiber glass filters were heated at 800°C for approximately 2 hr before being used for gravimetric analysis. Analysis of elementary and organic carbon on the filters was done with a C-mat (Ströhlein C-mat 5500, JUWE Laborgeräte GmbH, Viersen, Germany). For organic carbon measurement, the sample was first heated in an oxygen atmosphere to 550°C and the resulting gases were measured to calculate the amount of organic carbon. For elementary carbon measurements, the samples were subsequently cooled in an oxygen atmosphere and were then heated again to 900°C and the resulting gases were measured to calculate the amount of elementary carbon.

Clinical Signs and Body Weights

Animals were observed daily in their cage and a groupwise observation was made halfway during each exposure. The body weight of each animal was recorded prior to the start of the study, at day 0 prior to the first treatment, and weekly thereafter. In addition, animals were weighed before sacrifice for calculation of relative organ weights.

Respiratory Measurements

Breathing pattern, respiratory frequency, and tidal volume were monitored every minute for 20 sec/min at 1 day before the first challenge, during, and directly after the challenge, and again 24 hr after the challenge with 15-mg/m³ TMA (a nonirritating concentration; Arts et al. 2001).

Ventilatory flow was calculated from breathing frequency and tidal volume.

Hematology

At necropsy, blood was sampled from the abdominal aorta of all animals while under pentobarbital anesthesia. Ethylenediaminetetraacetic acid (EDTA) was used as an anticoagulant. In each sample hemoglobin, cell volume, red blood cell count, reticulocytes, white blood cell count, differential white blood cell count (manually determined), and thrombocyte count were automatically measured with an Advia 120 hematology analyzer (Bayer HealthCare, Ireland). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated.

Serum IgE Concentrations

Individual serum samples were prepared from blood withdrawn prior to sensitization and prior to the first challenge via the orbital plexus, and at necropsy via the abdominal aorta. Total serum immunoglobulin (Ig)E levels were measured by an enzyme-linked immunosorbent assay (ELISA, Bethyl Laboratories, Inc., Montgomery). Serum was diluted 200 times initially for the assay. The HRP conjugate was 1,000 times diluted. The IgE levels in serum are reported as relative OD values.

Bronchoalveolar Lavage

The right lung lobes were lavaged 2 times with a volume of 27-ml saline per kg body weight. The total amount of retracted lavage fluid was weighed, and total protein, lactate dehydrogenase (LDH), γ -glutamyl transferase (GGT), alkaline phosphatase (ALP), and *N*-acetyl glucosaminidase (NAG) were determined with an automatic analyzer (Hitachi 911, Hitachi Instruments Division, Japan). Total and differential cell numbers were measured, and cell viability was determined (acridine orange/ethidium bromide staining method).

Pathology

Organs were weighed and preserved in a neutral aqueous phosphate-buffered 4% solution of formaldehyde (Table 1). The left lung was infused with the fixative under ca. 15-cm water pressure. Preserved tissues were embedded in paraffin wax and 5- μ m sections were stained with hematoxylin and eosin for histopathological assessment. In addition, sections were stained with hematoxylin only to facilitate observation of the presence of MWCNT. The nasal tissues were examined at 6 levels, the larynx at 3 levels, the trachea at 3 levels (including the bifurcation), and the left lung lobe at 1 level across the main bronchi.

Scanning electron microscopy (SEM) was performed on deparaffinized, unstained 5- μ m lung sections, placed on carbon planchets. The sections were coated with gold (Au). Samples were analyzed with high-resolution field emission gun scanning electron microscopy in combination with energy

dispersive X-ray analysis (FEG-SEM/EDX; Tescan MIRA-LMH FEG-SEM). The microscope was operated at an accelerating voltage of 15 kV, working distance (WD) of 10 mm and spot size 5 nm. The EDX spectrometer is a Bruker AXS spectrometer with a Quantax 800 workstation and a XFlash 4010 detector with an active area of 10 mm² and super light element window (SLEW), which allows X-ray detection of elements higher than borium ($Z > 5$). The spectral resolution of the detector is 123 eV (Mn [10 keps] ave FWHM). After particle recognition, an X-ray spectrum was acquired. The software is equipped with a drift correction feature, which ensures that the electron beam will stay on the particle during EDX analysis. The X-ray acquisition time for a single analysis was 20 to 30 sec per particle (live acquisition time). For an EDX-mapping, the acquisition time was 5 min.

Statistics

Hematology parameters, parameters in bronchoalveolar lavage fluid (BALf), and relative lung and lymph node weights were analyzed with a two-way analysis of variance (ANOVA) for each parameter with TMA exposure and MWCNT exposure as factors. If significant factors were found, the Tukey's honestly significant difference (HSD) post hoc test was performed. Those with significant differences with respect to the reference groups—air control, TMA control or MWCNT control—were considered.

Changes in respiratory parameters and IgE levels were evaluated at each postexposure time point and compared with pre-exposure values, using an analysis of variance (two-way ANOVA) in a repeated measures design, using Greenhouse-Geisser correction for the degrees of freedom. Incidences of histopathological changes were evaluated by Fisher's exact probability test.

