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Inhalation Toxicity of Multiwall Carbon Nanotubes in Rats Exposed for 3 Months

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Carbon nanotubes (CNT) are of great commercial interest. Theoretically, during processing and handling of CNT and in abrasion processes on composites containing CNT, inhalable CNT particles might be set free. For hazard assessment, we performed a 90-day inhalation toxicity study with a multiwall CNT (MWCNT) material (Nanocyl NC 7000) according to Organisation for Economic Co-operation and Development test guideline 413. Wistar rats were head-nose exposed for 6 h/day, 5 days/week, 13 weeks, total 65 exposures, to MWCNT concentrations of 0 (control), 0.1, 0.5, or 2.5 mg/m^3. Highly respirable dust aerosols were produced with a proprietary brush generator which neither damaged the tube structure nor increased reactive oxygen species on the surface. Inhalation exposure to MWCNT produced no systemic toxicity. However, increased lung weights, pronounced multifocal granulomatous inflammation, diffuse histiocytic and neutrophilic inflammation, and intra-alveolar lipoproteinosis were observed in lung and lung-associated lymph nodes at 0.5 and 2.5 mg/m^3. These effects were accompanied by slight blood neutrophilia at 2.5 mg/m^3. Incidence and severity of the effects were concentration related. At 0.1 mg/m^3, there was still minimal granulomatous inflammation in the lung and in lung-associated lymph nodes; a no observed effect concentration was therefore not established in this study. The test substance has low dust-forming potential, as demonstrated by dustiness measurements, but nonetheless strict industrial hygiene measures must be taken during handling and processing. Toxicity and dustiness data such as these can be used to compare different MWCNT materials and to select the material with the lowest risk potential for a given application.

Key Words: **carbon nanotubes; MWCNT; pulmonary toxicity; 90-day inhalation study; granulomatous inflammation; lung; lymph nodes.**

Carbon nanotubes (CNT) are a new form of crystalline carbon which structurally resemble rolled-up graphene sheets with one end capped. These tiny tubes can have single or multiple concentric walls. Single-walled CNT (SWCNT) have diameters of 0.4–2 nm, whereas multiwalled CNT (MWCNT) consist of multiple concentric graphene cylinders of increasing diameter from 2 to 100 nm, depending on the number of walls (Lin *et al.*, 2004). The length of the tubes is usually several micrometers. Both SWCNT and MWCNT possess high strength, are ultralight weight and have excellent thermal and chemical stability. Due to their metallic and semiconductive electrical properties, they may have a plethora of industrial uses such as in high-strength materials, electronics and biomedical applications (Kohli and Martin, 2003). Many of the properties that make CNT remarkable for engineering applications have also caused concern about their biocompatibility, especially in the lung. Their aspect ratios (length/width ratio) of > 1000, reactive surface chemistry and poor solubility raise concerns linked to past experience with hazardous fibers (e.g., asbestos).

The potential health hazards of CNT were not investigated by the scientific community during the first decade after their discovery in 1991 (Iijima, 1991). Initially, CNT was produced either by arc discharge sublimation of a graphitic anode doped with catalyst (Ebbesen and Ajayan, 1992) or by laser ablation. Both methods are expensive and do not permit industrial-scale production. When chemical vapor deposition was established, particularly with the use of a floating-catalyst method (Endo, 1988; Lake, 2001), large quantities of CNT could be produced on an industrial scale. Since then, potential health effects have become a focus of attention in the scientific community. One of the major concerns about the use of CNT-based material is the unknown impact on the health of workers involved in its manufacture and handling. The majority of the work published has demonstrated that both SWCNT and MWCNT could pose potential health problems. The contribution of metal/metal oxide residual catalyst has also been discussed. However, information on the lung toxicity of CNT is limited and remains partly inconclusive.

The first study with SWCNT reported no significant signs of lung toxicity 4 weeks after intratracheal (i.t.) administration in guinea pigs (Huczko *et al.*, 2001). This finding was not confirmed by Lam *et al.* (2004), who observed persistent and dose-dependent epithelioid granulomas and interstitial

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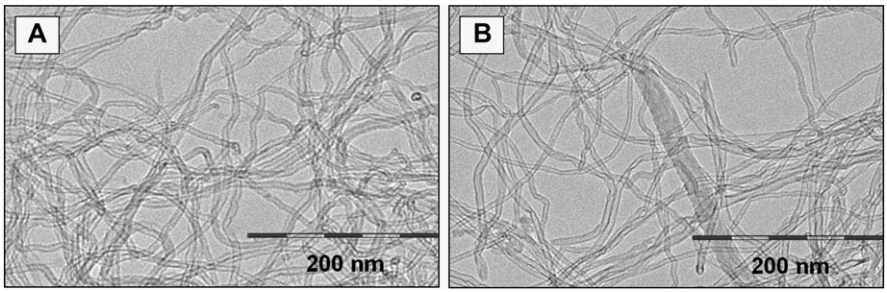

FIG. 1. Transmission electron microscopy images of the test material (A) as delivered (B) as collected in the inhalation chamber after dust generation.

inflammation in mice after a single i.t. treatment with 0.1 or 0.5 mg SWCNT per mouse. A study with SWCNT "soot" (generated by a laser ablation process) showed transient inflammation, cell injury and formation of multifocal granulomas centered around CNT in rats after intratracheal administration of 1 or 5 mg/kg body weight (Warheit *et al*., 2004), although this study had some inconsistencies, such as the apparent lack of a persistent effect in the lavage fluid and lack of a dose-response relationship. Later, Shvedova *et al.* (2005) reported unusual inflammation and fibrogenic potency in mice of pharyngeal-aspirated SWCNT (10–40 μg per mouse). Again, acute inflammation and granuloma formation were observed, accompanied by persistent interstitial fibrosis and alveolar wall thickening (up to 60 days). To more directly assess pulmonary exposure, the authors conducted an inhalation study with the same material in mice (Shvedova *et al*., 2008). Inhaled SWCNT was even more effective than pharyngeal aspiration in causing inflammatory response, oxidative stress, collagen deposition and fibrosis in C57BL/6 mice. Pharyngeally aspirated SWCNT in $ApoE^{-/-}$ mice fed an atherogenic diet (20 μg/mouse once every 2 weeks for 8 weeks) accelerated atherosclerotic plaque formation (this mouse strain lacks apolipoprotein E and consequently has elevated plasma levels of cholesterol and triglyceride and develop atherosclerotic plaques in a fashion similar to humans) (Li *et al*., 2007).

Concerning MWCNT, Muller *et al.* (2005) investigated the effects of intact or milled MWCNT by intratracheal instillation to female Sprague-Dawley rats (0.5, 2, and 5 mg per animal). They observed dose-dependent, persistent (60 days) inflammation and granuloma formation. Moreover, dose-related pulmonary fibrosis was diagnosed by measuring hydroxylproline in the lung tissue. When compared with asbestos and carbon black, the severity of the inflammatory effect induced by MWCNT was intermediate. These effects were not however observed by Mitchell *et al*. (2007) after whole-body inhalation exposure of mice to 0.3–5 mg/m^3 MWCNT (6 h/day for 7 or 14 days). Instead, immunosuppression was observed after 14 days, although without a clear concentration-response relationship.

Although the existing information on pulmonary toxicity of CNT is limited, summarizing the available data, the majority of the work published since the first study in 2001 has demonstrated that both SWCNT and MWCNT could pose potential health problems. Most studies to date have used intratracheal instillation; this route of administration is commonly used to assess the hazard potential of dust in the lungs. Both intratracheal instillation and pharyngeal aspiration assume that the particles can reach the lung in certain quantities, but do not consider dust deposition behavior and consequently its possible effects on the upper respiratory tract, and most importantly do not reflect the atmospheric concentration of potential worker exposure. Data obtained by these administration routes are necessary to identify potential hazard but are not sufficient for proper risk assessment.

