

Pulmonary Toxicity of Single-Wall Carbon Nanotubes in Mice 7 and 90 Days After Intratracheal Instillation

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Nanomaterials are part of an industrial revolution to develop lightweight but strong materials for a variety of purposes. Single-wall carbon nanotubes are an important member of this class of materials. They structurally resemble rolled-up graphite sheets, usually with one end capped; individually they are about 1 nm in diameter and several microns long, but they often pack tightly together to form rods or ropes of microscopic sizes. Carbon nanotubes possess unique electrical, mechanical, and thermal properties and have many potential applications in the electronics, computer, and aerospace industries. Unprocessed nanotubes are very light and could become airborne and potentially reach the lungs. Because the toxicity of nanotubes in the lung is not known, their pulmonary toxicity was investigated. The three products studied were made by different methods and contained different types and amounts of residual catalytic metals. Mice were intratracheally instilled with 0, 0.1, or 0.5 mg of carbon nanotubes, a carbon black negative control, or a quartz positive control and euthanized 7 d or 90 d after the single treatment for histopathological study of the lungs. All nanotube products induced dose-dependent epithelioid granulomas and, in some cases, interstitial inflammation in the animals of the 7-d groups. These lesions persisted and were more pronounced in the 90-d groups; the lungs of some animals also revealed peribronchial inflammation and necrosis that had extended into the alveolar septa. The lungs of mice treated with carbon black were normal, whereas those treated with high-dose quartz revealed mild to moderate inflammation. These results show that, for the test conditions described here and on an equal-weight basis, if carbon nanotubes reach the lungs, they are much more toxic than carbon black and can be more toxic than quartz, which is considered a serious occupational health hazard in chronic inhalation exposures.

Key Words: carbon nanotubes; pulmonary toxicity; epithelioid granulomas; nanotube toxicity.

Results of this study were presented as a poster at the Society of Toxicology annual meeting, (Salt Lake City, UT) in March 2003 and as invited talks at NanoDays 2002 (Rice University, Houston, TX) in October 2002, at the EPA/NIEHS-sponsored Nanotechnology and the Environment Symposium (American Chemical Society annual meeting, New Orleans, LA) in March 2003, and at the EPA/NSF-sponsored Interagency Grantees Workshop on Nanotechnology and the Environment (Arlington, VA) in September 2003.

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President Clinton established the National Nanotechnology Initiative in 2000 to lead this country into the next industrial revolution (White House, 2000). Nanomaterials are the building blocks of this new industry. One of the major objectives of the initiative calls for “developing materials that are 10 times stronger than steel, but a fraction of the weight for making all kinds of land, sea, air and space vehicles lighter and more fuel efficient.” This statement specifically implicates carbon nanotubes, a novel and lightweight material with the strongest tensile strength of all synthetic fibers (Ball, 1999). The presidential initiative directs NASA to search for applications of carbon nanotubes and other nanomaterials in aerospace.

Carbon nanotubes structurally resemble rolled-up graphite sheets with one end capped. These tiny tubes can have single or multiple walls. Single-wall carbon nanotubes (NTs), unlike graphite or carbon black, possess highly desirable electrical, mechanical, and thermal properties (Arepalli *et al.*, 2001; Ball, 2001) and have many potential applications in the electronics, computer, and aerospace industries. As stated by Ajayan *et al.* (1999), “It is rare to come across a material that has such a range of remarkable properties.” Enormous research efforts have been channeled into discovering applications of this novel material. Dr. R. Smalley (a Nobel laureate and a pioneer in carbon nanotube research) predicted that hundreds or thousands of tons of NTs could be produced in 5 to 10 years and “in time, millions of tonnes of nanotubes will be produced worldwide every year” (Ball, 2001; ISI, 2002). As the production and applications of NTs expand, potential human exposures will also increase.

NTs can be produced by deposition of carbon atoms vaporized from graphite by electric arc or by laser onto metal particles. More recently, they have been produced by chemical vapor deposition (CVD). High-pressure CO conversion (HiPco™, Rice University, TX) is a CVD process and is a more advanced method that uses carbon monoxide as carbon source; up to 97% of the carbon in the HiPco product ends up in NTs (Bronikowski *et al.*, 2001). All of the products produced by these methods contain residual catalytic metals; some also contain other non-NT carbon materials. An individual NT molecule is about 1 nm in diameter and several microns long

(Ajayan and Ebbesen, 1997). Microscopically, individual NT fibers aggregate into bundles or ropes, which in turn agglomerate loosely into small clumps.