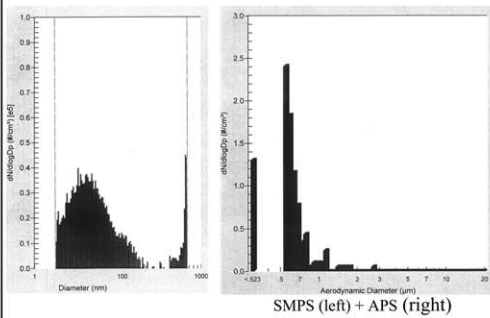
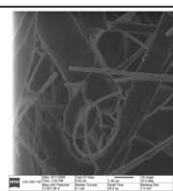
RESULTS

Characteristics of the Test Atmosphere with MWCNT

The characteristics are summarized in Table 2. The average surface area was $559 \pm 120 \mu\text{m}^2/\text{cm}^3$ for the low concentration and $1,394 \pm 426 \mu\text{m}^2/\text{cm}^3$ for the high concentration. The average mass concentrations of MWCNT were $11 \pm 2 \text{ mg}/\text{m}^3$ (low concentration) and $22 \pm 5 \text{ mg}/\text{m}^3$ (high concentration). Average particle number for the low concentration was 1.1×10^4 particles/cm³, the particle number of the high concentration could not be measured due to clogging of the particle counter. The low-concentration MWCNT test atmosphere had an average particle size (count median diameter, CMD) of $53.7 \pm 2.9 \text{ nm}$ with a GSD of 2 ± 0.4 , and at the high concentration an average particle size of 61.4 ± 4.7 with a GSD of 3.3 ± 0.6 .

As expected, most carbon on the filters collected during exposure of the high concentration was elementary carbon and not organic carbon. From the total amount of carbon, the concentration in air was calculated. Carbon analysis of the high concentration test atmospheres showed an average concentration of $19.2 \pm 2.3 \text{ mg}/\text{m}^3$ and for the low concentration test

TABLE 2.— Characteristics of Multiwalled carbon nanotubes (Nanocyl 7000) measured in test atmosphere and according to the supplier.

Characteristics	Low concentration High concentration average $\pm$ SD	Method
Particle mass concentration in test atmosphere	11 $\pm$ 2 22 $\pm$ 5 mg/m ³	Gravimetry
Particle count in test atmosphere	1.1 $\times 10^4$ nm ^A particles/cm ³	CPC
Surface area of test material/test atmosphere volume	559 $\pm$ 120 1394 $\pm$ 426 $\mu\text{m}^2/\text{cm}^3$	LQ1
CMD (count median diameter) in test atmosphere And geometric standard deviation (gsd)	53.7 $\pm$ 2.9 nm with gsd of 2.0 $\pm$ 0.4 61.4 $\pm$ 4.7 nm with gsd of 3.3 $\pm$ 0.6	
Morphology in atmosphere	Mainly agglomerates/ aggregates; also a few single, long and bended nanotubes (figure)	SEM 
Morphology bulk material	Mainly agglomerates/ aggregates	SEM (Figure of aggregates)
Surface area bulk material	250-300 m ² /g	BET (Supplier)
Purity	90% (10% metal oxides)	Supplier
Average diameter	9.5 nm	Supplier
Average length	1.5 μm	Supplier

^A The high concentration of nanotubes interfered with the operation of the CPC and reliable data could not be obtained.

atmospheres this was $8.1 \pm 1.6 \text{ mg/m}^3$. As expected, this is lower than the gravimetrically determined mass concentration, which is caused by the presence of other elements (10% metal oxides according to the supplier).

Clinical Signs, Body and Organ Weight, and Respiratory Measurements

No treatment-related clinical abnormalities were observed, and body weights were not affected. Relative and absolute lung weights and relative draining (mediastinal) lymph node weights were increased in the animals made allergic by

TABLE 3.—Relative organs weights and blood parameters in BN rats exposed to the respiratory allergen trimellitic anhydride and to multiwalled carbon nanotubes.

