

## Comparative Pulmonary Toxicity Assessment of Single-wall Carbon Nanotubes in Rats

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The aim of this study was to evaluate the acute lung toxicity of intratracheally instilled single-wall carbon nanotubes (SWCNT) in rats. The lungs of rats were instilled either with 1 or 5 mg/kg of the following control or particle types: (1) SWCNT, (2) quartz particles (positive control), (3) carbonyl iron particles (negative control), (4) phosphate-buffered saline (PBS) + 1% Tween 80, or (5) graphite particles (lung tissue studies only). Following exposures, the lungs of PBS and particle-exposed rats were assessed using bronchoalveolar lavage (BAL) fluid biomarkers and cell proliferation methods, and by histopathological evaluation of lung tissue at 24 h, 1 week, 1 month, and 3 months postinstillation. Exposures to high-dose (5 mg/kg) SWCNT produced mortality in ~15% of the SWCNT-instilled rats within 24 h postinstillation. This mortality resulted from mechanical blockage of the upper airways by the instillate and was not due to inherent pulmonary toxicity of the instilled SWCNT particulate. Exposures to quartz particles produced significant increases versus controls in pulmonary inflammation, cytotoxicity, and lung cell parenchymal cell proliferation indices. Exposures to SWCNT produced transient inflammatory and cell injury effects. Results from the lung histopathology component of the study indicated that pulmonary exposures to quartz particles (5 mg/kg) produced dose-dependent inflammatory responses, concomitant with foamy alveolar macrophage accumulation and lung tissue thickening at the sites of normal particle deposition. Pulmonary exposures to carbonyl iron or graphite particles produced no significant adverse effects. Pulmonary exposures to SWCNT in rats produced a non-dose-dependent series of multifocal granulomas, which were evidence of a foreign tissue body reaction and were nonuniform in distribution and not progressive beyond 1 month postexposure (pe). The observation of SWCNT-induced multifocal granulomas is inconsistent with the following: (1) lack of lung toxicity by assessing lavage parameters, (2) lack of lung toxicity by measuring cell proliferation parameters, (3) an apparent lack of a dose response relationship, (4) nonuniform distribution of lesions, (5) the paradigm of dust-related lung toxicity effects, (6) possible regression of effects over time. In addition, the results of two recent exposure assessment studies indicate very low aerosol SWCNT exposures at the work-

place. Thus, the physiological relevance of these findings should ultimately be determined by conducting an inhalation toxicity study.

**Key Words:** single-wall carbon nanotubes; SWCNT; pulmonary toxicity; nanoparticle toxicity; carbon nanotubes.

Carbon nanotubes are known to have superior mechanical, electrical, and magnetic properties. Single-wall nanotubes are a component of carbon nanotubes and are self-organized into ropelike structures, which can range in length up to several microns. The potential hazards related to inhalation of carbon nanotubes are unknown. Moreover, the toxicological database for most of the carbon-containing particulates is rather sparse. Recent experimental studies in rats indicate that inhaled carbon black particles may produce significant lung toxicity in rats, and the toxicity potential increases with decreasing particle size and increasing surface area. Thus, ultrafine carbon black particles are known to produce greater pulmonary toxicity in rats when compared to larger-sized carbon black particles [furnace black particles—mean diameter = 14 nm (surface area = 270 m<sup>2</sup>/g); lamp black particles—mean diameter = 95 nm (surface area = 22 m<sup>2</sup>/g)] (Driscoll *et al.*, 1996; Heinrich *et al.*, 1994, 1995; Nikula *et al.*, 1995).

There is also limited toxicological information on carbon fibers and on graphite particles. The results of several epidemiological studies indicated an increased incidence of pneumoconiosis among workers exposed to graphite-containing dusts. These include miners and millers, carbon electrode manufacturers, and molders. The pneumoconiosis reported in both synthetic and natural graphite workers resembles coal workers' pneumoconiosis. Similarly, there is a paucity of data on the health effects related to carbon nanotube exposures. As a consequence, no exposure guidelines have yet been proposed. Some preliminary exposure assessment studies have recently been reported, and the findings suggest that there are low aerosol exposure levels at the workplace (Baron *et al.*, 2002; Joseph, 2002).

This study was designed as a preliminary screen to determine whether the SWCNT particles impart significant toxicity

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in the lungs of rats, and more importantly, how the activity of the carbon-derived particulates compares with other reference particulate materials. Thus, the aim was to assess in rats, using a well-developed, short-term pulmonary bioassay, (1) the acute pulmonary toxicity effects of intratracheally instilled single-wall carbon nanotube (SWCNT) samples and to compare the lung toxicity of these samples with a low-toxicity particulate (negative control) and a cytotoxic particulate (positive control) sample, and (2) to bridge the results of these instillation studies with data previously generated from inhalation studies with quartz particles in the form of crystalline silica and with carbonyl iron particles as the inhalation/instillation bridge material. In this study, intratracheal instillation exposure was used as a surrogate for inhalation exposure.

## MATERIALS AND METHODS

**Animals.** Groups of male Crl:CD(SD)IGS BR rats (Charles River Laboratories, Inc., Raleigh, NC) were used in this study. The rats were approximately 8 weeks old at study start (mean weights in the range of 240–255 grams). All procedures using animals were reviewed and approved by the Institutional Animal Care and Use Committee, and the animal program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

**Particle types.** Quartz particles in the form of crystalline silica (Min-U-Sil 5) were obtained from Pittsburgh Glass and Sand Corporation. The particle sizes range from 1 to 3  $\mu\text{m}$ . Carbonyl iron particles were obtained from GAF Corp. The particle sizes range from 0.8  $\mu\text{m}$  to 3.0  $\mu\text{m}$ . SWCNT soot generated via a laser ablation process (Rinzler *et al.*, 1998) was obtained from GAM Reynolds and DH Roach of DuPont Central Research. The nominal size of single-wall nanotubes is 1.4 nm diameter  $\times$   $>1$   $\mu\text{m}$  length. However, the nanotubes rarely exist as individual units and exist primarily as agglomerated “ropes” of nanotubes of  $\sim 30$  nm diameter. The soot is composed of about 30–40% amorphous carbon (by weight) and 5% each of nickel and cobalt, with the balance being the carbon nanotube agglomerates. As a control sample, a mixture of graphite particles (Carbone of America Ultra Carbon Division UCP-1-M grade) and catalyst particles with the same percentages of catalysts as the carbon nanotube soot was also obtained from GAM Reynolds and DH Roach. The particle size range of the graphite was 3–10  $\mu\text{m}$ , and that of the cobalt and nickel particles was 2–3  $\mu\text{m}$ .