A study by the National Institute of Occupational Safety and Health on unprocessed NT samples demonstrated that fine particles of respirable sizes could be generated, albeit with some difficulty, from the bulk materials (Baron *et al.*, 2003; Maynard *et al.*, submitted). Fine particles may pose a health risk by inhalation. Because no toxicity information about NTs is available, and because the atoms in NTs and in graphite configure in the same molecular hexagonal/honeycomb pattern, Carbon Nanotechnologies, Inc (CNI, Houston TX), a major NT manufacturer and supplier, classified this new form of carbon as synthetic graphite. Its material safety data sheet (CNI, 2003) references the permissible inhalation exposure limit (PEL) set by the Occupational Safety and Health Administration (OSHA) for synthetic graphite at 15 mg/m³ of total dust and 5 mg/m³ for the respirable fraction (NIOSH/OSHA, 1988). NTs are rather unique in physical and chemical properties; hence no other dusts, except perhaps graphite, possess any properties similar to those of NTs. However, graphite does not possess the electrical properties and fibrous structure of NTs. It is well known that the geometry and surface chemistry of particulates can play an important role in causing lung toxicity (Lippmann, 1994). The absence of toxicity data for such an important commodity has concerned many (Gorman, 2002). Concern about the potential for its workers to be exposed to materials of unknown toxicity prompted NASA to sponsor the present pulmonary toxicity study.

The study was conducted on three NT products made by different methods and containing different types or amounts of residual metals; they were raw (RNT) and purified (PNT) iron-containing HiPco products of Rice, and CarboLex's nickel-containing electric-arc product (CNT). For the present study, we used intratracheal instillation, an accepted route of exposure commonly used to screen dusts for potential pulmonary toxicity (Leong *et al.*, 1998; Driscoll *et al.*, 2000). Intratracheal instillation studies also allow comparative toxicity investigation of several dusts simultaneously (Lam *et al.*, 2002a,b). However, the bolus dosing can exaggerate acute toxic effects and is more likely to overwhelm clearance mechanisms when compared to the same dose received by inhalation over a long period of time. These three NT products (RNT, PNT, and CNT), together with carbon black (CB, a low-toxicity dust) and quartz (fibrogenic in the lungs) as reference dusts, were studied in mice. The study followed a lung histopathological protocol similar to that of the National Toxicology Program for the subchronic study of dusts (NTP, 1995). This screening study focused on histopathology as an endpoint.

MATERIALS AND METHODS

Animals and animal care. Male mice (B6C3F₁, 2 months old), free of known rodent pathogens, were obtained from Charles River (Indianapolis, IN).

The types of pathogens screened can be found in Charles River's Rodent Health Monitoring Summary (Charles River, 1998). The animals were housed in groups of 4 or 5 in polycarbonate cages (with HEPA air filters) in the AAALAC-accredited vivarium at the Johnson Space Center (JSC). Animals were allowed to acclimate at this facility (with a 12-h light-dark cycle) for at least one week before being used in the study. The mice had free access to tap water and Purina Formulab Chow No. 50008 (Ralston Purina Co., St. Louis, MO). They were cared for and used humanely according to NASA Animal Care and Use Program guidelines. The animals weighed about 30 g when the dust treatments were administered.

Materials. The raw (RNT, Fig. 1A) and purified HiPco™ NTs (PNT, Fig. 1B) were generously provided by the Center for Nanoscale Science and Technology of Rice University (Houston, TX). A study on a raw HiPco™ NT sample revealed that gentle agitation (blowing air over the material shaken in a vortex) produced large airborne clumps. Very few small particles were present. At high agitation levels, more airborne NT particles were generated; most were respirable sizes, with some being in the ultrafine range (Fig. 1D, courtesy of Maynard *et al.*, submitted). The CNT sample (Fig. 1C) was donated by CarboLex, Inc. (Lexington, KY). The carbon black (Printex 90®) sample was a gift from Degussa Corporation (Germany) and was provided to us via G. Oberdorster; the quartz sample (Mil-U-Sil-5®) was generously provided by US Silica (Berkeley Spring, WV). All samples were used without further purification.

Determination of metal content of nanotube samples. The metal content of the NT and carbon black samples used in the present study was determined in our laboratory. Two to four samples of NTs or Printex 90 were placed in crucibles and ashed in a muffle furnace at 550°C for 3 h. The ash from each sample was dissolved in 1 ml of hot concentrated nitric acid and was then diluted to 30 ml with deionized water. The diluted solutions were first screened