Group	Blood								
	Relative left lung weight (g/kg body weight $\pm$ SD)	Relative mediastinal lymph node weight (g/kg body weight $\pm$ SD)	Total white blood cells (10 ⁸ /L $\pm$ SD)	Lymphocytes (10 ⁸ /L $\pm$ SD)	Neutrophils (10 ⁸ /L $\pm$ SD)	Eosinophils (10 ⁸ /L $\pm$ SD)	Basophils (10 ⁸ /L $\pm$ SD)	Monocytes (10 ⁸ /L $\pm$ SD)	IgE levels in serum (Mean OD $\pm$ SD)
2-Way ANOVA, Factor TMA	***	***		*	***	***			
2-Way ANOVA, Factor MWCNT						**	***		
Interaction									
TMA/low MWCNT	4.01 $\pm$ 0.41 ^{SSS,&&&}	0.12 $\pm$ 0.04	36.7 $\pm$ 11.0	19.7 $\pm$ 6.2	15.7 $\pm$ 8.9 ^{SS,&}	0.0 $\pm$ 0.0 ^{SSS}	0.0 $\pm$ 0.0 ^{&}	1.3 $\pm$ 0.5	nm ^a
TMA/high MWCNT	4.49 $\pm$ 0.41 ^{SSS,&&&}	0.17 $\pm$ 0.05 ^S	37.2 $\pm$ 5.2	17.8 $\pm$ 3.3	18.0 $\pm$ 3.1 ^{SS}	0.73 $\pm$ 0.34	0.13 $\pm$ 0.08 ^S	0.9 $\pm$ 0.3	day 20: 1.083 $\pm$ 0.359
Air control	2.52 $\pm$ 0.23 ^{###}	0.09 $\pm$ 0.04	38.7 $\pm$ 10.3	28.6 $\pm$ 8.8	8.0 $\pm$ 3.5	0.91 $\pm$ 0.24 [#]	0.01 $\pm$ 0.03 ^{&}	1.0 $\pm$ 0.4	day 44: 0.781 $\pm$ 0.158
TMA control	4.06 $\pm$ 0.50 ^{SSS, &&&}	0.13 $\pm$ 0.07	35.4 $\pm$ 12.2	21.0 $\pm$ 10.5	13.1 $\pm$ 4.9	0.36 $\pm$ 0.4 ^S	0.05 $\pm$ 0.05 ^S	1.0 $\pm$ 0.6	day 20: 0.171 $\pm$ 0.005 ^b
MWCNT control	2.51 $\pm$ 0.34 ^{###}	0.11 $\pm$ 0.03	36.5 $\pm$ 21.1	26.7 $\pm$ 16.9	8.0 $\pm$ 3.3	1.35 $\pm$ 0.56 ^{###}	0.13 $\pm$ 0.14 ^S	0.8 $\pm$ 0.4	day 44: 0.274 $\pm$ 0.113 ^b
									day 20: 0.857 $\pm$ 0.178
									day 44: 1.172 $\pm$ 0.217
									day 20: 0.212 $\pm$ 0.015
									day 44: 0.315 $\pm$ 0.046

Note: Tukey's honestly significant difference post hoc tests for 1-way analysis of variance (ANOVA): ^anm = not measured. ^bSerum of only 3 animals was measured.

^s, ^{ss}, ^{sss}Significantly different from air control ($p < .05$, $< .01$, $< .001$, respectively).

[#], ^{###}Significantly different from trimellitic anhydride (TMA) control ($p < .05$, $< .001$, respectively).

[&], ^{&&}, ^{&&&}Significantly different from multiwalled carbon nanotubes (MWCNT) control ($p < .05$, $< .01$, $< .001$, respectively), SD = standard deviation.

^{*}, ^{**}, ^{***}Significantly different ($p < .05$, $< .01$, $< .001$, respectively).

treatment with TMA when compared to the animals not treated with TMA (Table 3), regardless of MWCNT exposure.

As found earlier with this allergy model (Arts et al. 2003), TMA challenge in sensitized rats induced irregularly lengthened pauses between varying numbers of breaths. Breathing frequency decreased considerably during challenge, starting within a few minutes, and returned to normal after challenge. The frequency was increased at 24 hr after challenge. Upon the second TMA challenge, it was evident that MWCNT exposure had not affected the breathing pattern, frequency, tidal volume, or ventilatory flow of allergic animals after particle exposure. In addition, particle exposure had no effect on the pauses between breaths; these were similar in duration, in seriousness, and in time of occurrence (data not shown).

Hematology and Serum IgE Levels

Total white blood cell numbers were not affected in any of the groups. Main effects of treatment to TMA were seen for lymphocytes, neutrophils, and eosinophils and for treatment to MWCNT for eosinophils and basophils (Table 3). In allergic rats, the number of neutrophils was increased, which reached significance in animals also exposed to MWCNT. The number of eosinophils was significantly decreased (compared to air control) in animals treated with TMA and exposed to low MWCNT and animals treated with TMA only. Basophils were significantly increased in the TMA high MWCNT and MWCNT groups compared to air control. No effects were observed in red blood cell and coagulation parameters (data not shown).

As expected, total IgE levels in serum were increased in all animals sensitized by TMA, as measured 1 day before the first challenge with TMA and before exposure to MWCNT (day 20; Table 3). At day 44, after MWCNT exposure and the second challenge with TMA, IgE levels were increased even further in all animals, including the rats not treated with TMA, except in the animals exposed additionally to the high concentration of MWCNT. Remarkably, in all of the latter animals, IgE levels were decreased on day 44 (Figure 1).

Bronchoalveolar Lavage

The most pronounced effects of treatment with TMA in BAL cell parameters were seen in the number of total cells, lymphocytes, neutrophils, and eosinophils and in the biochemical parameters LDH and total protein (Table 4). The most pronounced effects of exposure to MWCNT were seen in the number of eosinophils and in ALP and GGT. A significant interaction between the two factors (treatment with TMA and exposure to MWCNT) was seen in the number of lymphocytes. Post hoc testing did not reveal significant group differences for the number of total cells. Exposure to TMA increased the number of lymphocytes, and the increase was counteracted by exposure to a high concentration of MWCNT, but not to a low concentration of MWCNT. Eosinophils were significantly increased (compared to air control) in the TMA control and the TMA/high MWCNT groups. Exposure to low MWCNT