**General experimental design.** The fundamental features of this pulmonary bioassay are (1) dose response evaluation and (2) time course assessments to determine the sustainability of any observed effect. Thus, the major endpoints of this study were the following: (1) time course and dose/response intensity of pulmonary inflammation and cytotoxicity, (2) alveolar macrophage function at 1 week postexposure (pe), (3) airway and lung parenchymal cell proliferation, and (4) histopathological evaluation of lung tissue.

Groups of rats were intratracheally instilled with 1 or 5 mg/kg of SWCNT, quartz-crystalline silica particles (Q), or carbonyl iron (CI) particles. All particles were prepared in a volume of 1.0% Tween 80 and phosphate-buffered saline (PBS) and subjected to polytron dispersment. Groups of PBS and PBS-Tween instilled rats served as controls. The lungs of PBS, PBS-Tween, and particle-exposed rats were evaluated by bronchoalveolar lavage at 24 h, 1 week, 1 month, and 3 months pe. For the morphological studies, additional groups of animals were instilled with the particle types listed above as well as PBS, PBS-Tween, and graphite particles. These studies were dedicated for lung tissue analyses and consisted of cell proliferation assessments and histopathological evaluations of the lower respiratory tract. Similar to the BAL fluid studies, the intratracheal instillation exposure period was followed by 24-hour, 1-week, 1-month, and 3-month recovery periods.

**Bronchoalveolar lavage studies.** Groups of male rats were exposed via intratracheal instillation to (1) vehicle control–PBS, (2) additional vehicle control–PBS + 1% Tween 80, (3) SWCNT in 1% Tween + PBS at 1 and 5 mg/kg, (4) Min-U-Sil crystalline quartz particles in 1% Tween + PBS at 1 and 5 mg/kg, and (5) carbonyl iron particles in 1% Tween + PBS at 1 and 5 mg/kg. All particle types were suspended in phosphate-buffered saline and 1% Tween 80.

**Pulmonary lavage and biochemical analyses.** The lungs of sham and particulate-exposed rats were lavaged with a warmed PBS solution as described previously (Warheit *et al.*, 1991, 1997). Briefly, the lungs were removed from the thoracic cavity and lavaged with a PBS solution that had been heated to  $\sim 37^\circ\text{C}$ . A 10-ml syringe was used to fill the lungs with  $\sim 8$  ml of PBS per wash. The lungs were gently manipulated after insertion of the PBS and during the withdrawal of lavage fluid. The first recovered 12 ml of lavaged fluids was used for BAL fluid analyses, and an additional 30 ml was collected for cell counts and differentials. Methodologies for cell counts, differentials, and pulmonary biomarkers in lavaged fluids were conducted as previously described (Warheit *et al.*, 1991, 1997). All biochemical assays were performed on BAL fluids using a Roche Diagnostics (BMC)/Hitachi 717 clinical chemistry analyzer using Roche Diagnostics (BMC)/Hitachi reagents. Lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and lavage fluid protein were measured using Roche Diagnostics (BMC)/Hitachi reagents. Lactate dehydrogenase is a cytoplasmic enzyme and is used as an indicator of cell injury. Alkaline phosphatase activity is a measure of Type II alveolar epithelial cell secretory activity, and increased ALP activity in BAL fluids is considered to be an indicator of Type II cell toxicity. Increases in BAL fluid protein concentrations generally are consistent with enhanced permeability of vascular proteins into alveolar regions.

**Chemotaxis Studies.** Chemotactic studies were conducted as previously described (Warheit *et al.*, 1986). Alveolar macrophages were collected from particulate-exposed or control rats by lavage as described above. The chemotaxis assay was carried out using three concentrations (i.e., 1, 5, and 10%) of normal heated sera (NHS) as the chemotactic stimulus. One hundred microliters of the activated sera was pipetted into the lower portion of a blind well chamber. A polycarbonate filter (pore size = 5  $\mu\text{m}$ ) was inserted between the lower and upper compartments. A concentration of  $2.0 \times 10^6$  lavage-recovered alveolar cells/mostly macrophages was pipetted into the upper compartment of the chamber. The chemotaxis assay was carried out for 3.5 h in an incubator at  $37^\circ\text{C}$  (5%  $\text{CO}_2$  concentration). Following termination of the assay, the polycarbonate filters were removed from the chambers, fixed with ethanol, and stained with Diff-Quik for light microscopy. The cells on the top of the filter were removed with a wet swab, and the filter was placed face down (lower end of the filter) on a coverslip. The numbers of macrophages which had migrated from the top to the bottom of the polycarbonate filter were counted by light microscopy ( $\times 1000$ ) of 20 predetermined high-power fields.