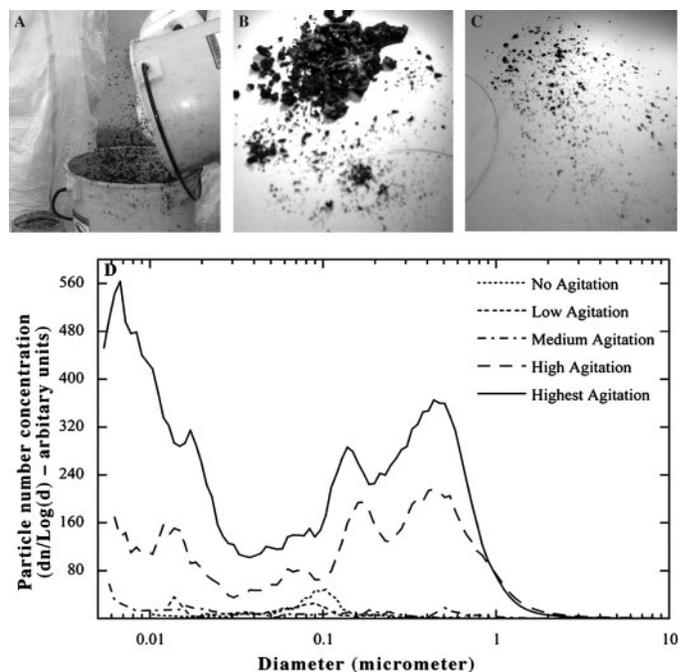


FIG. 1. (A) Pouring HiPco NT (raw) between containers (courtesy of Baron *et al.*, 2003). This product was similar to the RNT sample used in our study. (B) Purified HiPco NT. (C) CarboLex NT. Note that all these products contain fine particles [a human hair is shown in (B) and (C) for comparison]. (D) Size distribution of particles generated from a raw HiPco NT sample using a single component vortex-shaker fluidized bed; data have been smoothed for clarity (courtesy of Baron *et al.*, 2003).

for the presence of metals using ICP/MS (PE Sciex Elan 6000, Perkin Elmer, Norwalk, CT). Of the 70 elements scanned, only Fe, Ni, Y, Al, Cu, Mo, Zn, and Co were found in measurable concentrations. Quantitative analyses for these 8 metals were then conducted with the same instrument. Metals detected at weight percentage $\geq 0.01\%$ are shown in Table 1.

Preparation of fine-dust suspensions. NTs are neither water soluble nor wettable, and fine particle suspensions suitable for instillation must be prepared with a nontoxic dispersion vehicle (Driscoll *et al.*, 2000; Leong *et al.*, 1998). The products are extremely difficult to disperse even in the presence of a dispersing agent. A group at Rice University used "aggressive sonication of purified NT samples in surfactants such as Triton-X or highly polar solvents, like dimethyl formamide" to make fine-particle suspensions (10 mg/l) containing mostly individual fibers and a few small bundles (Walters *et al.*, 2001). We prepared the NT suspensions [2 mg/ml (0.1 mg/50 μ l) or 10 mg/ml (0.5 mg/50 μ l)] by briefly shearing (2 min in a small glass homogenizing tube) and subsequently sonicating (0.5 min) NT samples in heat-inactivated mouse serum (Sigma, St. Louis, MO). Brief sonication does not shorten or change the fundamental nature of NTs (Hauge, personal communication). Serum inactivation was performed in a water bath heated to 56°C for 0.5 h. Serum, used by Leong *et al.* (1998) and found in our pilot studies to be the best dispersing vehicle for NT, was also used to suspend other test dusts. Samples that were not freshly generated on the day of dosing were resonicated before the instillation. Each sample was vortexed just before an aliquot was drawn for instillation.

Intratracheal instillation. After being anesthetized with 3 to 5% isoflurane in a small chamber, individual mice were secured on an inclined plastic platform and anesthetization continued via a small nose cone. The trachea was exposed by a 1-cm incision on the ventral neck skin for instillation of the dust suspension (Lam *et al.*, 2002a). The instillation procedures for mice reported by these authors and the intratracheal fast instillation/nebulization procedure for rats used by Leong *et al.* (1998) were modified to ensure that instilled material was delivered into the lungs of mice with a good distribution. Because it was difficult to eliminate air bubbles from a small NT-serum sample drawn into the syringe and confidently measure the intended volume, the test sample, containing 0, 0.1 (low dose, LD) or 0.5 mg (high dose, HD) in a 50- μ l aliquot, was drawn (up to a premarked location) into a 30-cm fine silicone tube connected to the back metal end of a 24-gauge blunted needle. A small hole was made in the trachea close to the larynx, and a 24-gauge plastic catheter was

inserted through the hole to the distal end of the trachea; the blunted needle was then inserted inside the plastic catheter. A 1-ml syringe prefilled with 150 μ l of air and 20 μ l of saline was then connected to the free end of silicone tubing to rapidly propel the test sample from the tubing and needle into the lungs. The neck incision was then sutured, swabbed with Povidone iodine, and anesthetized with a drop of lidocaine. The mice recovered and were active within 10 min after removal from the inhalation anesthetic. The incision healed within two days, and the animals were observed daily until their scheduled termination.

Lung collection and histopathological examination. Seven or 90 days after instillation of the test material, each mouse was injected intraperitoneally with a lethal dose (0.1 ml) of pentobarbital sodium solution (Nembutal, Abbott, North Chicago, IL). Body weights were determined in the 90-d groups. An incision on the neck skin was made to expose the trachea for inserting a catheter; formalin (10% in neutral phosphate buffer) was allowed to drip by gravity (from a 25-ml syringe barrel hanging 1.5 feet above the neck) through the catheter into the lung for about 10 min. The trachea was then tied, and the isolated lung was placed in a glass vial containing about 10 ml of the same fixative (Lam *et al.*, 2002a). For the 90-d groups, each vial was assigned a number, with the treatment unknown to the pathologist. The lungs were fixed for at least 7 days before further processing. The formalin-fixed mouse lungs were embedded in paraffin, thin-sectioned coronally, and mounted on glass microscope slides using standard histopathological techniques. Sections were stained with hematoxylin-eosin and examined by light microscopy.