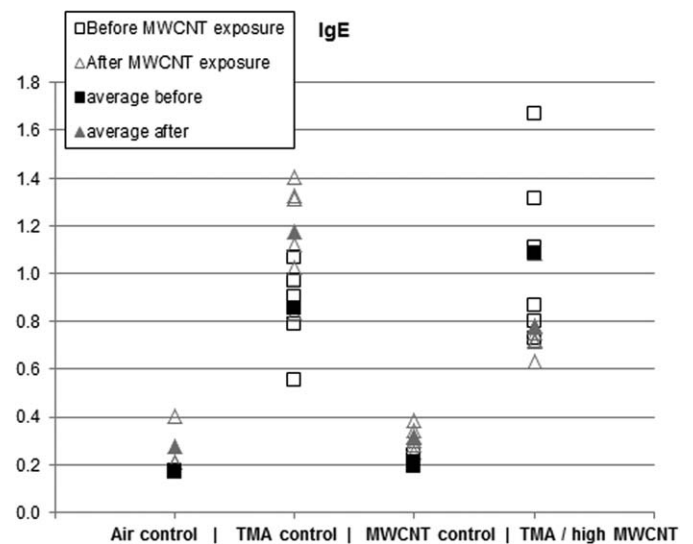


FIGURE 1.—Serum IgE levels at day 20 (after sensitization and before the first challenge with TMA) and at day 44 (after exposure to MWCNT and the second challenge with TMA). IgE was increased in the sensitized animals at day 20 and increased further at day 44, with the exception of the group of animals exposed to MWCNT of which IgE levels were decreased. IgE, Immunoglobulin E; TMA, trimellitic anhydride; MWCNT, multiwalled carbon nanotubes.

seemed to partially counteract the increase by TMA treatment. ALP was increased in the TMA/low MWCNT group and the increase was significant in comparison with all 3 control groups (air control, TMA control, and MWCNT control). GGT was increased in the TMA/low MWCNT group compared to the air control group. Microscopic evaluation of differential cells showed clusters of cells, presumably macrophages, in relation to meshworks of MWCNT in allergic and nonallergic animals. In addition, cells with complete as well as incomplete uptake of agglomerated/aggregated tubes were found.

Pathology

Untreated (non-allergic) BN rats

A minimal pulmonary granulomatous inflammation is common in BN rats (Germann et al. 1998; Kuper et al. 2008). The number of microgranulomata or macrophage aggregates observed in the left lung of the untreated rats varied from 0 to 2 to a small area with multiple macrophage aggregates (covering less than 5% of the lobe). The latter animals had in addition some goblet cell hyperplasia in the bronchi and larger bronchioli.

Allergic (TMA-sensitized and TMA-challenged) rats. The two inhalation challenges with TMA in the TMA-sensitized rats induced inflammation and ulceration at the base of the epiglottis in the larynx and inflammation in the lungs, as described previously (Arts et al. 1998).

MWCNT in nonallergic rats. MWCNT in the lungs were observed as black dots and black fibers or meshworks of

TABLE 4.—Bronchoalveolar parameters in BN rats exposed to the respiratory allergen trimellitic anhydride and to multiwalled carbon nanotubes.

Group	Bronchoalveolar lavage cells					Bronchoalveolar lavage enzymes and protein				
	Total cells (105/L $\pm$ SD)	Lymphocytes (105/L $\pm$ SD)	Neutrophils (105/L $\pm$ SD)	Eosinophils (105/L $\pm$ SD)	Monocytes/ macrophages (105/L $\pm$ SD)	ALP (U/L $\pm$ SD)	GGT (U/L $\pm$ SD)	LDH (U/L $\pm$ SD)	NAG (U/L $\pm$ SD)	Total protein (mg/L $\pm$ SD)
Factor TMA, 2-way ANOVA	*	**	***	***				***		***
Factor MWCNT, 2-way ANOVA				*		**	*			
Interaction		*								
TMA/low MWCNT	26.8 $\pm$ 9.5	0.3 $\pm$ 0.25	5.3 $\pm$ 2.0 ^{ss}	2.0 $\pm$ 1.1 ^{##}	19.3 $\pm$ 8.2	102.80 $\pm$ 19.31 ^{ss, #, &}	17.90 $\pm$ 2.31 ^{ss}	431.20 $\pm$ 62.32 ^{ss, & &}	7.00 $\pm$ 0.85	1182.80 $\pm$ 309.63 ^{ss, & &}
TMA/high MWCNT	20.8 $\pm$ 1.3	0.06 $\pm$ 0.5 ^{##}	4.6 $\pm$ 1.6 ^s	5.0 $\pm$ 2.7 ^{ss, & &}	11.1 $\pm$ 3.0	41.40 $\pm$ 21.90	10.80 $\pm$ 7.39	384.20 $\pm$ 223.63 ^{ss, & &}	4.95 $\pm$ 2.39	1084.80 $\pm$ 645.32 ^{ss, &}
Air control	19.3 $\pm$ 12.4	0.03 $\pm$ 0.05 ^{##}	0.2 $\pm$ 0.3 ^{###}	0.4 $\pm$ 0.7 ^{###}	18.7 $\pm$ 12.0	48.00 $\pm$ 20.33	6.83 $\pm$ 2.12	90.75 $\pm$ 53.41 ^{###}	3.95 $\pm$ 1.37	221.75 $\pm$ 124.47 ^{##}
TMA control	24.4 $\pm$ 8.2	0.55 $\pm$ 0.37 ^{& & ss}	6.7 $\pm$ 4.0 ^{ss, &}	5.8 $\pm$ 2.8 ^{ss, & & &}	11.4 $\pm$ 5.7	55.40 $\pm$ 25.09	12.05 $\pm$ 3.78	357.70 $\pm$ 112.07 ^{ss, & &}	5.39 $\pm$ 1.80	1041.90 $\pm$ 447.98 ^{ss, & &}
MWCNT control	11.2 $\pm$ 2.8	0.04 $\pm$ 0.09 ^{##}	0.7 $\pm$ 0.2 ^{##}	0.1 $\pm$ 0.1 ^{ss, ###}		54.00 $\pm$ 36.93	13.76 $\pm$ 9.92	93.40 $\pm$ 45.54 ^{##}	4.40 $\pm$ 2.23	220.20 $\pm$ 112.72 ^{##}