Therefore, we have developed a test system which is capable of reliably generating dust aerosols with nanomaterials (Ma-Hock *et al*., 2007), and have performed inhalation toxicity studies with test design strictly according to the Organisation for Economic Co-operation and Development (OECD) test guideline 413 (OECD, 1981), and additional exposure assessments based on nanomaterial-specific analytical data. The study reported in this paper is the first inhalation toxicity study with MWCNT conducted according to OECD 413 under Good Laboratory Practice (GLP) compliance (OECD, 1998). It is intended to provide data to support risk assessment for the workplace.

MATERIAL AND METHODS

Test Material and Characterization

The test substance, Nanocyl NC 7000 (Fig. 1), was thin MWCNT provided by Nanocyl S.A. (Sambreville, Belgium). The purity was 90% C and 10% metal oxide, of which 9.6% was aluminum oxide with traces of iron and cobalt. The tubes have diameters of 5–15 nm and length 0.1–10 μm. The specific surface area (Brunauer Emmet Teller method) was 250–300 m^2/g according to the manufacturer.

The test material (both as delivered, and sampled from the inhalation atmosphere after dust generation) was examined morphologically by transmission electron microscopy. In addition, it was tested for the presence of reactive oxygen species (ROS) which might be formed on the test material surface during the dust generation process, using the radical scavenger 2,2-diphenyl-1-picrylhydrazyl (DPPH). Five milliliters of 50μM DPPH in methanol was added to a test tube containing 1.5 mg of test material or material sampled from the test atmosphere of MWCNT (i.e., after dust generation); in the presence of ROS, DPPH turns from purple to yellow. To demonstrate the

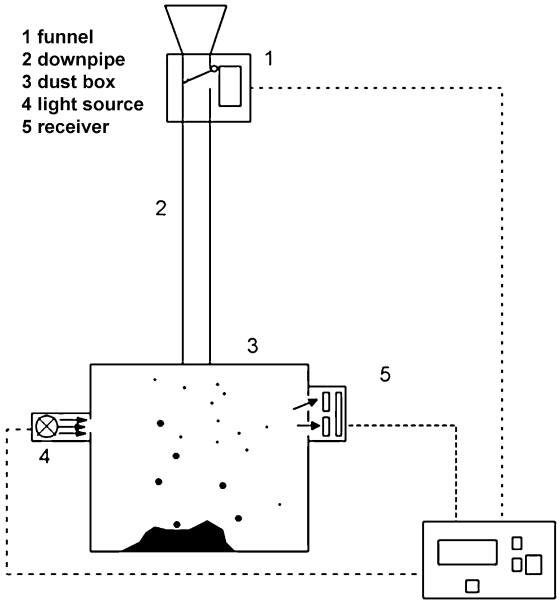

FIG. 2. Schematic view of the ''Dust View'' system used to measure the dustiness of the MWCNT.

sensitivity of the method, ball-milled Nanocyl NC 7000 (Nanocyl S.A., Sambreville, Belgium) was used as positive control substance.

Separately from the inhalation study, the inherent dust-forming potential of the test substance was measured according to modified DIN 55992-2 (German Industry Norm for pigments and extenders) after dropping 35, 55, 70, and 140 ml of test substance through a 780-mm-high cylinder into a 6.6-l dustbox (PALAS DustView, Palas GmbH, Karlsruhe, Germany, Fig. 2) using comparable methods to those in the inhalation study (SMPS Scanning Mobility Particle Sizer by Grimm Aerosol Technik, Germany; condensation particle counter by TSI USA; scattered light spectrometer WELAS 2010 by Palas, Germany). The particle size range was 10–1083 nm with the SMPS and 0.3–40 μm with the WELAS 2010. In addition, after the dustiness experiment, scanning electron microscopy was performed on a gold-coated capillary filter through which air had been sampled from the dustbox.

Study Design

To select test concentrations for the 90-day inhalation study, a 5-day rangefinding study was performed. The endpoints and examination time points of this rangefinding study were strictly according to the previously recommended short-term inhalation test protocol for poorly soluble nanoparticles (Ma-Hock *et al*., 2009). Groups of male Wistar rats were head-nose exposed to MWCNT dust aerosol for 6 hours per day on 5 consecutive days at target concentrations of 0, 2, 8, and 32 mg/m^3. Three days after the last exposure (study day 8) bronchoalveolar lavage (five rats per concentration), blood clinical chemistry, and hematology as well as histological examination of the respiratory tract (three rats per concentration) were performed. To follow the potential reversibility or progression of the effects, all examinations were performed again after a 24-day recovery period (study day 28).

Based on the results of the rangefinding study, concentrations of 0.1, 0.5, and 2 mg/m^3 were chosen for the main study. The design of the main study was based on the OECD 90-day inhalation toxicity study guideline 413 (OECD, 1981). Groups of 10 males and 10 females Wistar rats were head/nose exposed to dust atmospheres for 6 h a day, from Monday to Friday, for 13 weeks (total 65 exposures). On exposure days, checks for clinical signs of toxicity were performed once prior to and once after exposure, and several times during exposure. On exposure-free days, clinical observation was performed once a day. Body weight and food consumption were determined weekly. Detailed clinical observation, ophthalmological examination, functional observation battery and motor activity tests were performed towards the end of the exposure. Blood clinical-chemical and hematological parameters and organ weights were determined, and the organs and tissues were examined by light microscopy at the end of exposure according to OECD 413.

The study was performed in an International Association for Assessment and Accreditation of Laboratory Animal Care-approved laboratory in accordance with the German Animal Welfare Act, European Council Directive 86/609/EEC, and the OECD Principles of GLP (OECD, 1998).

Animals

Male and nonpregnant nulliparous female Wistar rats (Crl:WI; Charles River, Germany), 8–9 weeks old at the start of exposure, free from clinical signs of disease, were allocated randomly, stratified by weight (separately for males and females), to one of the treatment groups. When not exposed they were housed three to five per sex per cage in 0.2-m^2 polycarbonate cages (TECNIPLAST H-Temp [PSU], Hohenpeissenberg, Germany) with dust-free bedding (Lignocel FS 14 fibers; SSNIFF, Soest, Germany) and wooden gnawing blocks (NGM E-022; Abedd Lab. and Vet. Service GmbH, Vienna, Austria). The animal quarters were air-conditioned (20–24°C, 30–70% relative humidity) with a 12-h light/12-h dark cycle. Certified feed (Kliba GLP rat/mouse maintenance diet meal, Provimi Kliba SA, Kaiseraugst, Switzerland) and tap water (human drinking quality) were available *ad libitum* except during treatment. After acclimatization to the inhalation apparatus (on 2 consecutive days for 6 h/day with clean supply air, in the week before start of exposure), all animals were exposed for 6 h/day for the exposure period. During exposure, the animals had no access to water or feed.

Aerosol Generation

The dust aerosol generation and exposure apparatus (Fig. 3) is described in Ma-Hock *et al*. (2007). Dust aerosols were produced with a brush generator developed by the Technical University of Karlsruhe, Germany, and BASF (Ma-Hock *et al*., 2007) with 5–200 mg test substance/h (adjusted by varying the speed of the piston feed and rotation speed of the brush) mixed with compressed air (filtered air pressurized to about 6 bar, flow rate 1.5 ± 0.3 m^3/h) in a glass tube, diluted with conditioned air (activated charcoal-filtered air, 22 ± 2°C, 50 ± 20% relative humidity, flow rate 4.5 ± 0.3 m^3/h) passed via a cyclone into the inhalation system, a 90-l cylindrical stainless steel inhalation chamber with cone-shaped inlet and exhaust at opposite ends. The desired inhalation chamber concentrations were achieved by withdrawing/exhausting and replacing a portion of the dust aerosol air with conditioned supply air immediately before the chamber (0–6 m^3/h, depending on target concentration). Mean flow rate through the inhalation chamber, measured at exhaust air, was 5.4 ± 0.3 m^3/h for all concentrations, that is, air was changed in the inhalation chambers about 67 times per hour.