RESULTS

Effects of Carbon Nanotubes

All animals treated with 0.1 mg per mouse (low dose, LD) of CNT (containing Ni and Y) showed no overt clinical signs. However, 5 of the 9 mice treated with 0.5 mg (high dose, HD) of this product died (2/4 in the 7-d group and 3/5 in the 90-d group). All deaths occurred 4 to 7 days after instillation of the CNT. The deaths were generally preceded by lethargy, inactivity, and body-weight losses. These symptoms were also seen

TABLE 1
Metal Content of Test Samples and Experimental Design of the Intratracheal Instillation Study in Mice

Test materials	Metal contents (% by Wt.)*				Dust dose (mg/mouse)	Number of mice		
	Fe	Ni	Y	Others		7 d	90 d	
Raw nanotubes (RNT), Rice product	26.9	0.78	0.00	Cu: 0.36; Mo: 0.95; Zn: 0.01	LD	0.1	4	5
Purified nanotubes (PNT), Rice product	2.14	0.00	0.00	None	HD	0.5	4	5
CarboLex nanotubes (CNT)	0.53	25.99	5.01	Al: 0.15; Zn: 0.15; Co: 0.02	LD	0.1	4	5
Carbon black (Printex-90)	0.00	0.00	0.00	None	HD	0.5	4	5
Quartz (Min-U-Sil-5)	ND	ND	ND	ND	LD	0.1	4	5
Vehicle control (mouse serum)	ND	ND	ND	ND	HD	0.5	4	5
					—	0.0	4	5

Note. Mice (4 or 5 per group) were each intratracheally instilled once with 0, 0.1, or 0.5 mg dust in mouse serum and euthanized after 7 or 90 days for histopathology study.

Note. ND: Not determined.

*Metals with concentrations $< 0.01\%$ by weight are not shown.

in the HD mice that survived. Mice in the HD 90-d CNT group (including those that died within the first week) lost about 27% of their body weight (pretreatment: 30.9 ± 1.1 g; posttreatment: 22.5 ± 0.9 g) by the first week. Symptoms in the two surviving mice disappeared after one week, and the animals started to gain weight.

The iron-containing NTs (RNT and PNT) did not cause deaths in the mice. Mild signs of inactivity, hypothermia (felt cold to the touch), piloerection, and occasionally shivering (when provoked by picking up and laying down) were most noticeable 8 to 12 h after treatment with the HD RNT; these symptoms disappeared soon after this time. These clinical signs were not observed in the mice treated with PNT. Body weight losses, seen in the first week with the HD CNT, were not observed with RNT or PNT.

The distribution of black particles in lungs 90 d after they were instilled with 0.5 mg of CB or NTs is illustrated in Figure 2. Some of the lungs had a relatively uniform particle distribution, while others did not. At the microscopic level, the lungs of dead animals of the HD CNT groups showed congestion and postmortem histopathological changes. The lungs of the 4 (2 from the 7-d and 2 from the 90-d group) surviving mice treated with HD CNT had large aggregates of particles in macrophages in the alveolar space; some of these aggregates were also found in the interstitium, forming granulomas (Figs 3D and 4C). Some interstitial inflammation was apparent. Granulomas were not detected in the LD groups (7 d and 90 d) given CNT. The lungs of mice in the HD 7-d study that were treated with either RNT or PNT showed prominent granulomas (Figs 3E and 3F).

Most of these microscopic nodules were located beneath the bronchial epithelium and were present throughout most of the lung fields. Some appeared to extend into the bronchi as polyps. The granulomas consisted of macrophages laden with black particles, and had very few lymphocytes, neutrophils, eosinophils, or other inflammatory cells. The macrophages had abundant granular cytoplasm with indistinct borders, a characteristic of activated macrophages or epithelioid cells. The black particles were almost entirely contained within these granulomas. Some of the lungs from HD 90-d NT-treated groups appeared grossly abnormal (Figs 2C and 2F). The micrographs of lungs from these groups showed the persistence of granulomas that contained particle-laden macrophages and NT particles (Figs 4C–E). The lung lesions were generally more pronounced than those of the HD 7-d groups (Figs 3D–F); some also had necrosis, interstitial inflammation that had extended into the alveolar septa, and peribronchial inflammation (Figs 4E and 4F). Granulomas and other pulmonary lesions were also seen in some of the LD HiPco-NT-treated mice (Table 2), but to a mild degree.