**Pulmonary cell proliferation studies.** This experiment was designed to measure the effects of particle exposures on airway and lung parenchymal cell turnover in rats following 24-h, 1-week, and 1- or 3-month pe periods. Groups of particulate-exposed rats and corresponding controls were pulsed 24 h after instillation, as well as 1 week, 1 month, and 3 months pe, with an intraperitoneal injection of 5-bromo-2'-deoxyuridine (BrdU) dissolved in a 0.5 N sodium bicarbonate buffer solution at a dose of 100 mg/kg body weight. The animals were euthanized 6 h later by pentobarbital injection. Following cessation of spontaneous respiration, the lungs were infused with a neutral buffered formalin fixative at a pressure of 21 cm  $\text{H}_2\text{O}$ . After 20 min of fixation, the trachea was clamped, and the heart and lungs were carefully removed *en bloc* and immersion-fixed in formalin. In addition, a 1-cm piece of duodenum (which served as a positive control) was removed and stored in formaldehyde. Subsequently, parasagittal sections from the right cranial and caudal lobes and regions of the left lung lobes, as well as the duodenal sections, were dehydrated in 70% ethanol and sectioned for histology. The sections were embedded in paraffin, cut, and mounted on glass slides. The slides were stained with an anti-BrdU antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) and with an AEC (3-amino-9-ethyl carbazole) marker and were counter-

stained with aqueous hematoxylin. At least 1000 cells/animal were counted each in terminal bronchiolar and alveolar regions. For quantification of airway cells, random terminal bronchioles were selected at low magnification, and airway cells were counted at higher magnification. For quantification of lung parenchymal cells, random bronchoalveolar junctions were selected at low magnification, and moving distally toward the direction of the pleura, the cells were counted at higher magnification. For each treatment group, immunostained nuclei in airways (i.e., terminal bronchiolar epithelial cells) or lung parenchyma (i.e., epithelial cells, interstitial cells, or macrophages) were counted by light microscopy at  $\times 1000$  magnification (Warheit *et al.*, 1991, 1997).

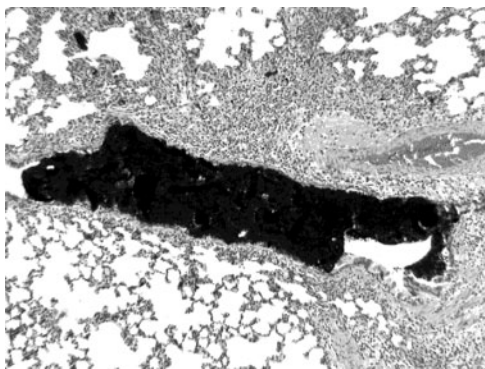
**Morphological studies.** The lungs of rats exposed to particulate-exposed or PBS-Tween controls were prepared for microscopy by airway infusion under pressure (21 cm H<sub>2</sub>O) at 24 hours, 1 week, 1 month, and 3 months pe. Sagittal sections of the left and right lungs were made with a razor blade. Tissue blocks were dissected from left, right upper, and right lower regions of the lung and were subsequently prepared for light microscopy (paraffin embedded, sectioned, and hematoxylin-eosin stained) and evaluated (Warheit *et al.*, 1991, 1997).

**Statistical analyses.** For analysis, each of the experimental values was compared to its corresponding sham control value for each time point. A one-way analysis of variance (ANOVA) and Bartlett's test were calculated for each sampling time. When the *F* test from ANOVA was significant, the Dunnett test was used to compare means from the control group and each of the groups exposed to particulates. Significance was judged at the 0.05 probability level.

## RESULTS

### SWCNT-Related Mortality

Exposure to high-dose (5 mg/kg) carbon nanotubes produced mortality in  $\sim 15\%$  of the SWCNT-instilled rats within 24 h postinstillation. Upon further extensive investigation, this mortality was determined to result from mechanical blockage of the upper airways by the instillate and was not due to inherent pulmonary toxicity of the instilled SWCNT particulate (Fig. 1). Thus, it is believed that the mortality caused by exposure to SWCNT 5 mg/kg was an artifact of the exposure regimen, due, in large part, to the nature of the carbon nanotubes, which are highly electrostatic and do not disperse into



**FIG. 1.** Light micrograph of lung tissue from a rat exposed to 5 mg/kg SWCNT (a few h after exposure). The major airways are mechanically blocked by the SWCNT instillate. This led to suffocation in 15% of the CNT-exposed rats and was not evidence of pulmonary toxicity of SWCNT.

individual nanotubes (1 nm diameter  $\times$  1  $\mu$ m length), but instead form "nanoropes" that consist of agglomerates of 10–100 nanotubes. In addition, careful observations of the animals indicated that the rats that survived the 5 mg/kg SWCNT dose for a 24-hour period appeared normal throughout the duration of the study, as evidenced by subsequent normal eating behavior and weight gain throughout the pe period. Instillation of SWCNT at 1 mg/kg or exposures to any other particle types did not produce mortality in exposed rats.