Monitoring and Characterization of the Test Atmosphere

Compressed and conditioned supply air and exhaust air flow rates, chamber temperature and humidity were measured automatically with appropriate sensors/orifice plates; data were saved every 10 s and retained for analysis. MWCNT substance flow was read once per exposure from the display of the brush generator. To ensure the stability of the MWCNT dust aerosols, the inhalation chambers were monitored continuously during exposure using scattered light photometers (VisGuard; Sigrist-Photometer AG, Switzerland). These photometers were not calibrated and therefore these data indicate the stability of the aerosol in the test atmosphere during the exposure period, not the actual atmospheric concentration. The photometers were cleaned when oscillation of signals occurred. Comparable signal height for the same concentration group during the course of an exposure day or from day to day indicated stability of the dust atmosphere. To quantify the atmospheric dust concentration, gravimetric measurements of air samples taken adjacent to the animals' breathing zone were performed (probe internal diameter 7 mm). A defined volume of sample air was drawn by vacuum pump across a binder-free glass-fiber filter paper (Macherey-Nagel MN 85/90 BF, diameter 4.7 cm).

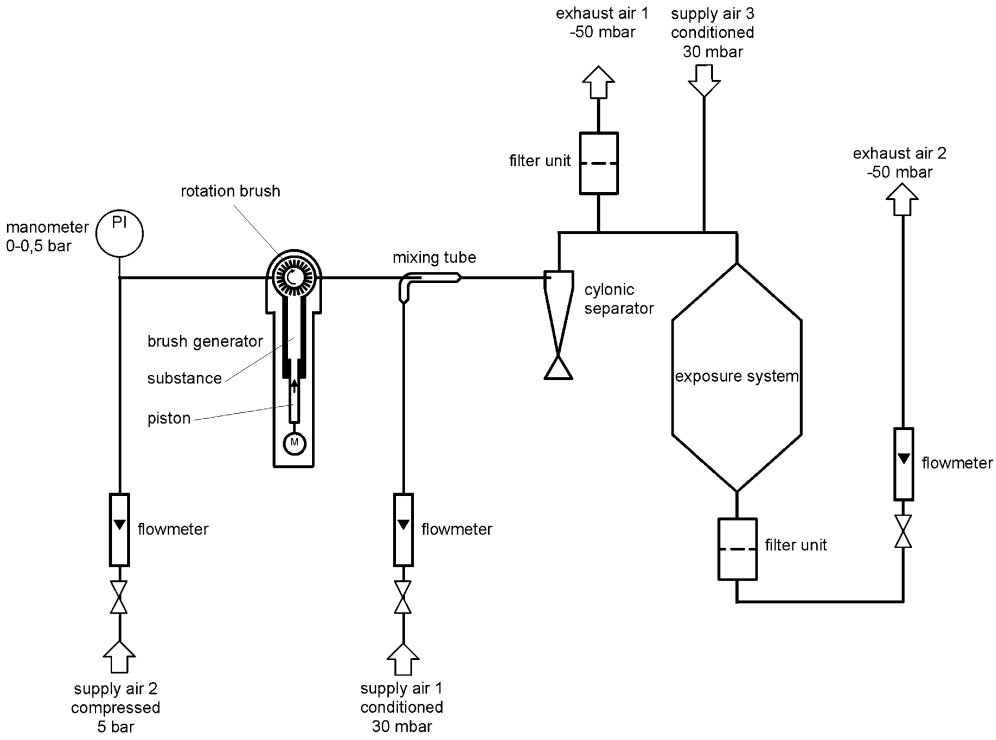

FIG. 3. Schematic view of the dust generation and exposure system.

Aerosol dust concentration in mg/m^3 was calculated as the increase in weight of the filter after sampling, divided by sample volume at test conditions (22°C, atmospheric pressure, 50% relative humidity). As a rule, two samples were taken per exposure and concentration group. The duration of sampling was adjusted to the test substance concentration in the chamber to obtain a total sample weight of 1–5 mg. Thus, the volume of the air samples varied with the atmospheric concentration. In the rangefinding study, the sampling volumes were 450, 120, and 30 l in test groups 2, 3, and 4. In the 90-day study, they were 450, 180, and 90 l, respectively.

To determine the aerodynamic diameter, cascade impactor measurements were performed with a Sierra Marple 298 cascade impactor. The effective aerodynamic cut-off diameters were 21, 15, 10, 6.5, 3.5, 1, 0.7, and 0.4 μm. To capture the particles mass median aerodynamic diameter (MMAD) (the calculated aerodynamic diameter which divides the size distribution in half when measured by mass) < 0.4 μm, the impactor was equipped with a backup filter. The deposition on each impactor stage as well as on the backup filter was determined gravimetrically. Particle size distributions were calculated according to DIN 66141 and DIN 66161. Particle sizes measured by cascade impactor are expressed as MMAD, and geometrical standard deviation (GSD; the ratio of the estimated 84th to 50th percentile, which indicates the width of the cumulative particle size distribution curve).

Additionally, a light-scattering spectrometer (WELAS 2100; Palas, Karlsruhe, Germany) was used for particle sizes from 0.24 to 10 μm (at least 10 repeats). In the submicrometer range (11–1083 nm), size distribution was measured with a Scanning Mobility Particle Sizer equipped with a condensation particle counter (SMPS + C) (Grimm Aerosol Technik GmbH, Ainring, Germany). SMPS classify the particles by electrostatic fractionation of the different sized particles. Particle counts in each of the fractions were counted by condensation particle counter.

To ensure that content of metal oxide in the test atmosphere did not differ from that in the test substance container, supplementary analysis was carried out to determine aluminum content in the air. To assess whether the dust generation procedure damaged the MWCNT tube structure, aerosol samples were taken on a transmission electron microscopy grid and on a gold-coated capillary filter (25 mm diameter, capillary size 0.2 μm) for dust sampling (APC GmbH, Eschborn, Germany). The morphology of the aerosol particles was examined by transmission and scanning electron microscopy.

Animal Exposure

During exposure, rats were restrained in glass tubes fixed to the inhalation chamber walls with their snouts projecting into the inhalation chamber (head/nose exposure). Overpressure was maintained inside the inhalation chamber to ensure that the aerosol in the animals' breathing zone was not diluted by laboratory air. The exposure systems were kept under exhaust hoods in an air-conditioned room.

Examinations in the Rangefinding Study

Clinical observation was performed at least three times during exposure days (prior to, during and after exposure) and at least once daily on exposure-free days. Body weight was determined weekly. To obtain the bronchoalveolar lavage fluid (BALF), animals were killed by exsanguination under Narcoren anesthesia and the lungs lavaged twice with 6 ml (22 ml/kg body weight) of 0.9% (wt/vol) saline. The two washes were combined (an average of 11 ml of lavage fluid was recovered per animal) and aliquots of the combined washes were used for the determination of the total protein concentration, total cell count, differential cell count as well as activities of the BALF enzymes as described elsewhere (Ma-Hock *et al*., 2009). Prior to the lung lavage, blood was sampled from the retroorbital venous plexus of fasted animals under isoflurane (Isoba, Essex GmbH Munich, Germany) anesthesia. The blood samples were used for hematology and differential cell count. In satellite animals, gross necropsy was carried out and lung, liver, kidney, spleen, thymus, testes, epididymides, brain, heart, adrenals, and thyroid gland were weighed. Light microscopic examination was performed on the respiratory tract comprising nasal cavity (four levels), larynx (three levels), oropharynx, trachea (longitudinal with carina), lung (five lobes), and mediastinal lymph nodes.

Examinations in the 90-Day Study

Clinical and functional observations. Health status and cage-side clinical signs were checked at least once daily (three times on exposure days). Food consumption and body weights were measured weekly.

Detailed clinical observations in a standard arena (50 × 37.5 cm with sides 25 cm high) were recorded in all animals prior to and weekly during the exposure period (abnormal behavior during handling, fur, skin, posture, salivation, respiration, activity/arousal level, tremors, convulsions, abnormal movements, gait impairment, lacrimation, palpebral closure, exophthalmus, feces appearance/consistency, urine, pupil size).

The eyes of group 1 (control) and 4 (high concentration) animals were examined with an ophthalmoscope (HEINE Optotechnik, Herrsching, Germany) for any changes in the refracting media before (day −6) and at the end of the exposure period (day 84).