Effects of Serum, Carbon Black, and Quartz

As expected, heat-inactivated mouse serum did not produce any clinical signs, or gross and microscopic lesions (Figs 2A and 3A). The mice of the negative (CB) or positive (quartz) control groups also did not show any clinical signs that could be attributed to treatment. Aside from the presence of black particles that appeared predominantly in alveoli, the lungs of

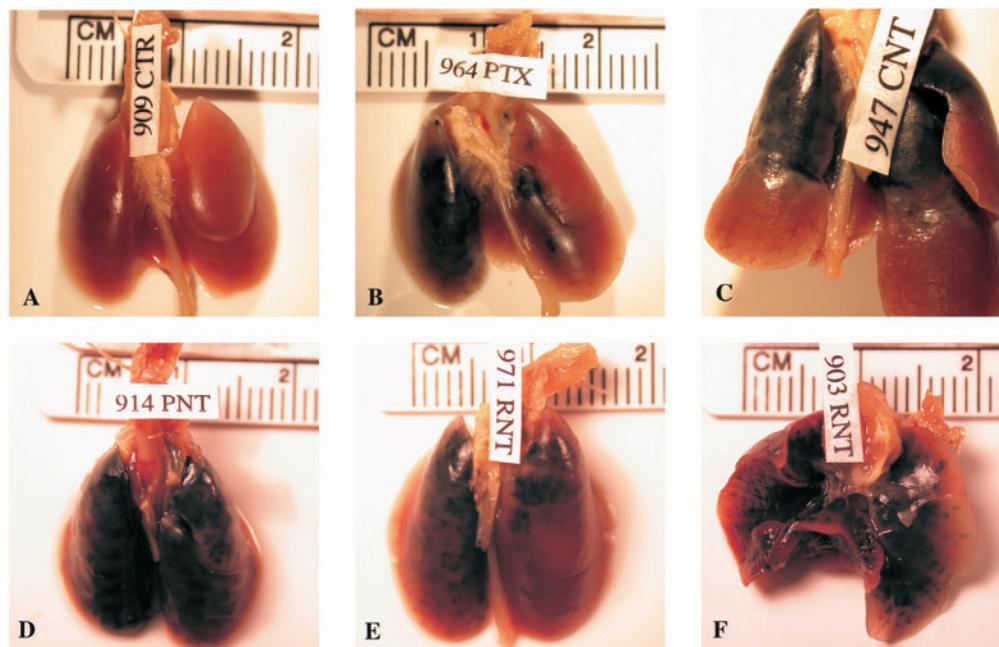


FIG. 2. Lungs from mice instilled with 0.5 mg of a test material per mouse and euthanized 90 d after the single treatment. (A) Serum control. (B) Carbon black (Printex). (C) CNT. The portions of the lung receiving NT had an abnormal appearance. (D) PNT. Particle distribution was even. (E) RNT. Clusters of black pigment probably correspond to granulomas. (F) RNT. Dorsal view shows some necrotic changes.

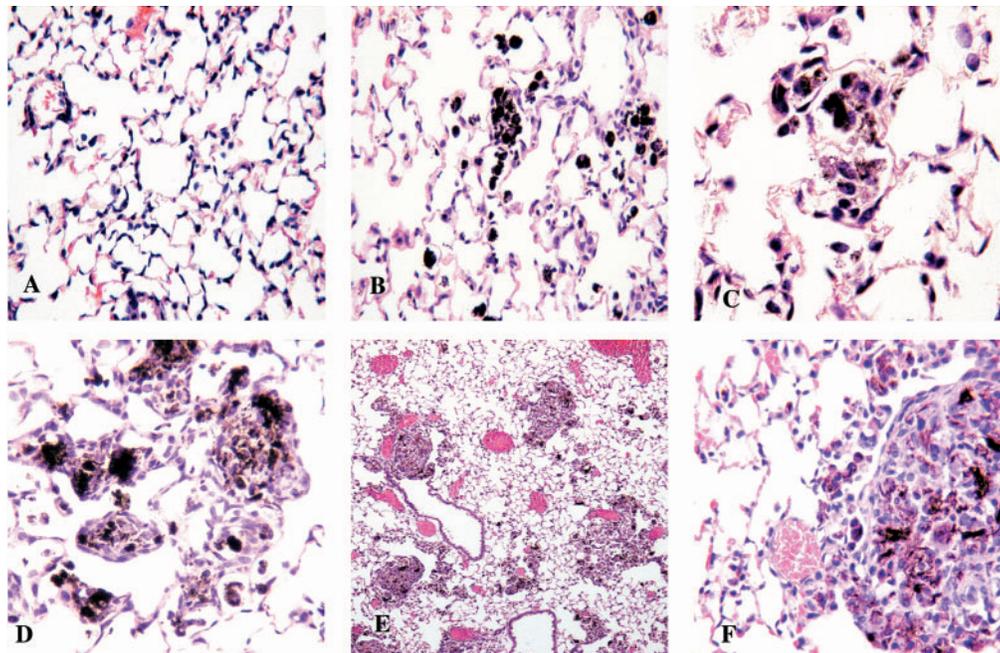


FIG. 3. Lung tissue from mice instilled with 0.5 mg of a test material per mouse and euthanized 7 d after the single treatment. (A) Serum control. (B) Carbon black. Particles and particles contained in macrophages were scattered in the alveoli. (C) Silica quartz. A low-grade granuloma. (D) CNT. Particles were predominately inside the granulomas, and none were in the area of normal alveolar tissue on the right side. (E) RNT. Black particles were predominately inside granulomas. (F) PNT. Close-up view of granulomas. Magnifications varied from 40 to 200 \times .