### Lung Weights

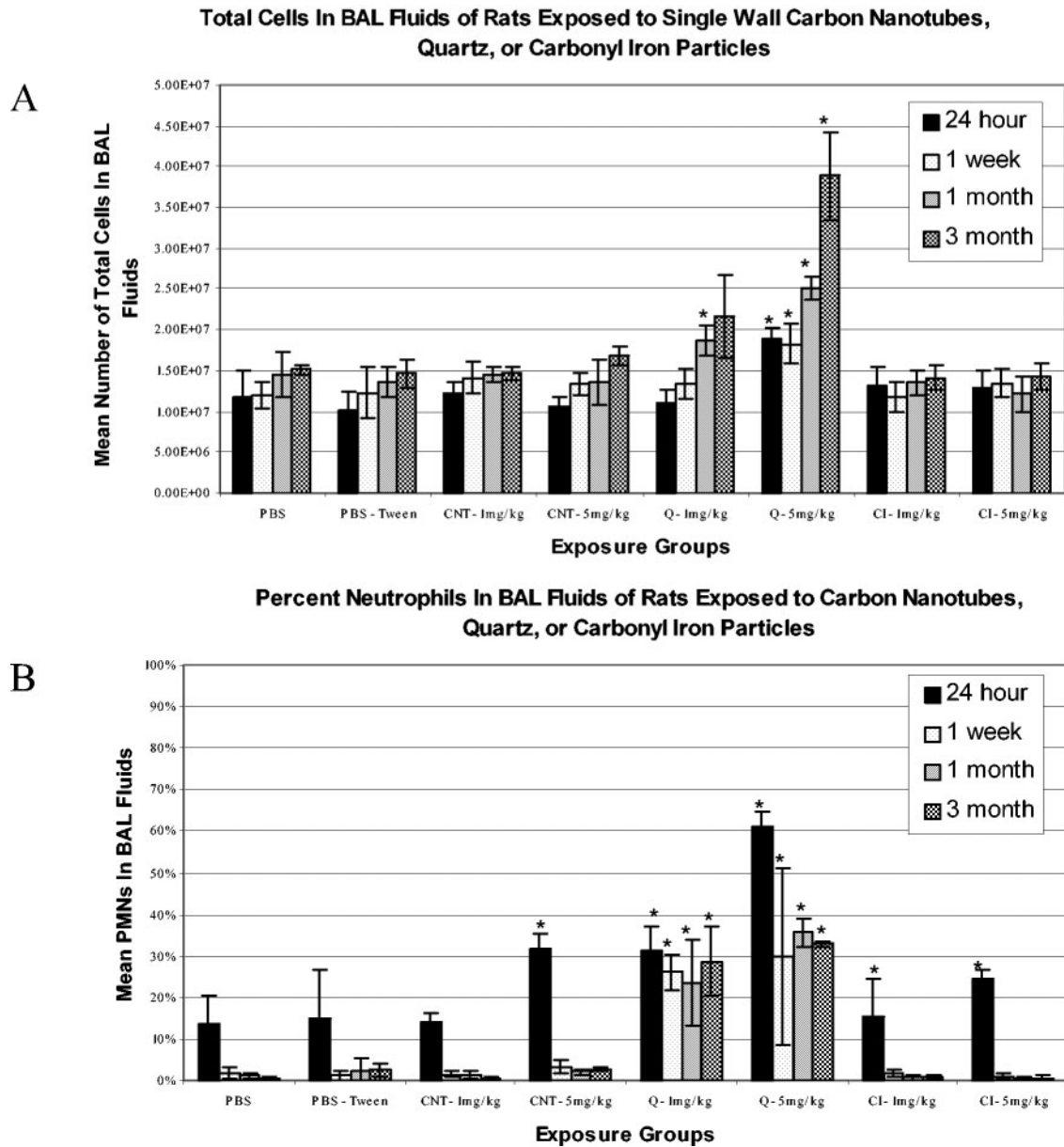
Lung weights of rats increased with increasing age (i.e., increased pe time periods following instillation). Lung weights in high-dose SWCNT-exposed rats were significantly increased versus controls at 24 h, 1 week, and 1 month but not at 3 months pe (data not shown).

### Pulmonary Injury and Inflammation

The numbers of cells recovered by bronchoalveolar lavage from the lungs of high-dose quartz-exposed (5 mg/kg) groups were significantly higher than any of the other groups for all pe time periods (Fig. 2A). Intratracheal instillation exposures of several particle types produced a short-term, pulmonary inflammatory response, as evidenced by an increase in the percentages/numbers of BAL-recovered neutrophils, measured at 24 h postexposure. However, only the exposures to quartz particles (1 and 5 mg/kg) produced sustained pulmonary inflammatory responses, as measured through 3 months pe (Fig. 2B).

Transient increases in BAL fluid lactate dehydrogenase values were measured in the lungs of high-dose (5 mg/kg) SWCNT-exposed rats at 24 h postexposure, but were not sustained through the other pe time periods. In contrast, exposures to 5 mg/kg quartz particles produced a sustained increase in BAL fluid LDH values through the 3-month pe period (Fig. 3A). Transient increases in BAL fluid microprotein values were measured in the lungs of high-dose (5 mg/kg) SWCNT-exposed rats at 24 h pe, but were not different from controls at 1 week pe. In contrast, exposures to 5 mg/kg quartz particles produced a sustained increase in BAL fluid microprotein values at 24 h, 1 month, and 3 months pe (Fig. 3B). Transient increases in BAL fluid alkaline phosphatase values were measured only in the lungs of quartz-exposed rats at 24 h pe (1 mg/kg) and at the 1-week (5 mg/kg) pe time periods (data not shown).

Chemotaxis studies conducted at 1 week pe demonstrated that alveolar macrophages exposed to quartz particles (5 mg/kg) were impaired in their chemotactic responses to normal heated sera when compared to controls (data not shown). This deficit in cell motility represents a deficiency in macrophage function and clearance capacity and is due, in part, to the cytotoxicity and inflammation caused by exposure to quartz particles. Exposures to SWCNT or carbonyl iron particles did



**FIG. 2.** (A) Numbers of cells recovered in BAL fluids from particulate-exposed rats and corresponding controls at 24 h, 1 week, 1 month, and 3 months pe. Values are means  $\pm$  SD. Exposures to 5 mg/kg quartz (Q)-particles produced significantly increased numbers of cells at all pe time points.  $*p < 0.05$ . (B) Pulmonary inflammation in particulate-exposed rats and controls as evidenced by % neutrophils (PMN) in BAL fluids at 24 h, 1 week, 1 month, and 3 months pe. Instillation exposures resulted in transient inflammatory responses for nearly all groups at 24 h pe. However, exposures to quartz particles at 1 and 5 mg/kg produced a sustained lung inflammatory response.  $*p < 0.05$ .