A functional observational battery (FOB) was conducted on the first 5 males and 5 females in each group on day 84 (no inhalation exposure for these animals on this day). The FOB examinations, comprising home-cage and open field observation, sensorimotor and reflex tests and automated motor activity measurement, were conducted in a treatment-randomized sequence. Feed but not drinking water was withdrawn before the tests. For home-cage observation, animals were observed at least one hour after transfer to individual polycarbonate cages (floor area about 800 cm^2), in the cage rack with closed lid. Avoiding cage or rack contact and loud noises, particular check of posture abnormalities, movement, gait, tremors or convulsions was performed. Animals were then removed from their cages and placed in a 50 × 50 cm open field arena to assess behavior on removal from the cage, fur, skin, salivation, nasal discharge, lacrimation, eyes/pupil size, posture, palpebral closure, respiration, tremors, convulsions, abnormal movements/stereotypies, gait abnormalities, activity/arousal level, fecal pellets (number/appearance/consistency), urine (amount/color), and rearing within 2 min.

Sensorimotor and reflex tests were conducted after removal from the open field arena: approach, touch and visual placing response, pupillary and pinna reflex, auditory startle response, righting response, behavior during handling, vocalization, tail pinch, fore- and hindlimb grip strength, and landing foot splay.

Motor activity was quantified in polycarbonate cages each with four infrared beams to measure horizontal (locomotor) and vertical (rearing) movement (Multi-Varimex, Coulbourn Instruments, Allentown, PA). Testing was conducted afternoons, with no feed or water. For each animal, the distance traveled and the number of rearings were quantified over 12 consecutive 5-min periods.

Clinical chemistry and hematology. On the morning after the last day of exposure, blood samples for hematology and clinical chemistry were taken from all animals (fasted overnight) by retroorbital venous plexus puncture under isoflurane anesthesia. Hematology (ADVIA120 Instrument, Siemens, Germany) comprised white blood cell, red blood cell, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelets, differential blood cell counts. Clinical chemistry (Hitachi 917; Roche, Mannheim, Germany) comprised alanine and aspartate aminotransferase, alkaline phosphatase and gamma-glutamyltransferase activity, sodium, potassium, chloride, phosphate, calcium, magnesium, urea, creatinine, glucose, total bilirubin, total protein, albumin, globulins, triglycerides and cholesterol levels, and prothrombin time (AMAX Destiny Plus; Trinity Biotech, Germany).

Pathology. Animals were necropsied after exsanguination under sodium pentobarbital anesthesia. According to OECD 413, selected organs were weighed. All organs and tissues listed in OECD 413 (including brain frontal and parietal lobes, olfactory bulb, pituitary, cervical, thoracic and lumbar spinal cord, sciatic nerve, and eyes with optic nerve, heart, and aorta) were fixed in 4% buffered formaldehyde (corresponding to 10% formalin), paraffin embedded, sectioned and stained with hematoxylin-eosin for histopathology. Full histopathological examinations were performed in animals of the control and high concentration group. The respiratory tract was examined in all test groups. To clarify findings in the lung, periodic acid Schiff (PAS) stain was used on single lung lobes from four animals. Peer review of histopathological findings in the lungs of representative animals was performed by internal reviewer.

Statistical Analysis

Data are reported as mean ± standard deviation. Dunnett's test (Dunnett, 1955, 1964) was used for simultaneous comparison of all concentration groups with the control group for food consumption (g/animal), body weights and body weight changes. Kruskal-Wallis (two-sided) tests, followed if significant by pairwise Wilcoxon tests (Hettmansperger, 1984; Miller, 1981; Nijenhuis and Wilf, 1978; Siegel, 1956) were used for clinical chemistry and hematology, functional observation battery and automated motor activity data. Statistical significance was defined as $p \leq 0.05$ compared with the control group.

RESULTS

Characterization of the Test Substance

Electron microscopic examinations of the test material before and after the dust generation procedure revealed no damage to the tube structure (Fig. 1). The positive control ball-milled material clearly increased ROS (attributable to mechanical stress during the milling process) as indicated by a DPPH color change from purple to yellow. The same test performed on MWCNT before and after the brush generator dust generation procedure did not show any increase in ROS.

The dustiness measurements (DIN 55992-2, Drop method) detected no nanoscale particles above ambient levels, and only a small number of microscale agglomerates. This lack of respirable dust is consistent with the macroscopic appearance of the test substance. It is composed of millimeter-sized granules; when held in the hand, it has the consistency of wool, with a bulk density (density of uncompressed material) of about 0.043 g/ml. Lam *et al.* (2006) also summarized The National Institute for Occupational Safety and Health data which found very low airborne concentrations of MWCNT in the working environments of facilities (<53 $\mu g/m^3$).

Results of the Rangefinding Study

The targeted atmospheric concentrations were 2, 8, and 32 mg/m^3. Actual analyzed concentrations were 2.4 ± 0.7, 8.4 ± 1.6, and 29.8 ± 2.1 mg/m^3. Cascade impactor measurements of particle size resulted in MMAD between 0.5 and 1.3 μm with GSDs between 3.1 and 5.4. The calculated mass fractions of particles below 3 μm aerodynamic size ranged between 77.4 and 86.3%.

No exposure-related clinical signs or altered body weight development or food consumption were noted in animals exposed to 2 or 8 mg/m^3. Body weight gain was reduced in animals exposed to 32 mg/m^3.

In BALF, there were treatment-related increases in total cell counts (due to significantly increased polymorphonuclear neutrophils), total protein content, and enzyme activities in all treated groups (Fig. 4A) on study day 8 (3 days after the last exposure). Lymphocyte counts of the individual animals varied markedly and therefore the increases were not statistically

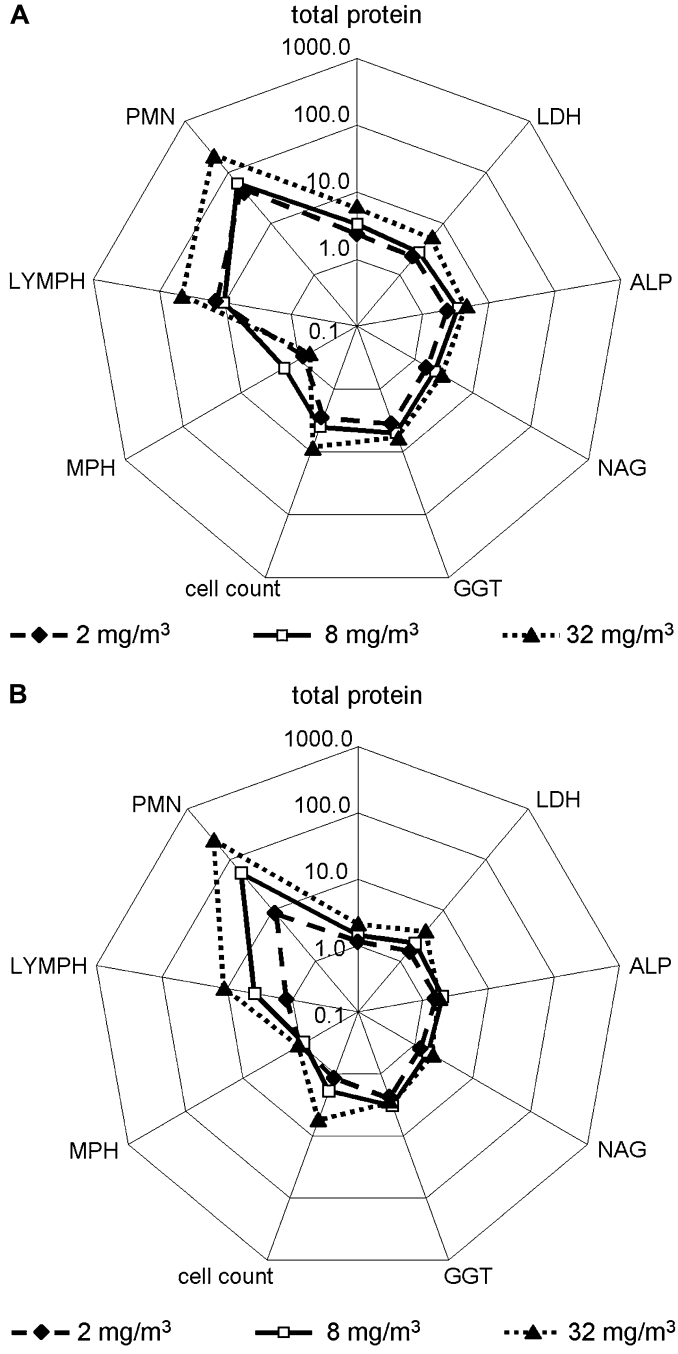

FIG. 4. Changes (fold of control) of the BALF parameters (A) 3 days after the last exposure and (B) 21 days after the last exposure. Abbreviations: LDH: lactate dehydrogenase, ALP: alkaline phosphatase, NAG: N-Acetyl glucosaminidase, GGT: γ-Glutamyltransferase, MPH: macrophage, LYMPH: lymphocytes.

significant. Inhalation exposure had no significant effects on BALF eosinophil or macrophage cell counts.