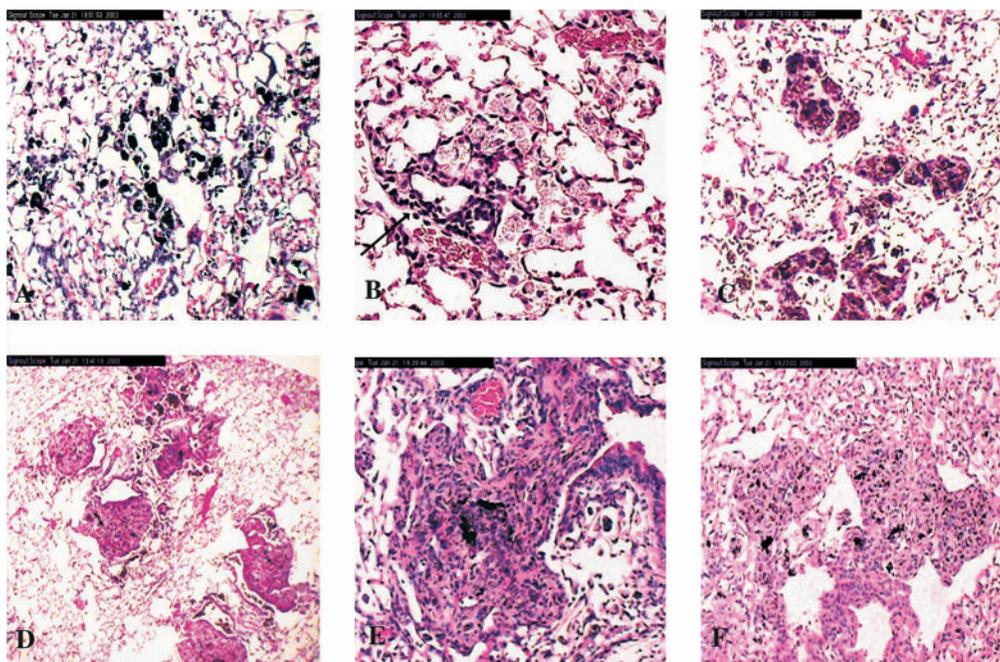


FIG. 4. Lung tissue from mice instilled with 0.5 mg of a test material per mouse and euthanized 90 d after the single treatment. (A) Carbon black. Particles were scattered in alveoli. (B) Quartz. \rightarrow shows an aggregate of inflammation cells (lymphocytes) around an area surrounded by quartz particle-containing macrophages. (C) CNT. Granulomas contained black particles. (D) RNT. Granulomas at low magnification. (E) RNT. A granuloma at a high magnification. (F) PNT. A large granuloma underwent degeneration with necrosis. Magnifications varied from 40 to 200 \times .

TABLE 2
Incidence of Pulmonary Lesions in Mice 90 Days After
Intratracheal Instillation with Nanotubes*

Dust dose (mg)	Type of lung lesion	Carbon black	Quartz	RNT	PNT	CNT
0.1	Inflammation	0	1	3	2	0
0.1	Granulomas	0	0	5	2	0
0.5	Inflammation	0	4	3	5	0
0.5	Granulomas	0	0	5	5	5**

*Mice (5/group) were each instilled with 0.1 or 0.5 mg of a test material and euthanized 90 d after the single treatment. Lungs were microscopically examined by a pathologist who had no knowledge of the treatment of each animal.

**Including 3 mice that died in the first week.

the mice in CB groups were microscopically normal (Figs 3B and 4A). The lungs of the LD quartz groups were also normal. Quartz at HD induced an increase in the number of alveolar macrophages in the lungs, and some of these cells contained particles. Quartz also produced mild to moderate alveolar and interstitial inflammation. The results for the 7-d and 90-d groups were generally similar. One of the mice in the 7-d group had a low-grade granulomatous reaction (Fig. 3C). The HD 90-d group had increased clusters of peribronchiolar lymphocytes (Fig. 4B).

DISCUSSION

The present study shows that all three NT products, regardless of the type and amount of metal impurities they contained, induced dose-dependent lung lesions characterized chiefly by interstitial granulomas. Our finding that the purified NTs (PNTs), which were prepared by rigorous treatment (45-h reflux) with concentrated acids (2 M to 3 M nitric acid) to remove metal impurities (Rinzler *et al.*, 1998), and contained only a small amount (2 % by weight) of residual iron, produced prominent granulomas, along with the fact that insoluble iron and iron compounds are low in toxicity and have not been shown to produce these lung lesions (Warheit *et al.*, 1991), strongly indicates that NTs themselves induced the granulomas. NT-induced granulomas were also observed in a similar study in which rats were intratracheally instilled with a laser-produced NT product sonicated and suspended in Tween 80 (Warheit, 2003). The NT dosage of 5 mg/kg in Warheit's rat study was comparable to that given to our low-dose (0.1 mg/30 g mouse or 3.3 mg/kg) groups of mice. At this dose we saw granulomas in the mice treated with the HiPco products (RNT and PNT), but not with the CNT.