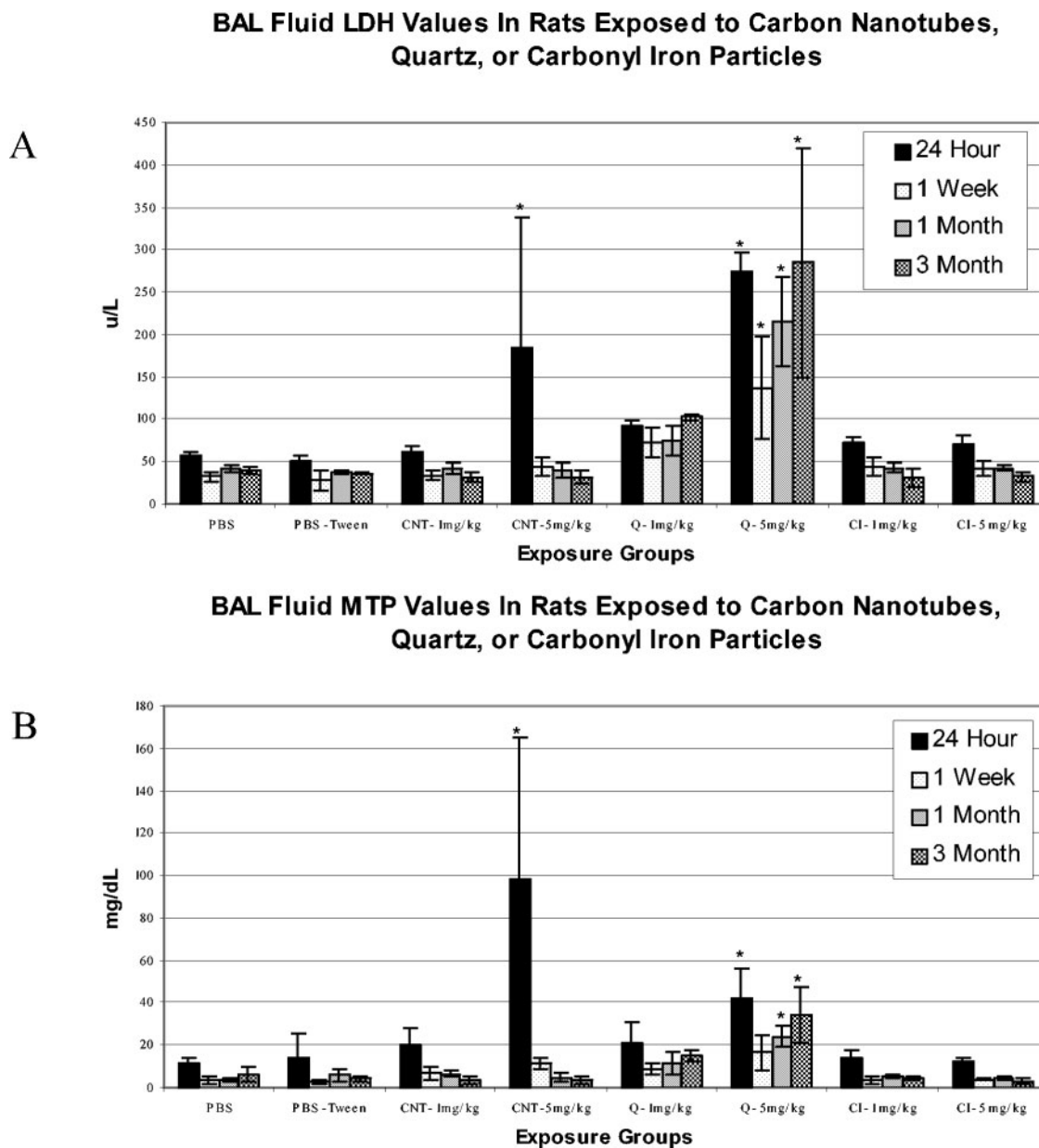
not result in functional deficits of bronchoalveolar lavage-recovered macrophages.

Analyses of lung tissues revealed that pulmonary exposures to carbonyl iron or graphite particles in rats produced no significant adverse effects when compared to PBS-Tween exposed controls, as evidenced by the normal lung architecture observed in the exposed animals at 1 month postinstillation exposure.

Histopathological evaluation of lung tissues revealed that

pulmonary exposures to quartz particles in rats produced a dose-dependent lung inflammatory response characterized by neutrophils and foamy (lipid-containing) alveolar macrophage accumulation. In addition, lung tissue thickening as a prelude to the development of fibrosis was evident and progressive (Fig. 4, left and right panels).

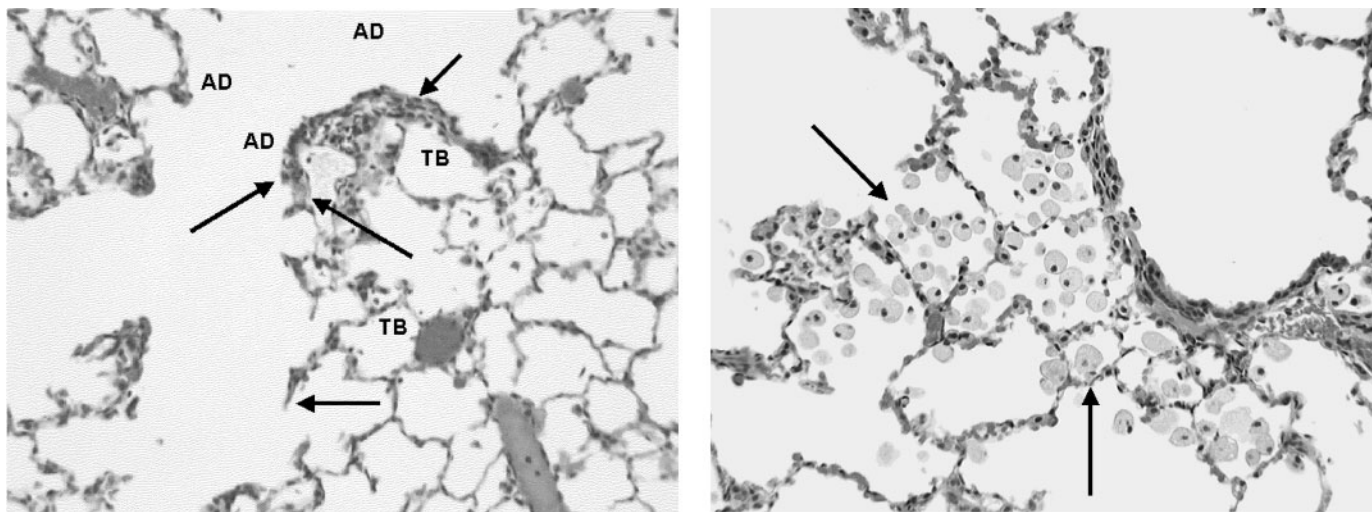
Pulmonary exposures to SWCNT in rats produced a non-dose-dependent series of multifocal granulomas, and this was evidence of a foreign tissue body reaction. The early develop-