At the end of the 24-day recovery period, the same pattern of BALF findings was found (Fig. 4B). In the animals exposed to 2 mg/m^3 (the low concentration), slight recovery was observed; protein content and N-acetyl glucosaminidase activity returned to control levels, but the other parameters were still significantly increased ($p < 0.05$).

Treatment had no effect on hematological parameters at either of the two examination time points (end of treatment or end of recovery).

In addition to the changes in BALF, increased lung weights were noted in animals exposed to 8 or 32 mg/m^3 test item. In these animals, mostly small alveolar septal granulomas composed mainly of macrophages were observed. Moreover, irritation of the upper respiratory tract was observed at high concentrations. At 32 mg/m^3, there was minimal to mild diffuse pulmonary histiocytosis and minimal infiltration with neutrophils (granulocytes). Diffuse or focal histiocytosis, particle laden macrophages and bronchoalveolar hypertrophy and hyperplasia were also noted at 2 mg/m^3. These effects were not reversible during the recovery period. Due to the changes in the BALF parameters and the respiratory tract histopathology, a No Observed Effect Concentration (NOEC) after 5-day inhalation exposure could not be established.

Results of the 90-Day Study

Characterization of the test atmosphere. Test substance concentrations and particle size data are summarized in Table 1. Achieved concentrations were close to target concentrations. The online surveillance photometer data showed that the dust aerosol concentrations were maintained throughout all exposures, and air flows were in the desired range. Mean relative humidity in the inhalation system ranged between 39 and 49%, mean temperatures were 21.5–21.6°C. Cascade impactor measurements revealed mass median aerodynamic diameters of between 0.7 and 2.0 μm. The calculated mass fractions of particles below 3 μm aerodynamic size ranged between 66.2 and 90.4%. Thus the dust aerosol particles generated were highly respirable for rats, and a very high proportion was capable of reaching the alveolar region of the lung. Sampling from the test atmosphere on the gold-coated capillary filter showed particles of wool-like structure, which was also the electron microscopic appearance of the test material before dust generation (Figs. 1 and 5).

The particle size distribution in the test atmospheres was also measured by SMPS particle sizer and WELAS scattered light spectrometer. Median particle diameter in the submicron fraction measured by SMPS was about 60 nm. By scattered light spectrometer, the median particle size was 0.6 μm, close to the size measured by cascade impactor. Both SMPS particle sizer and WELAS scattered light spectrometer are only to a very limited extent appropriate for measuring particle size of CNT, due to their particular physical property and shape. SMPS assumes that particles are spherical and measures the mobility of the particles in the electric field. The structure of CNT, however, is not regular and spherical (Fig. 5). Moreover, the charging efficiency of such complex

TABLE 1
Characterization of the Test Atmospheres

Parameter	Target concentration MWCNT		
	0.1 mg/m^3	0.5 mg/m^3	2.5 mg/m^3
Measured concentration ± SD	0.1 ± 0.03	0.5 ± 0.12	2.5 ± 0.45
MMAD (μm)/GSD (three measurements)	2.0/2.8	1.5/2.1	0.7/3.6
	1.3/3.0	1.2/2.7	0.8/4.1
	1.5/3.6	0.9/4.1	0.8/2.8
Count concentration of particles in OPC (number of particles ± SD/cm^3)	30 ± 3	156 ± 9	1182 ± 57
OPC (nm): Count median diameter (Q_0)	580	580	540
Count concentration of particles in SMPS(Number of particles ± SD/cm^3	365 ± 28	5527 ± 241	31,695 ± 3708
SMPS (nm): Count median diameter ± GSD (Q_0)	58 ± 1.8	63.7 ± 1.7	62.8 ± 1.7

Note. Data are mean ± SD unless otherwise noted. SMPS = Scanning Mobility Particle Sizer apparatus, WELAS = light-scattering spectrometer (see ''Method'').

agglomerate structures is not adequately characterized. The light-scattering spectrometer measures the light scattered by the particles and calculates particle size based on a default calibration curve using latex particles which were provided by the manufacturer. As the refraction index of CNT particles is very different to that of the latex particles used for the calibration, the size measured by the spectrometer may not accurately represent the real size. The particle size distributions measured by SMPS and WELAS 2100 can be used to monitor the aerosol spectrum within the test groups. These data are also useful references for other studies with the same test material. However, the SMPS and WELAS data should not be used in a quantitative way, for example, to derive the volume or mass distribution, or to compare the aerosol spectra with other test materials.

Clinical examinations. Exposure to the MWCNT produced no premature mortalities and had no effects on clinical signs, body weights, food consumption, ophthalmoscopy, FOB, or motor activity.

Clinical pathology. At the end of the exposure, total white blood cell counts were significantly increased in males and females exposed to 2.5 mg/m^3 MWCNT. These increases were due to raised neutrophil counts (relative and absolute) and correspondingly decreased relative lymphocyte counts, indicating slight systemic inflammation. All other statistically significant changes in hematological values were within the historical normal ranges of these parameters, and were therefore regarded as nonadverse. There were no treatment-related changes in clinical chemistry or prothrombin time.

Pathology. At necropsy, all animals exposed to the high and mid-concentration had diffuse grey discolored lungs, presumed due to deposition and accumulation of test substance. In addition, discoloration of the mediastinal lymph node was seen in all females and one male exposed to the high concentration as well as in one female and one male exposed to the mid-concentration. Macroscopic lung foci were seen at the high concentration in three males and three females; these were attributed to macrophage accumulation and/or lipoproteinosis. Lung weights (Table 2) were increased concentration dependently.

Histopathology revealed treatment-related lesions in lungs (Table 2), draining mediastinal lymph nodes (Table 3), nasal cavity (Table 4), and larynx and trachea (Table 5). No histopathological abnormalities were observed in any other organ or tissue including brain, olfactory bulb, spinal cord, heart, aorta, liver, kidney, spleen, thymus, gastrointestinal tract, male and female sexual organs, urinary bladder, adrenals, thyroids, skin, muscle, bones (sternum, femur with joint), and bone marrow.

Effects in Lung

Diffuse inflammation in the lungs was seen in all high concentration animals, mainly represented by alveolar

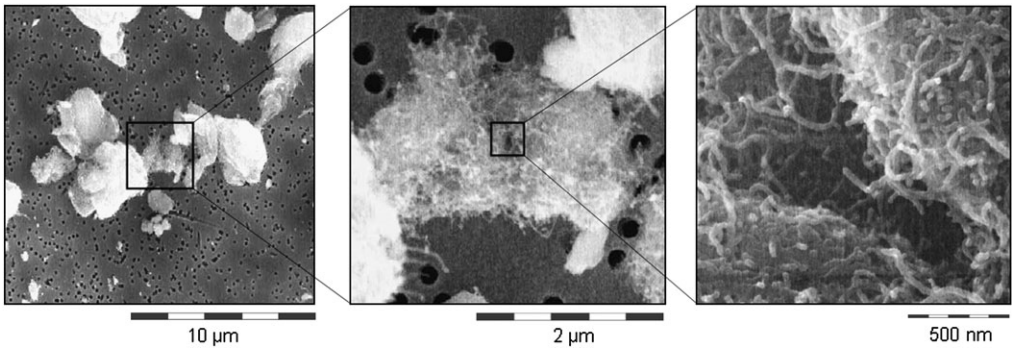

FIG. 5. Scanning electron microscopy images of particles collected from the test atmosphere after dust generation, showing the wool-like clumps of agglomerated MWCNT with ''hairy'' surface at high magnification.