We observed mortality (5/9) in animals treated with the high dose of CNT, suggesting that any subsequent intratracheal studies with CNT should not involve doses this high. The CNT product contains substantial amounts of nickel and yttrium. Nickel and its compounds are highly toxic. Benson *et al.*

(1987) reported that all 10 mice exposed to 10 mg/m³ Ni₃S₂ died before the end of the 12-day study. The lung burden of nickel in this group was not available, but mice exposed to 2.5 mg/m³ of the nickel compound for 12 days (6 h/d) accumulated 4 μg/lung. Assuming that the lung burden of this insoluble nickel compound was proportional to exposure concentrations, the amount of nickel in the lungs of animals that died would be ≤16 mg/lung. In our study, treatment with a CNT dose of 0.5 mg/mouse would load 130 μg of nickel and 25 μg of yttrium into the lung. We could not rule out the possibility that some of the nickel and yttrium, surrounded or coated by NTs, was freed by ultrasonication and subsequently contributed to the acute lethality. If sonication-freed nickel contributed to the acute toxicity, then deaths, observed in bolus-dosed animals, should not occur in inhalation-exposed animals. Further studies would be needed to elucidate the cause of death with certainty.

Although both types of carbon particles (CB and NTs) were taken up by alveolar macrophages, their fate and reactions in the lung tissue were very different. CB-laden macrophages scattered in the alveolar space, but NT-laden macrophages moved rapidly to centrilobular locations, where they entered alveolar septa and clustered to form epithelioid granulomas. It is well known that, if the lung is not dust-overloaded, dust-laden macrophages on the alveolar surface will migrate upward and be carried by the escalator/mucociliary system up the trachea, and cleared into the esophagus. However, when dusts enter the interstitial or subepithelial space, they are very difficult to clear from the lung. Thus, if a biopersistent dust is irritating or toxic, the lesions resulting from the persistent interaction between the cells and the dust trapped in the interstitium will generally worsen with time, as is the case with NTs. As seen in the present study, the lung lesions of HD 90-d groups were generally more pronounced than those of the HD 7-d groups in animals treated with NTs. These findings indicate that NTs and CB have different intrinsic toxicities in the lungs.

The findings that all four NT products (three in our study and one in Warheit's study) were capable of inducing granulomas in mice or rats, together with the findings (in our studies and others) that granulomas were not observed in rodents exposed to carbon black, point to the fundamental difference between the unique physicochemical properties of NTs and those of CB. In addition to its unique surface chemistry (NTs are an excellent electrical conductor, while carbon black is not), the fibrous structure of NT products must be considered. Microscopically, thin NT fibers pack tightly and in parallel to form ropes or rods (ISI 2002; Unrau, 1996). As defined in *Comprehensive Toxicology*, "Fibers are a special class of particles defined as elongated objects whose aspect ratio, the ratio of the object's length to its diameter, is greater than three" (McClellan, 1997). Therefore, toxicologically, individual NT molecules, and assembled NT ropes, rods, and bundles are fibers. Some of the NT particles were clearly seen in the lungs as fiber or rope structures (Figs 5A–C). This physical structure would make NTs toxicologically different from carbon black. NTs are to-

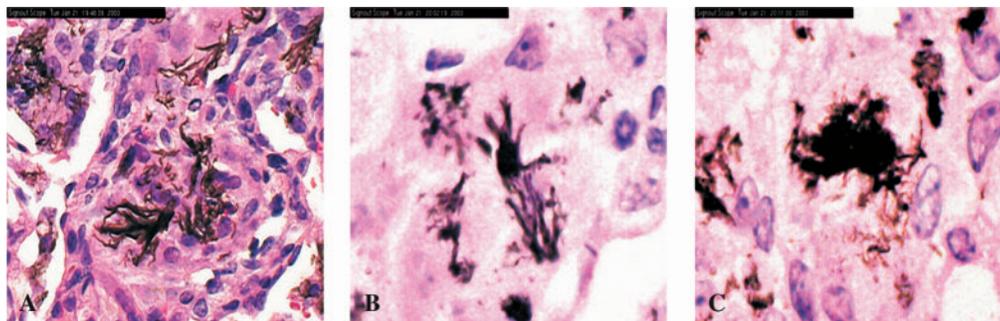


FIG. 5. Lung tissues from mice instilled with 0.5 mg of NT per mouse and euthanized 90 d after the single treatment showing presence of NT fibers. (A) RNT. NT fibers in a granuloma. (B) PNT. NT fibers in a granulomas. (C) PNT. Clumps of NT fibers in a granuloma. (Magnification 900 \times).

tally insoluble and probably one of the most biologically non-degradable man-made materials. It is well established that the pathogenicity of a fiber in the lungs directly correlates with its biopersistence (Oberdorster, 2000). Granulomas could impair cellular and physiological (gas exchange) lung functions and give rise to fibrosis, more defined nodules, and other lesions. Determining how the NT-induced granulomas progress would require a longer-duration study with this biopersistent material.