**FIG. 3.** (A) BAL fluid LDH values for particulate-exposed rats and corresponding controls at 24 h, 1 week, 1 month, and 3 months pe. Significant increases in BALF LDH versus controls were measured in the 5 mg/kg SWCNT-exposed group at 24 h pe and the 5 mg/kg quartz (Q)-exposed animals at all 4 time periods pe. \* $p < 0.05$ . (B) BAL fluid protein (MTP) values for particulate-exposed rats and corresponding controls at 24 h, 1 week, 1 month, and 3 months pe. Significant increases in BALF MTP vs. controls were measured in the 5 mg/kg SWCNT-exposed group at 24 h pe and the 5 mg/kg quartz-exposed animals at 3 time periods pe. \* $p < 0.05$ .

ment of lesions was first observed at 1 week pe, wherein the lesions surrounded the instilled SWCNT, and this was associated with a nonuniform, diffuse pattern of carbon nanotube particulate deposition in the lung (Fig. 5, top panel). Subsequently, at 1 month pe, a diffuse pattern of multifocal macrophage-containing granulomas was present. It was interesting to note that few lesions existed in some lobes, while other lobes contained several granulomatous lesions. This was likely due to the nonuniform deposition pattern following SWCNT instil-

lation (Fig. 5, middle panel). At higher magnification, one could discern the discrete multifocal mononuclear granulomas centered around the carbon nanotube material, most likely in the form of nanoropes (Fig. 5, bottom panel). There seemed to be little if any progression of the lesion at 3 months pe, as the number of lesions was either reduced or not increased and the distribution pattern continued to be uneven/nonuniform. In some cases, the SWCNT bolus had not exited out of the airways (terminal bronchioles) into the alveolar regions, but



**FIG. 4.** (Left panel) Higher magnification light micrograph of lung tissue from a rat exposed to quartz particles (5 mg/kg) at 1 month postinstillation exposure. Note the prominence of tissue thickening (arrows) at the junction at the terminal bronchiole and alveolar duct bifurcation. Magnification =  $\times 400$ . (Right panel) Light micrograph of lung tissue from another rat exposed to quartz particles (5 mg/kg) at 3 months postinstillation exposure. Note the tissue accumulation of foamy multinucleated alveolar macrophages (arrows) within alveolar spaces. The macrophages have migrated to the sites of quartz particle instillation at the terminal bronchiolar alveolar junctions. The accumulation of lipid-filled macrophages and lack of clearance is a common feature of the progressive nature of silica induced lung disease. Magnification =  $\times 100$ .

remained as a large bolus of nanoropes in the airways. The pulmonary response was to surround the nanotube/nanorope bolus with mononuclear cells. This is highly unusual, and the granulomatous response represents an attempt to sequester the instilled carbon nanotubes.

#### *Pulmonary Cell Proliferation*

Tracheobronchial cell proliferation rates (percentage immunostained cells taking up BrdU) were measured in particulate-exposed rats and corresponding controls at 24 h, 1 week, 1 month, and 3 months pe. Although increases in cell labeling indices were noted in SWCNT (5 mg/kg at 24 h pe) and quartz-exposed animals (1 and 5 mg/kg at 24 h–3 months pe), no statistically significant increases in BrdU immunostained cells were measured in these groups versus controls at any time point pe (data not shown).

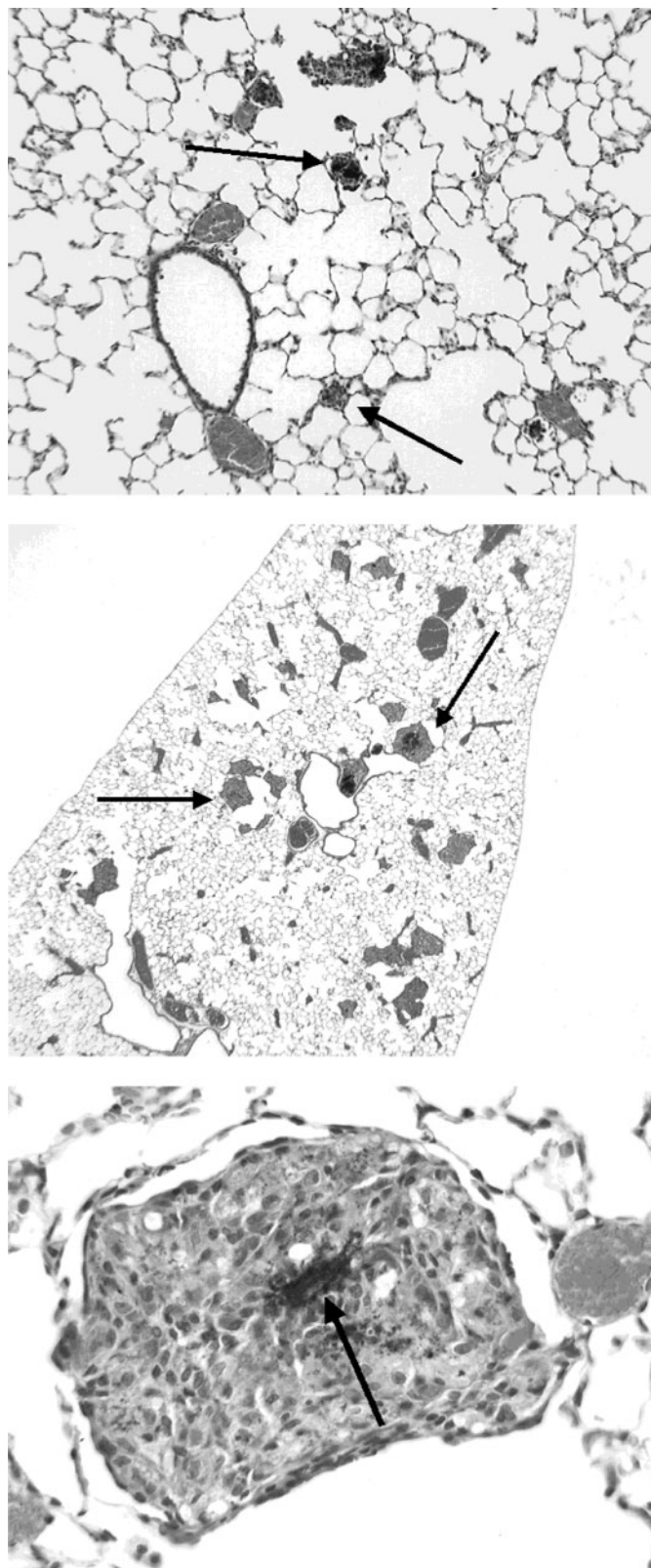
Lung parenchymal cell proliferation rates (percentage immunostained cells taking up BrdU) were measured in particulate-exposed rats and corresponding controls at 24 h, 1 week, 1 month, and 3 months pe. Significant increases in cell proliferation indices were measured in the high-dose quartz-exposed rats at 24 h and 1 month pe (Fig. 6).