Note: Tukey's honestly significant difference (HSD) post hoc tests for 1-way analysis of variance (ANOVA).

s, ss, sss Significantly different from air control ($p < .05$, $< .01$, $< .001$, respectively).

#, ### Significantly different from trimellitic anhydride (TMA) control ($p < .05$, $< .001$, respectively).

&, &&, &&& Significantly different from multiwalled carbon nanotubes (MWCNT) control ($p < .05$, $< .01$, $< .001$, respectively).

ALP = alkaline phosphatase, LDH = lactate dehydrogenase, GGT = γ -glutamyl transferase.

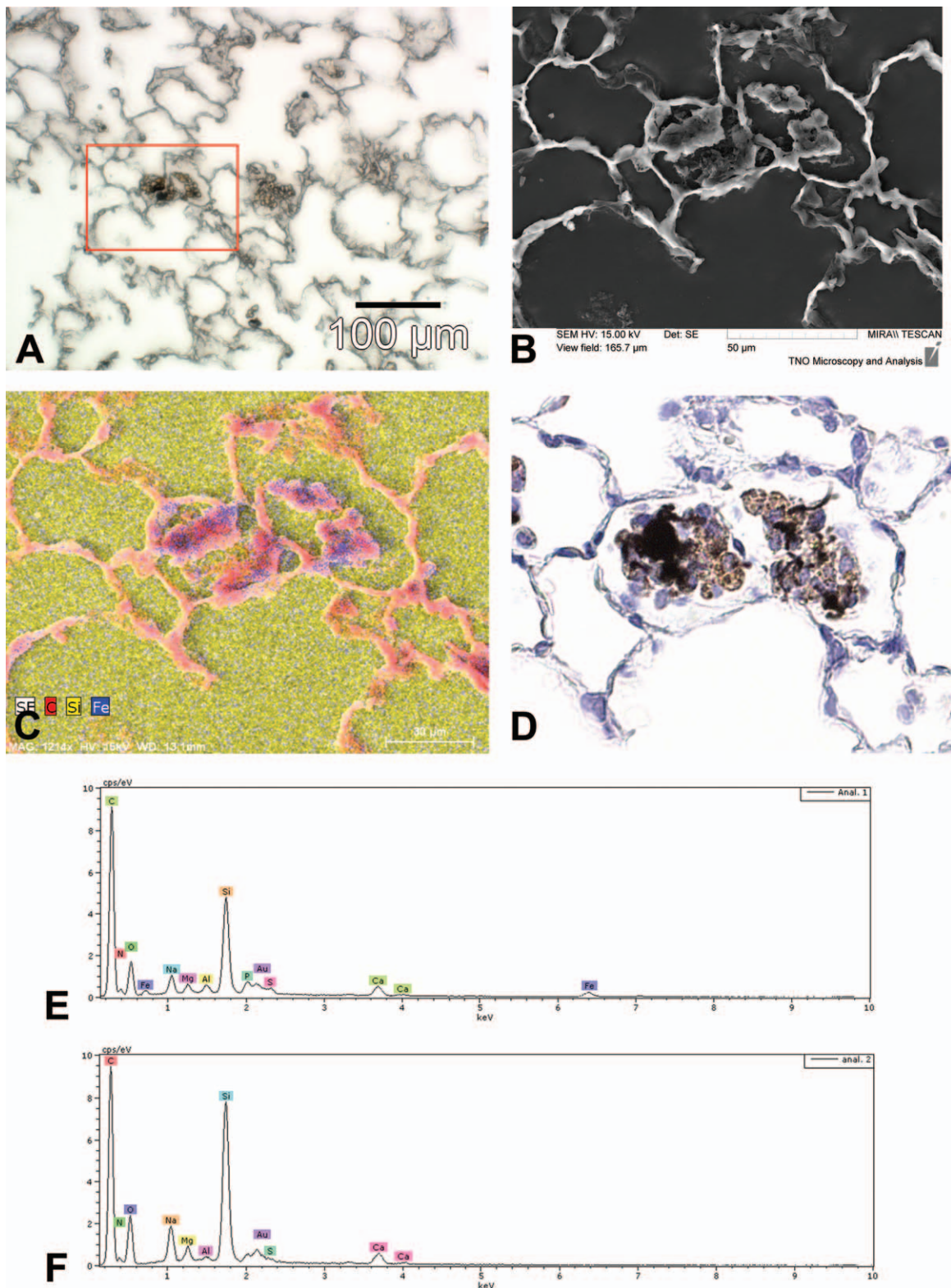


FIGURE 2.—Overview of a deparaffinized, unstained and uncovered lung section (A) from a non-allergic rat, containing black agglomerates. (B) SEM of the area, indicated in (A) by the rectangle. (C) Element analysis of the SEM area, demonstrating high Fe content. (D) A light microscopic view of the same area as above in another (step) section, H-stained; magnification 400x. Graph of the element analysis of (C) in the spots with black agglomerates (E) and control area (F). The control area illustrates the element analysis of lung tissue itself, of the object glass and of the gold (Au) coating.

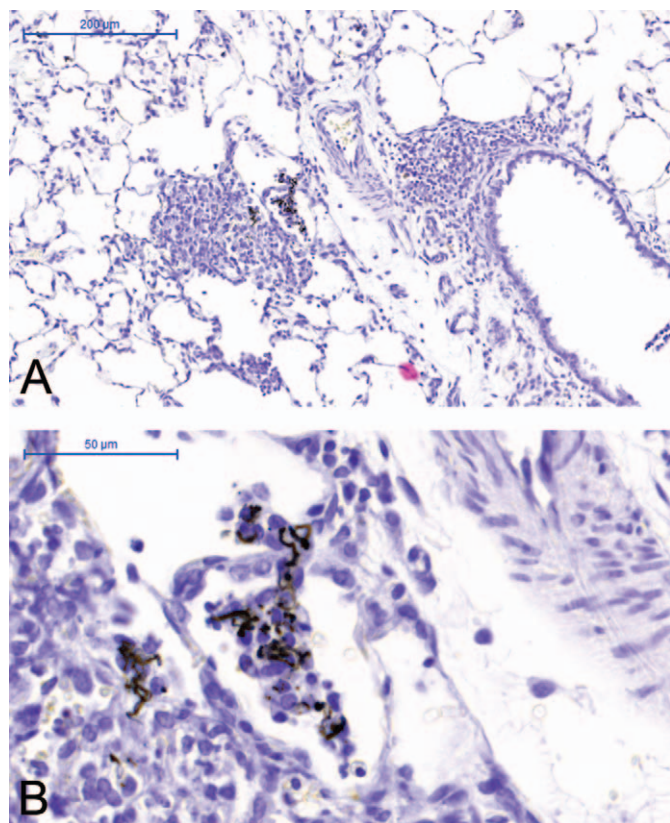


FIGURE 3.—Overview (A) and detail (B) of macrophages clustered around agglomerated/aggregated MWCNT, in an allergic rat exposed to MWCNT by inhalation. The process involved 2 alveoli. MWCNT in lung: indications of frustrated phagocytosis. H&E-stained sections, at 10 $\times$ (A) and 40 $\times$ (B) magnification. MWCNT, multiwalled carbon nanotubes.