TABLE 2
Effects in Lung

		Male				Female			
	Severity grade	Control	0.1 mg/m^3	0.5 mg/m^3	2.5 mg/m^3	Control	0.1 mg/m^3	0.5 mg/m^3	2.5 mg/m^3
Number of animals		10	10	10	10	10	10	10	10
Relative lung weight		100%	+ 1%	* + 23%	** + 81%	100%	4%	** + 34%	** + 90%
Diffuse histiocytosis	1	—	8	—	—	—	10	—	—
	2	—	—	9	1	—	—	10	—
	3	—	—	1	9	—	—	—	8
	4	—	—	—	—	—	—	—	2
Granulomatous inflammation	1	—	1	—	—	—	4	3	0
	2	—	—	10	—	—	—	7	1
	3	—	—	—	10	—	—	—	9
Intra-alveolar lipoproteinosis	1	—	—	4	—	—	—	1	—
	2	—	—	3	7	—	—	8	—
	3	—	—	—	3	—	—	1	7
	4	—	—	—	—	—	—	—	3
Diffuse neutrophilic inflammation	1	—	—	10	10	—	—	10	10

Note. Severity grades: 1 = minimal, 2 = slight, 3 = moderate, 4 = marked, *$p < 0.05$ versus control, **$p < 0.01$ versus control.

macrophages and—to a lesser extent—neutrophils. The inflammation was accentuated in the centrilobular region in the area of the bronchoalveolar junction. In addition, intraseptal granulomas were observed, composed mainly of alveolar macrophages, occasional multinucleated giant cells, fibroblasts and connective tissue. Many of these macrophages and/or giant cells contained black particles in their cytoplasm which appeared to be MWCNT test substance. These particles were also seen in the cytoplasm of alveolar macrophages in the alveolar lumen and unbound within the alveoli. Despite the fibrotic changes within the focal granulomatous inflammation, there was no sign of pulmonary fibrosis, for example, thickening of alveolar septae, based on the hematoxylin and eosin (H&E) histopathological evaluation. Alveoli contained multifocal eosinophilic, granular material. This material stained PAS-positive in treated animals (single high concentration and control animals were tested; $n = 1$ per sex per group), and is therefore considered to represent lipoproteinosis (Figs. 6E and 6F). The black particles were also seen within bronchial epithelial cells overlying the bronchoalveolar lymphatic tissue (BALT) or within BALT cells in treated animals (females in all concentration groups, males in mid and high concentration groups). There was however no sign of cytotoxicity in this area. Findings in the mid-concentration group were similar but less severe than those in the high concentration group. Most low concentration animals had a minimal increase in alveolar macrophages (8 males, 10 females) and a few had minimal granulomatous inflammation (one male, four females). Alveolar macrophages also contained the same fibrous structures, apparently intracytoplasmic, as in the other treatment groups.

Effects in Mediastinal Lymph Nodes

Minimal to moderate lympho-reticulocellular hyperplasia of the mediastinal lymph nodes (increased lymph node size and

TABLE 3
Effects in Mediastinal Lymph Nodes

		Male				Female			
	Severity grade	Control	0.1 mg/m^3	0.5 mg/m^3	2.5 mg/m^3	Control	0.1 mg/m^3	0.5 mg/m^3	2.5 mg/m^3
Number of animals examined		10	10	10	10	10	10	10	10
Lymphoreticocellular hyperplasia	1	—	—	1	2	—	—	—	1
	2	—	—	1	6	—	—	—	6
	3	—	—	—	—	—	—	—	3
Granulomatous inflammation	1	—	2	4	6	—	4	1	—
	2	—	—	1	4	—	1	8	10
Particles in macrophages		—	5	9	10	—	9	10	10

Note. Severity grades: 1 = minimal, 2 = slight, 3 = moderate, 4 = marked.

TABLE 4
Effects in Nasal Cavity Levels II–IV

			Male				Female			
		Severity grade	Control	0.1 mg/m^3	0.5 mg/m^3	2.5 mg/m^3	Control	0.1 mg/m^3	0.5 mg/m^3	2.5 mg/m^3
Number of animals examined			10	10	10	10	10	10	10	10
Goblet cell hyperplasia	Level II	1	—	—	3	3	—	—	2	6
		2	—	—	1	6	—	—	—	4
Intraepithelial eosinophilic inclusions	Level II	1	—	—	—	3	—	—	5	4
		2	—	—	—	—	—	—	—	3
	Level III	1	—	—	7	4	—	—	7	2
		2	—	—	1	5	—	—	—	8
		3	—	—	—	1	—	—	—	—
	Level IV	1	—	—	3	4	—	—	3	6
		2	—	—	—	5	—	—	—	2
Submucosal inflammatory infiltration	Level II	1	—	—	—	—	—	—	—	3
	Level III	1	—	—	3	7	—	—	2	9
	Level IV	1	—	—	—	2	—	—	—	—

Note. Severity grades: 1 = minimal, 2 = slight, 3 = moderate, 4 = marked.

lymphocyte number) was seen in most high concentration animals, and in one mid-concentration male. Almost all treated animals had fibrous, black staining particles within macrophages, presumed to be MWCNT, located in the mediastinal lymph nodes. In most animals, the formation of small granulomas by particle-loaded macrophages was noted (Fig. 7). The formation of these granulomatous nodules suggests that MWCNT is probably not or only slowly degraded/digested by the macrophages and persists for a relatively long time in the lymph nodes.

Effects in Nasal Cavity, Larynx, and Trachea

In the nasal cavity, the mid and high concentration groups displayed concentration-related multifocal hyperplasia of the mucous-producing goblet cells and an increase in intraepithelial eosinophilic inclusions, mainly in the respiratory epithelium of levels II–IV (Fig. 8). No cytotoxicity was observed. These findings are normal reactions to an unspecific mild irritation (McInnes and Miller, 2007).

In the larynx (level I), minimal epithelial alteration was observed in a small focal area at the base of the epiglottis (epithelium flattened rather than normal cuboidal to columnar) with slightly increased incidence in treated groups. This alteration is regarded as a treatment-related effect but it is also occasionally observed in control animals and is considered to have no adverse functional consequences (Kaufmann *et al.*, 2009).

In the larynx (level III) and trachea of some animals in the mid and high concentration groups, black particles presumed to be CNT, as in the lungs, were observed in the epithelium or in associated macrophages in the submucosa. We cannot rule out the possibility that the particle material was stuck to the surface of the cells, but it did appear at high visual magnification to be intracellular. As no cytotoxicity or cellular degeneration was

TABLE 5
Effects in Larynx and Trachea

			Male				Female			
		Severity grade	Control	0.1 mg/m^3	0.5 mg/m^3	2.5 mg/m^3	Control	0.1 mg/m^3	0.5 mg/m^3	2.5 mg/m^3
Number of animals examined			10	10	10	10	10	10	10	10
Larynx, focal epithelial flattening	Level I	1	2	1	10	6	3	5	9	9
Larynx, intracellular particles	Level III	1	—	—	1	2	—	—	2	3
Trachea, intracellular particles		1	—	—	1	3	—	—	1	6

Note. Severity grades: 1 = minimal, 2 = slight, 3 = moderate, 4 = marked.