### DISCUSSION

The objective of this pulmonary bioassay study was to assess the acute lung toxicity of intratracheally instilled SWCNT in rats. SWCNT exposure at 5 mg/kg produced mortality in  $\sim 15\%$  of the exposed rats. This was due to the impact of agglomerating the major airways in the rat and not due to

inherent toxicity of SWCNT. Positive control quartz particle exposures produced pulmonary inflammation, cytotoxicity, enhanced parenchymal cell proliferation, foamy macrophage accumulation, and tissue thickening (i.e., fibrosis). Exposures to SWCNT produced transient lung inflammation and subsequent multifocal granulomas. The finding of granulomas, in the absence of adverse effects measured by pulmonary biomarkers and cell proliferation indices, is very surprising and does not appear to follow the normal paradigm generated by toxic dusts such as quartz, asbestos, and silicon carbide whiskers. Exposure to those dusts produces progressive lesions, which can be 'tracked' using bronchoalveolar lavage analyses and other pulmonary biomarkers of cell injury, inflammation, and fibrosis.

There exist only a few preliminary toxicology studies investigating the effects of fullerenes or nanostructures on pulmonary cells and/or tissues. Adelman *et al.* (1994) studied the effects of fullerenes on alveolar macrophages *in vitro*. In that study, the fullerene material, C<sub>60</sub> and C<sub>60-70</sub>, was prepared in an arc between two graphite electrodes in a helium atmosphere. The fullerene material was incubated in cell culture for 4 and/or 20 h with either a human macrophage cell line preparation or with bovine alveolar macrophages. After 4 and 20 h of incubation, the C<sub>60</sub> fullerenes produced decreases in viability of both macrophage cell types to about 60% of control values. In addition, three inflammatory cytokines, used as biomarkers of lung injury, were measured in the supernatant of the cell cultures following incubation with fullerenes. There were increased levels of tumor necrosis factor (TNF), interleukin-6 (IL-6), and interleukin-8 (IL-8) in the supernatant of macrophage cell cultures exposed to fullerenes when compared to



**FIG. 5.** (Top panel) Light micrograph of lung tissue from a rat exposed to single-wall carbon nanotubes (1 mg/kg) 1 week pe. Note the early development of lesions surrounding the instilled SWCNT (arrows) and the nonuniform,

control macrophage cell cultures. The investigators concluded that the fullerene preparation used in their experiments was toxic to alveolar macrophages. They claimed that the reactions were similar to those they had experienced with quartz (crystalline silica) particles.

In another preliminary study, Huczko et al. (2001) tested fullerene soot containing  $C_{60}$  or carbon nanotubes on skin irritation, allergen risks, and lung toxicity.

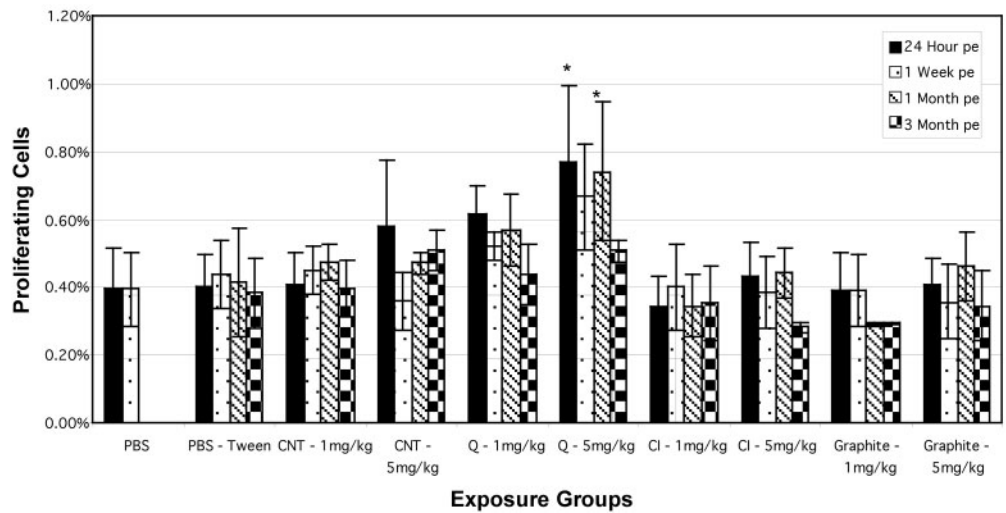
Two methods were utilized for the skin irritation tests. First, 40 volunteers reporting various irritation and allergy susceptibilities were subjected to a patch test for a period of 96 hours. In the second study, the modified Draize rabbit eye test was conducted, wherein one eye of a tested rabbit was instilled with a suspension of soot while the other eye was used as a control. The authors reported that the patch test showed negative results after testing for 96 hours, while no eye irritation was observed for both the  $C_{60}$  and carbon nanotube-containing soot.