different sizes and widths (the length of the agglomerated/aggregated tubes or “fibers” in the alveoli was up to 30 μ m; their diameter was up to 2 μ m). The fibers appeared as bended or even curled structures, but some of them were rod-like, suggesting stiffness. SEM of the black material in the lung did not reveal single nanotubes, but only agglomerates (Figure 2). Element analysis demonstrated that iron (Fe) was the best indicator of the presence of MWCNT, because the carbon content could not reliably distinguish between the background (tissue) carbon content and the MWCNT (Figure 2). The material was found within or in close association of cells, presumably macrophages, throughout the entire lung including close to the pleura. The bulk of the material was observed in the alveolar duct and alveolar lumina and in the tissue at the transition between the terminal bronchiole and alveolar ducts, but MWCNT were also found in the bronchiolar lumina. Frustrated phagocytosis was indicated by partial uptake of some fibers and rods, the presence of clusters of cells surrounding black-pigmented meshworks, and the presence of an inflammatory response. Minimal fibrosis was observed especially at the transition of the terminal bronchioles and alveolar duct. Some small black-pigmented structures were observed in the cortex

of the lung-draining mediastinal lymph nodes. They were not found in the nasal tissues, larynx, or trachea.

MWCNT in allergic rats

Exposure to the high- or low-concentration MWCNT did not increase the already extensive allergy-related inflammation in the larynx and the lung. As in nonallergic rats, frustrated phagocytosis was apparent (Figure 3). The MWCNT appeared to accumulate in sizable agglomerates in the granulomas in the lungs of allergic rats (Figure 4). Some small black-pigmented structures were observed in the cortex area of the lung-draining lymph nodes, similar to the lymph nodes in the nonallergic rats. A single black structure was observed in BALF of 1 animal.