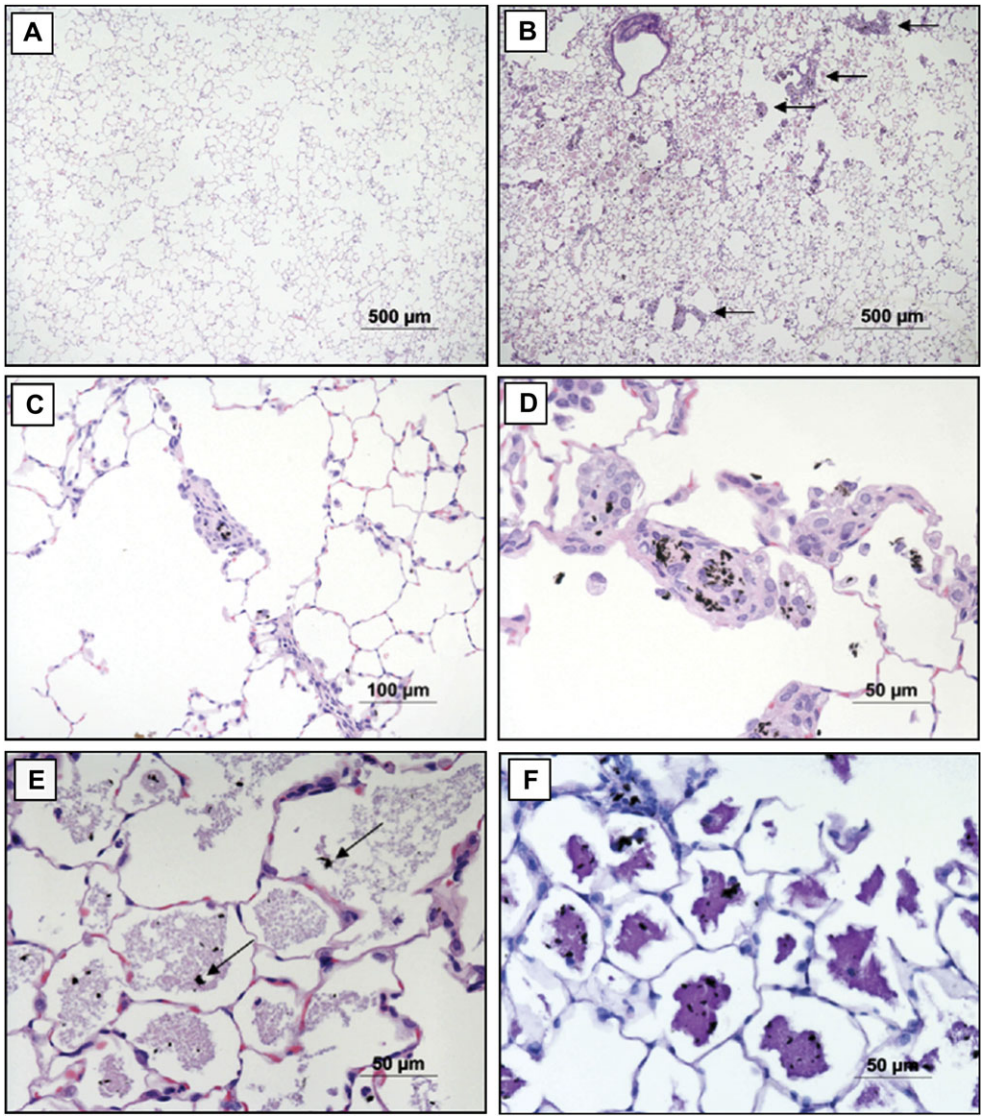

FIG. 6. Overview of the lung section of (A) control rat and (B) rat exposed to 2.5 mg/m^3 MWCNT; arrows indicate multifocal granulomatous inflammation. (C) is a detail view of focal granulomatous inflammation observed in a rat exposed to 0.1 mg/m^3 MWCNT, and (D) in a rat exposed to 2.5 mg/m^3 MWCNT. The dark particles, appear to be intra lesional-located MWCNT. (E) A lung section of a rat exposed to 2.5 mg/m^3 showing intra-alveolar eosinophilic granular material which was diagnosed as lipoproteinosis. The intermingled black staining clumps (arrows) appear to be CNT. A few alveolar macrophages and neutrophils are present. (F) This intra-alveolar eosinophilic granular material was PAS-positive (A–E: H&E stain, F: PAS).

observed in the region of these particles, so this localization is considered unlikely to represent an adverse effect.

DISCUSSION

This study was conducted to obtain sound data on inhalation hazard. There were concerns that the proprietary brush generator might alter the test material, but we found no sign of radical oxygen species generation or damage to the tube walls.

The 5-day rangefinder study demonstrated multifocal granulomatous inflammation and severe diffuse pulmonary histiocytosis with influx of neutrophils and bronchoalveolar hyperplasia at 8 and 32 mg/m^3. Based on the lavage parameters and the histological findings at 2 mg/m^3, a no effect concentration could not be established. The effects were not reversible within the 3-week recovery period. These findings were qualitatively in line with those published by Muller *et al.* (2005) and Lam *et al.* (2004) (even though exposure and recovery times were briefer).

In contrast to previous studies, this 90-day study focused not only on the lung but covered the whole range of potential systemic toxicity with special emphasis on the respiratory tract, including nasal cavity, larynx, trachea, lung, and lung-associated lymph nodes. Moreover, we chose inhalation

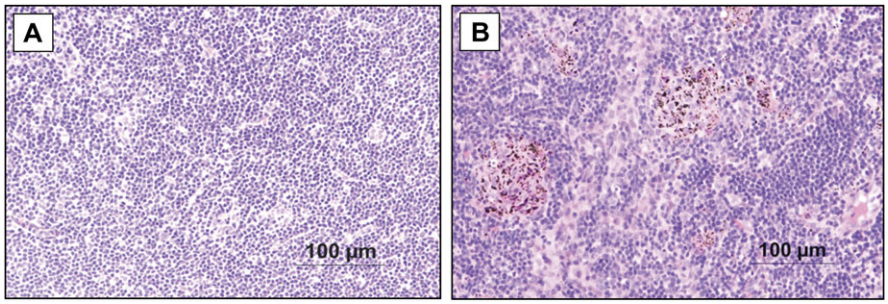

FIG. 7. Mediastinal lymph node of (A) control rat, and (B) rat exposed to 2.5 mg/m^3 MWCNT. In the treated rat there is multifocal granuloma formation with macrophages containing intracytoplasmatic CNT (H&E stain).

exposure, which is considered to be a physiological route for dust entering the host. Although high energy input was required to disperse the substance, this procedure can be regard as simulating the worst case exposure scenario.

The 90-day inhalation exposure to 0.1, 0.5, and 2.5 mg/m^3 MWCNT did not lead to any substance-related clinical signs of toxicity. Clinical pathology revealed only a slight blood neutrophilia in the rats of both sexes exposed to 2.5 mg/m^3 MWCNT, indicating a slight systemic inflammation. Histological examinations of the heart and aorta did not reveal any pathological changes, for example, formation of atherosclerotic plaque in aorta. This finding is not contradictory to that reported by Li *et al.* (2007), because their pharyngeally instilled SWCNT increased plaque area only in the genetically susceptible ApoE mice which were fed on atherogenic diet, but not in those fed on normal diet. It seems this effect cannot be easily induced in healthy animals fed on normal diet.

As previously demonstrated for intratracheally instilled SWCNT (Lam *et al.*, 2004) and MWCNT (Muller *et al.*, 2005), the present study shows that inhalation exposure to MWCNT induces concentration-dependent granulomatous inflammation of the lung and the lung draining lymph nodes. As findings specific to inhalation exposure, inflammation was also observed in the nasal cavity, larynx, and trachea, where the particles deposited during the inhalation exposure.