For the pulmonary studies, a group of 5 guinea pigs was intratracheally instilled with 25 mg of carbon nanotube-containing soot. A control group of 5 guinea pigs was instilled with saline. After 4 weeks following instillation exposure, the animals were tested noninvasively for pulmonary function parameters and sacrificed for bronchoalveolar lavage examination. (Note that this is not a standard protocol for evaluation and no justification for the experimental protocol was provided in the paper). The results of the lung function measurements and bronchoalveolar lavage studies demonstrated no differences between the experimental and control groups. The authors concluded that the fullerene soot with a high content of carbon nanotubes did not induce measurable inflammation in the respiratory tract of guinea pigs (Huczko *et al.*, 2001).

Lam et al. (2003) investigated the pulmonary toxicity of three nanotube (NT) products in mice, using intratracheal instillation as the method of exposure. The three nanotube materials were manufactured by different methods and contained different metal catalysts. Metal analysis showed that the HiPCO-prepared nanotubes contained 27% (w/w) iron in the raw form, and 2% iron after purification. Carbolex electric-arc product contained 26% nickel and 5% yttrium. Groups of male B6C3F1 mice each were intratracheally instilled once with 0, 0.1, or 0.5 mg of NT suspended and ultrasonicated in 50  $\mu$ l of mouse serum. Mice were also exposed to carbon black and

diffuse pattern of single-wall carbon nanotube particulate deposition in the lung. Magnification =  $\times 100$ . (Middle panel) Low-magnification micrograph of lung tissue from a rat exposed to single-wall carbon nanotubes (1 mg/kg) at 1 month postinstillation. Note the diffuse pattern of granulomatous lesions (arrows). It was interesting to note that few lesions existed in some lobes while other lobes contain several granulomatous lesions—and this was likely due to the nonuniform deposition pattern following carbon nanotube instillation. Magnification =  $\times 20$ . (Bottom panel) Higher magnification light micrograph of lung tissue from a rat exposed to single-wall carbon nanotubes (1 mg/kg) at 1 month postinstillation exposure. Note the discrete, multifocal mononuclear granuloma centered around the carbon nanotube material (arrows). Magnification =  $\times 400$ .

### Lung Parenchymal Cell Proliferation Rates of Rats Exposed to Carbon Nanotubes, Quartz, or Carbonyl Iron Particles



**FIG. 6.** Lung parenchymal cell proliferation rates (BrdU) in particulate-exposed rats and corresponding controls at 24 h, 1 week, 1 month, and 3 months pe. Significant increases in BrdU immunostained cells were measured in the 5 mg/kg quartz (Q)-exposed rats versus controls at 24 h and 1 month pe.  $p < 0.05$ .

quartz particles, two standard reference dusts. Carbon black exposure produced minimal effects, and high-dose quartz produced moderate inflammation in the lung. The investigators reported that all of the NT products, regardless of the type or amount of metal, induced a dose-dependent formation of epithelioid granulomas in the centrilobular alveolar septa and, in some cases, interstitial inflammation in the animals of the 7-day pe groups. These lesions persisted and, in some cases, became worse in the 90-day pe groups. The granulomas in NT-treated mice consisted of aggregates of macrophages laden with black NT particles. Lam *et al.* concluded that, if single-wall nanotubes reach the lung, they can be more toxic than quartz.

Shvedova *et al.* (2003) investigated the effect of SWCNT on basic cellular processes involved in the induction of adverse responses in targeted human keratinocytes. After 18 h of SWCNT exposure, oxidant generation and cellular toxicity (as indicated by the formation of free radicals, accumulation of peroxidative products, antioxidant depletion, and loss of cell viability) were detected. Exposure to SWCNT also resulted in ultrastructural and morphological changes in cultured human cells. These investigators concluded that dermal exposure to unrefined SWCNT can result in accelerated oxidative stress and toxicity in the skin of exposed workers.

The finding of granulomas in the lungs of SWCNT-exposed rats in the absence of pulmonary biomarkers of inflammation, cell proliferation, and cytotoxicity is perplexing. Clearly, the individual carbon nanotubes have “nanoscale” dimensions, however their predilection for electrostatic attraction and consequent agglomeration into nanoropes and “nanomats” may significantly reduce the potential for aerosol exposure and, as a consequence, the potential health risks. On the other hand, the mechanism(s) of granuloma formation following intratracheal

instillation exposures to likely nonrespirable forms of SWCNT are unclear but should be investigated.

In summary, we have demonstrated that under the conditions of this test, pulmonary exposures to carbon nanotubes in rats produced a non-dose-dependent series of multifocal granulomas, evidence of a foreign tissue body reaction. These multifocal granulomas consisted of macrophage-like multinucleate giant cells and appeared to be associated with and surrounded/sequestered a black carbon nanotube bolus. The distribution of these lesions was nonuniform, with some lobes containing many granulomas and others containing few if any granulomas. There appeared to be no dose-response relationship, concomitant with a possible regression of lesions occurring from the 1-month to 3-month pe periods. The development of these lesions is not consistent with a normal dust-related paradigm, wherein lesions are noted initially at the sites of particle deposition, i.e., the junctions of the terminal bronchiole and adjacent alveolar ducts. Moreover, the pulmonary bioassay/ bronchoalveolar lavage biomarker results were not predictive of this lesion. Two recent exposure assessment studies have been conducted at the workplace where carbon nanotubes are either manufactured or utilized. Both assessments have reported very low aerosol exposure levels of respirable-sized carbon nanotubes, ranging from not detectable to  $< 0.1 \text{ mg/m}^3$  (Baron *et al.*, 2002; Joseph, 2002; Maynard *et al.*, 2003). These findings give credence to the hypothesis that, due to their electrostatic nature and tendency to agglomerate into nanorope structures, exposures at the workplace to respirable-sized carbon nanotubes are extremely low. As a consequence, the pulmonary toxicity study findings of multifocal granulomas that we have reported herein may not have physiological relevance, and may be related to the instillation of a bolus of agglomerated nanotubes (i.e., nanoropes). Therefore, to reconcile the unusual



adverse findings of this study with the potential risk associated with inhaling carbon nanotubes, it seems clear that the pulmonary effects of carbon nanotubes must be evaluated by generating aerosols of SWCNT and, thus, conducting an inhalation toxicity study of carbon nanotubes in rats.

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