DISCUSSION

The present study examined the interaction between repeated inhalation exposure to MWCNT and TMA-induced respiratory allergy in the high IgE-responding BN rat, focusing on exposure-induced effects on airways, lung parenchyma, and local lymph nodes. The allergic condition was expected to interfere with the clearance of MWCNT, because less efficient clearance mechanisms have been observed in individuals with lung disease (reviewed by Donnelly and Barnes 2012; Bennett et al. 2011). MWCNT exposure in sensitized animals was expected to aggravate the TMA-induced allergy, based on epidemiologic evidence in humans (Knol et al. 2009) and studies in protein-allergic animals (Inoue et al. 2009; Nygaard et al. 2009; Li et al. 2010; Inoue 2011).

Single nanotubes cannot be observed by light microscopy, because they are under the detection limit (Hubbs et al. 2011). This may lead to underestimation of respiratory tract deposition. However, nanoparticles in air mostly occur as aggregates or agglomerates due to cohesive forces, enhanced by the high humidity in the airways (Holsapple et al. 2005; Ma-Hock et al. 2007). Schulz et al. (2011) described the morphology of Nanocyl 700 nanotubes as large grains (up to 500 μ m), constituted of densely packed tubes. In the present study, agglomerated/aggregated tubes (fibers) were observed in the larger and smaller airways but were found predominantly in the alveolar duct, alveoli, and interstitium. The length of the agglomerated/aggregated fibers in both the alveoli and the bronchioli was quite large, whereas the fibers in the interstitium were much smaller. Some systemic exposure was evident by the presence of MWCNT in the lung-draining lymph nodes. This is in agreement with a 3-month inhalation study by Ma-Hock et al. (2007). The dominant presence in the alveoli may reflect the slow clearance of the MWCNT by alveolar macrophages, in contrast to the rapid mucociliary clearance in the bronchial tree. The presence of MWCNT in the lungs at marginal overload at the low concentration and at overload conditions at the high concentration (Prof. Dr. J. Pauluhn, personal communication, February 19, 2013) led to local inflammation and fibrosis, as found in other inhalation studies with nanoparticles in nonallergic animals (e.g., Ma-Hock et al. 2008). Excessive inflammation may lead to DNA damage