Overall the histological changes in the respiratory tract were consistent and qualitatively comparable with the previously reported toxicological pattern for MWCNT after intratracheal instillation (Muller *et al.*, 2005) and for SWCNT after intratracheal instillation (Lam *et al.*, 2004; Shvedova *et al.*, 2005) and inhalation (Shvedova *et al.*, 2008). In contrast to the intratracheal MWCNT study, we did not observe diffuse pulmonary fibrosis but pronounced alveolar lipoproteinosis. To evaluate whether this difference is due to different substance dose in the lung, the lung burden in the present study should be compared with the instilled doses used in the intratracheal studies. There is no appropriate method for quantifying the deposited MWCNT, so we made estimates based on the form and the aerodynamic size of the particles. As regards the form, electron microscopic examination of the dust particles in the test atmospheres revealed particle clumps (agglomerates) of a few hundred nanometers to a few micrometers diameter, with a "hairy" surface consisting of numerous ends of MWCNT (Fig. 5). This means that although individual CNT have "nanoscale" dimensions, their predilection for electrostatic attraction and consequent agglomeration make their aerodynamic behavior particle-like rather than fiber like. The cascade impactor measurement showed MMADs between 0.7 and 2.0 µm. For particles in this aerodynamic size range, pulmonary deposition for rat after inhalation exposure was estimated at approximately 10% by Snips (1989), which is in

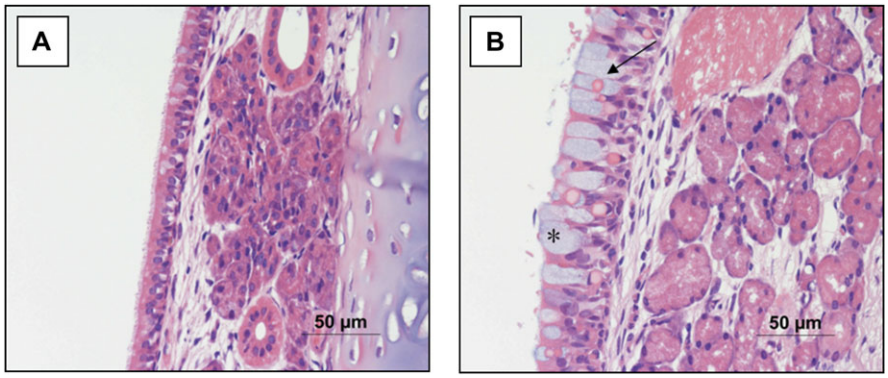

FIG. 8. Nasal cavity of (A) control rat and (B) rat exposed to 2.5 mg/m^3 MWCNT. The treated rat has goblet cells hyperplasia (star) and increased incidence of eosinophilic intraepithelial inclusions (arrow) in the respiratory epithelium (H&E stain).

good agreement with our experience (Ma-Hock *et al*., 2009). Assuming deposition of 10%, disregarding any clearance, and given the mean measured atmospheric concentrations and a respiratory minute volume of 0.2 l/min (Snips, 1989), we estimate pulmonary deposition of 46.8 μg, 243 μg, and 1170 μg per lung after 90 days (65 exposures) inhalation exposure to 0.1, 0.5, and 2.5 mg/m^3 MWCNT, respectively. According to this calculation, only the high concentration of 2.5 mg/m^3 resulted in pulmonary deposition in the "overload" range (Snips, 1989). These calculated pulmonary doses are lower than the intratracheal doses used in previous studies (Lam *et al*., 2004; Muller *et al*., 2005; Warheit *et al*., 2004).

With regard to pulmonary fibrosis, Muller *et al.* (2005) reported significantly increased hydroxylproline in lung tissue at dose groups 2 and 5 mg per rat, but not at 0.5 mg per rat. The doses which caused fibrogenic effect were at least twice those deposited in the current study, thus this difference is likely to be a consequence of the different dose range. We observed only fibrotic changes within the focal granulomatous inflammation, but no diffuse pulmonary fibrosis, based on routine H&E diagnostic. This diagnosis may however miss minimal diffuse increases of fibrous tissue. A more collagen-specific stain (e.g., Sirius red) was not performed because we observed only fibrous tissue within the granulomatous inflammation but no sign of any diffuse fibrosis, for example, alveolar septal thickening. Summarizing these considerations, we conclude that no pronounced diffuse fibrous change was observed in the lung.

Alveolar lipoproteinosis has not been previously reported in association with exposure to CNT materials despite the higher doses in the instillation studies. Although it is mechanistically not well understood, this effect seems to be substance specific. As lipoproteinosis has been observed in rats after inhalation exposure to quartz dust (Heppleston, 1975; Porter *et al*., 2004) as well as in humans in acute silicosis (Hoffmann *et al*., 1973), we consider this effect as highly relevant for health hazard assessment.

The contribution of the metal content in MWCNT materials to their toxicity has been discussed controversially since the very beginning of MWCNT materials. In fact, all manufacturing techniques require the present of metal catalyst. Unpurified CNT product invariably contains various types and amounts of residual metal, for example, iron, nickel, cobalt. The majority of commercially available MWCNT, including that examined in the present study, is produced by the vapor deposition technique. For SWCNT, the first comparison of materials with different metal content was performed by Lam *et al.* (2004). They found comparable toxicity for all materials regardless of metal content, and concluded that granulomatous inflammation was due to the CNT. Muller *et al.* (2008) reported that MWCNT is clastogenic in rat lung cells *in vitro* and *in vivo*. The possible contribution of cobalt as residual (1%) catalyst was discussed, because cobalt is clastogenic according to the same investigators (De Boeck *et al*., 2003). The material tested in the present study contains mainly aluminium oxide (9.6%) as impurity, with less than 0.2% cobalt. To date, there has been no indication that any of these impurities is capable of inducing progressive granulomatous inflammation in the lung after inhalation exposure at concentrations as low as tested in this study (Lindenschmidt *et al*., 1990; Warheit *et al*., 1991). This strongly implies that MWCNT induced the lung lesions.

At the lowest tested concentration of 0.1 mg/m^3, the incidence of granulomatous inflammation was low and the severity grade minimal; only single granulomas were observed (versus multifocal at higher concentrations). Although these findings at the low concentration are subclinical and unlikely to be associated with functional effects, they mean that a NOEC could not be established; 0.1 mg/m^3 is the LOEC.

MWCNT toxicity appears to differ significantly from that of the well-characterized nanostructured industrial carbon black Printex 90 (Degussa, Germany), a form of amorphous carbon with large surface area (> 300 m^2/g). After 90-day inhalation exposure to this material, there was pneumocyte type II cell hyperplasia/hypertrophy, lipoproteinosis and pulmonary fibrosis 3 and 11 months postexposure at an atmospheric concentration of 50 mg/m^3, corresponding to a measured lung burden of 3.5 mg per animal (Elder *et al*., 2005). Although the achieved dose of carbon black (lung burden) was significantly higher than the highest lung burden calculated in the present study (about 1 mg per animal), no granulomatous inflammation were observed with carbon black. At 7 mg/m^3, which corresponds to a lung burden of approximately 1 mg per animal, some effects were still evident but much less severe, and some effects were not observed at all (e.g., type II cell hyperplasia/hypertrophy, influx of neutrophile, and pulmonary fibrosis). The NOEC for carbon black after 90-day inhalation exposure was 1 mg/m^3, 10 times higher than the LOEC for MWCNT in the present study. This comparison demonstrates that the MWCNT we tested possesses higher toxic potency per unit mass than nanostructured carbon black Printex 90. The mechanism for this needs further investigation.

In conclusion, the results clearly show that inhaled MWCNT produces lesions (inflammation and granuloma formation) in the lung and associated lymph nodes similar to those after intratracheal administration. Apart from a slight neutrophilia at the highest tested concentration of 2.5 mg/m^3 MWCNT, there was no substance-related systemic toxicity at any tested concentration. Only nonspecific adaptive responses are seen in the upper respiratory tract (nasal cavity and larynx). However, because of minimal granulomatous inflammation in the lung, the NOEC is less than 0.1 mg/m^3. In parallel to the hazard assessment, the dust-forming potential of these MWCNT measured by standardized drop methods was found to be relatively low. These hazard and potential exposure data are essential for the risk assessment procedure, and demand strictest industrial hygiene measures for handling these MWCNT. The experimental data also provide a basis for comparing toxicity and dustiness potencies of various

MWCNT and thus allow the selection of MWCNT associated with the lowest risk for given applications.

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