Because some NTs are derived from graphite and may contain graphite as an impurity, and toxicological data indicate that graphite may induce granulomas, the toxicity of graphite warrants a discussion. Graphite pneumoconiosis, which is characterized by granulomas, interstitial fibrosis, perifocal emphysema, necrosis, and severe vascular sclerosis, is a lung disease that has long been recognized in a large number of workers involved in mining and processing graphite (Gaensler *et al.*, 1966; Hanoa, 1983; Jaffé, 1951; NRC, 1999). A study (Okutani *et al.*, 1964), showing 112 cases of pneumoconiosis among 256 workers who had been exposed to an average of 60 mg/m³ of graphite that contained little (<0.1%) quartz (equivalent to <0.1 mg/m³ in the air), and another study (Aranyi *et al.*, 1992), revealing graphite-containing granulomas in lung lymphoid tissue in rats exposed to pure synthetic graphite at 100 mg/m³ for 13 weeks, strongly suggest that pure graphite at high concentrations and prolonged exposures could also induce granulomas. However, the granulomas observed in the present study were not caused by graphite as an impurity. CNTs, which were made from graphite and may contain more graphite impurities than the CO-derived HiPco-NTs (Bronikowski *et al.*, 2001), actually were less potent in producing granulomas. The RNT and PNT samples, which contained more carbon nanotubes than the CNT product on an equal-weight basis, produced granulomas in some animals of the LD groups, whereas no animals in the LD CNT group had granulomas. In the HD groups, HiPco NTs produced more prominent granulomas than did the CNT. These data argue against the possibility that a graphite impurity induced granulomas in our study.

The comparative toxicity of NTs and quartz, which was used as a positive control for the present study, also warrants a discussion. Granulomas, along with other lung lesions, have

long been observed in animals chronically exposed to quartz. A granulomatous reaction in the lungs was observed in mice exposed to 1.5 to 2.1 mg/m³ for 150 or 300 d (Wilson *et al.*, 1986), in rats exposed to 25 mg/m³ for 100 d (Eden and von Seebach, 1976), and in rats intratracheally instilled once with 50 mg quartz and examined 1 to 12 months after treatment (Reiser *et al.*, 1982). In our study, in which mice were exposed to relatively less quartz than the animals in the above studies, one mouse had low-grade granulomas; the rest of the HD groups had only mild to moderate inflammation in the lungs. At the doses used in the present study, no fibrosis was observed in the lung.

One may ask the relationship of these intratracheal-instillation NT doses that produced lung lesions to an inhalation exposure. NT particles are neither soluble nor biodegradable, and particles in the interstitium could not be removed from the lung by the macrophage-mucociliary clearance mechanism. Therefore, the lung burden from an intratracheal dose could be used to roughly estimate a burden that could be achieved by inhalation exposures (Lam *et al.*, 2002a). Using data from the International Commission of Respiratory Protection Task Group, showing that the fractional deposition of particles deep into the lung is about 30% for 3- μ m particles and increases to 55% for 0.05- μ m particles (Bates *et al.*, 1966), we can estimate the pulmonary deposition of respirable dust. If we assume that 40% of the inhaled respirable NT particles deposit in the pulmonary region, and that a 30-g mouse breathes in 30 ml of air per min (Parent, 1992), then a mouse breathing respirable NT dust at 5 mg/m³ for 8 h daily would accumulate 0.029 mg NT/day. Then, a lung burden equivalent to that instilled with 0.1 mg or 0.5 mg could be attained by a mouse inhaling a respirable NT aerosol concentration for about 3.5 or 17 days, respectively. The exposure concentration of 5 mg/m³ is the same concentration as the PEL that OSHA set for respirable synthetic graphite dust, and is the same exposure concentration referenced by a major U.S. nanotube manufacturer and supplier (CNI, 2003). OSHA's PELs, which are time-weighted concentrations (40 h/week), are set for lifetime occupational exposures. It can be inferred from the exposure-risk estimation information presented here that, if workers were chronically

exposed to respirable NT dust at a fraction of the PEL concentration for synthetic graphite, they would likely develop serious lung lesions. Therefore, the PEL for synthetic graphite must not be used to protect workers exposed to NTs. The current TLV® for graphite (2 mg/m³) explicitly excludes graphite fibers (ACGIH, 2001). Our study provides data needed to guide additional toxicity studies and to support the design of industrial hygiene procedures (Baron *et al.*, 2003).

In conclusion, this study shows, for the test conditions described here and on an equal-weight basis, that if NTs reach the lungs, they are much more toxic than carbon black and can be more toxic than quartz, which is considered a serious occupational health hazard in chronic inhalation exposures. If fine NT dusts are present in a work environment, exposure protection strategies to minimize human exposures should be implemented.

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