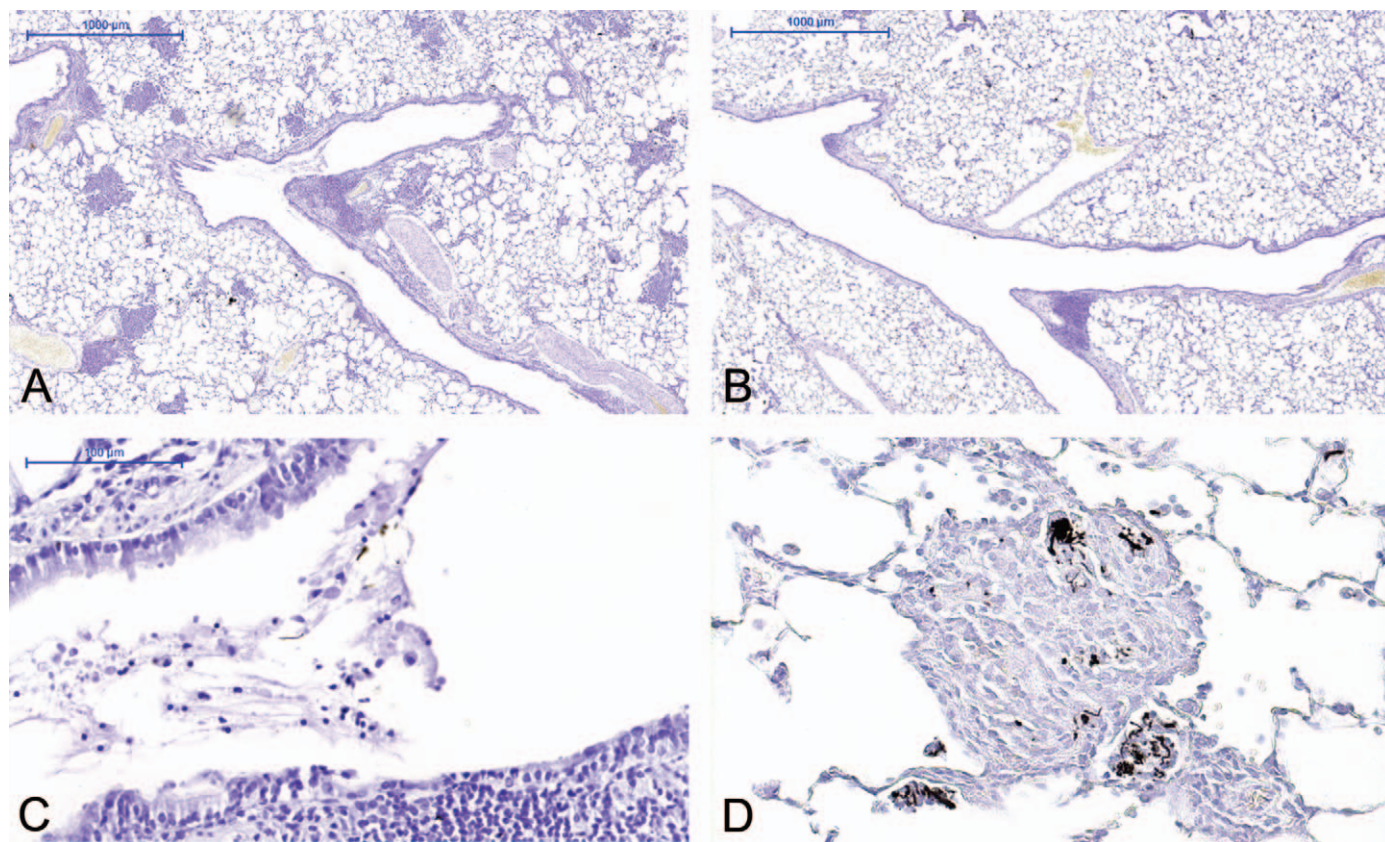


FIGURE 4.—(A–F). Overview of the left lung lobe of an allergic (A) and a nonallergic BN rat (B), exposed to MWCNT by inhalation. Several microgranulomata are present in the lungs of the allergic rat. Detail of the bronchiole show a mucus plug in the airway, with a few MWCNT agglomerates/aggregates, in the allergic rat (C); no mucus abundance was observed in the nonallergic rat. Large MWCNT agglomerates/aggregates were present in some of the granulomas in the lungs of the allergic rat (D). H&E- and H-stained sections, at 5× (A, B) and 20× (C–F) magnification. MWCNT, multiwalled carbon nanotubes.

(Trouiller et al. 2009). Indeed, markedly increased numbers of comets were observed in lung and BAL cells of the allergic rats. Exposure to MWCNT did not influence the number of comets in the allergic rats nor did it induce DNA breaks in the lungs of healthy rats (unpublished data). This is in contrast to some studies on the genotoxicity of MWCNT (*in vivo* bone marrow cells upon intraperitoneal injection: Patlolla et al. 2010; *in vitro* human lung cells: Ursini et al. 2012; *in vitro* hamster lung cells: Asakura et al. 2010). Overall, the direct genotoxic potential of nanotubes is still considered inconclusive (Aschberger et al. 2010).

Although the breathing pattern of the allergic rats differed from the healthy rats, this was only true during challenge with TMA, shortly after challenge, and 24 hr after challenge. It is therefore not surprising that the distribution of MWCNT throughout the lungs appeared not to be affected by the allergic state. Remarkably, the lungs of the allergic rats contained in general large MWCNT agglomerates/aggregates. The persistence of MWCNT, especially in the allergic rats, may be due to the already present allergy-associated inflammation and the increase in mucus-producing cells, leading to an increase in mucus. Such preexisting conditions may affect clearance times,

which have been found in smokers and chronic obstructive pulmonary disease patients (Moller et al. 2008).

Exposure of the allergic rats to the MWCNT did not result in effects on the breathing pattern, or on the amount and type of the extensive allergy-associated inflammation, but decreased the number of lymphocytes in BAL and decreased IgE levels in blood. This is in contrast to exacerbation of the allergic response reported previously with nano- and micro-sized particles (Alessandrini et al. 2006; Shvedova et al. 2008; Inoue 2011; Li et al. 2010; Nygaard et al. 2009; Sakai et al. 2009). However, a few, mostly recent, studies have shown quite the opposite, namely, a reduction or even inhibition of allergy. Hardy et al. (2012) found inhibition of airway and parenchymal inflammation, airway mucus production, and distinct decrease in allergen-specific IgE and Th2 cytokines by inert polystyrene nanoparticles. Comparable results were observed by Ryan et al. (2007; fullerene nanomaterial), Rossi et al. (2010; nano- and micro-sized titanium dioxide), and Tahara et al. (2012; biodegradable poly-lactide-co-glycolide nanomaterial). Interestingly, the reductions of the allergic response were especially consistent for IgE levels in these studies, which all used a protein-induced respiratory allergy model. Although chemically

induced respiratory allergy is a less prominent IgE-related allergy, our study with MWCNT in TMA-allergic rats also demonstrated a decrease in serum IgE levels when compared to TMA-allergic rats not exposed to MWCNT.

The decrease in IgE levels may very well be related to macrophage activity. Depletion of alveolar macrophages by clodronate-containing liposomes led to a reduction in IgE levels in serum and lung lavage fluid in our TMA model (Valstar et al. 2006a, 2006b). At the same time, it augmented the allergic lung inflammation by increasing neutrophil and eosinophil cell numbers in BAL. Similar results were obtained in a protein-induced asthma model (Bang et al. 2012). This dual role of macrophages in respiratory allergy has been reviewed by Balhara and Gounni (2012), and it may offer an explanation for the diverse results found in the different studies of the influence of particulate material on respiratory allergy. A complicating factor can be attributed to variations in timing of particle exposure, namely, during the induction/sensitization phase or during the provocation/challenge phase, in combination with the characteristics and concentrations of the particle.

In conclusion, high IgE-responding rats with chemically (TMA)-induced respiratory allergy appeared to retain more MWCNT in the lungs as compared to the nonallergic (healthy) rats. MWCNT exposure of these animals did not exacerbate the allergic response. Instead, serum IgE levels and the numbers of lymphocytes in BAL were decreased.

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