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A Review of Carbon Nanotube Toxicity and Assessment of Potential Occupational and Environmental Health Risks

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A Review of Carbon Nanotube Toxicity and Assessment of Potential Occupational and Environmental Health Risks

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Nanotechnology has emerged at the forefront of science research and technology development. Carbon nanotubes (CNTs) are major building blocks of this new technology. They possess unique electrical, mechanical, and thermal properties, with potential wide applications in the electronics, computer, aerospace, and other industries. CNTs exist in two forms, single-wall (SWCNTs) and multi-wall (MWCNTs). They are manufactured predominately by electrical arc discharge, laser ablation and chemical vapor deposition processes; these processes involve thermally stripping carbon atoms off from carbon-bearing compounds. SWCNT formation requires catalytic metals. There has been a great concern that if CNTs, which are very light, enter the working environment as suspended particulate matter (PM) of respirable sizes, they could pose an occupational inhalation exposure hazard. Very recently, MWCNTs and other carbonaceous nanoparticles in fine ($<2.5 \mu\text{m}$) PM aggregates have been found in combustion streams of methane, propane, and natural-gas flames of typical stoves; indoor and outdoor fine PM samples were reported to contain significant fractions of MWCNTs. Here we review several rodent studies in which test dusts were administered intratracheally or intrapharyngeally to assess the pulmonary toxicity of manufactured CNTs, and a few *in vitro* studies to assess biomarkers of toxicity released in CNT-treated skin cell cultures. The results of the rodent studies collectively showed that regardless of the process by which CNTs were synthesized and the types and amounts of metals they contained, CNTs were capable of producing inflammation, epithelioid granulomas (microscopic nodules), fibrosis, and biochemical/toxicological changes in the lungs. Comparative toxicity studies in which mice were given equal weights of test materials showed that SWCNTs were more toxic than quartz, which is considered a serious occupational health hazard if it is chronically inhaled; ultrafine carbon black was shown to produce minimal lung responses. The differences in opinions of the investigators about the potential hazards of exposures to CNTs are discussed here. Presented here are also the possible mechanisms of CNT pathogenesis in the lung and the impact of residual metals and other impurities on the toxicological manifestations. The toxicological hazard assessment of potential human exposures to airborne CNTs and occupational exposure limits for these novel compounds are discussed

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in detail. Environmental fine PM is known to form mainly from combustion of fuels, and has been reported to be a major contributor to the induction of cardiopulmonary diseases by pollutants. Given that manufactured SWCNTs and MWCNTs were found to elicit pathological changes in the lungs, and SWCNTs (administered to the lungs of mice) were further shown to produce respiratory function impairments, retard bacterial clearance after bacterial inoculation, damage the mitochondrial DNA in aorta, increase the percent of aortic plaque, and induce atherosclerotic lesions in the brachiocephalic artery of the heart, it is speculated that exposure to combustion-generated MWCNTs in fine PM may play a significant role in air pollution-related cardiopulmonary diseases. Therefore, CNTs from manufactured and combustion sources in the environment could have adverse effects on human health.

Keywords Cardiopulmonary Diseases, Fibrosis, Fullerenes, Granulomas, Intratracheal Instillation, Multi-Wall Carbon Nanotubes, Nanomaterials, Nanotechnology, Natural Gas Combustion, Particulate Matter, PM2.5, Pulmonary Toxicity, Risk Assessment, Single-Wall Carbon Nanotubes

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I. INTRODUCTION

A. Nanotechnology and Carbon Nanotubes

The rapid growth in research and development involving materials of nanoscale size has propelled nanotechnology to the forefront of science and technology development. The anticipation that nanotechnology would become the strategic and dominating science and engineering field of the 21st century prompted the U.S. President and the National Science and Technology Council to promote nanotechnology and to predict that it would lead the United States to the next industrial revolution (National Science and Technology Council, 2003; White House Press Secretary, 2000). Of the materials associated with the inception and progression of nanotechnology, fullerenes and carbon nanotubes (CNTs) are the two most important and noted ones. It is the potential applications of these two nanomaterials, especially CNTs, that helped trigger the rush to nanotechnology research and development.

In 1985, the discovery of C_{60} molecules by Harold Kroto, James Heath, Sean O'Brien, Robert Curl, and Richard Smalley (Kroto et al., 1985) opened a whole new frontier in the chemistry of carbon. C_{60} is a spherical molecule with carbon atoms arranged in a pattern like that of a geodesic dome; the molecule was given the name "buckminsterfullerene." Since the discovery of C_{60} , fullerenes of larger size, as well as derivatives of C_{60} fullerene, have been synthesized and intensively investigated (Kroto and Walton, 1993; McKeith, 2002). These cagelike nanostructures and other related molecules have been projected to have wide industrial and medical applications. For the discovery of fullerenes, Curl, Kroto, and Smalley were awarded the Nobel Prize in Chemistry in 1996 (Kungl. Vetenskapsakademien [Royal Swedish Academy of Sciences], 1996).

B. Manufactured Carbon Nanotubes

1. Discovery of Carbon Nanotubes

In a graphite arc process that formed fullerenes from atomized carbon, Sumio Iijima in 1991 discovered multiwall CNTs (MWCNTs, Figure 1) deposited at the graphite anode (Iijima, 1991, 2004). Shortly after, Iijima succeeded in synthesizing single-wall CNTs (SWCNTs) (Figure 1) in the presence of metal catalyst and found that the yield of SWCNTs could be increased by increasing the amount of cobalt or other catalytic transition metals (Iijima and Ichihashi, 1993). Adapting the laser ablation process that was used to make fullerenes, Richard Smalley's group at Rice University (Houston, TX) succeeded in synthesizing SWCNTs with a much higher yield and greater purity than the arc process produced (Arepalli et al., 2004a; Guo et al., 1995; Thess et al., 1996). Compared with SWCNTs, MWCNTs are more heterogeneous and difficult to characterize and study. Theoretical calculations and results of experiments on SWCNTs show that SWCNTs have highly desirable mechanical, thermal, photochemical, and electrical properties (Wikipedia, 2005).

Seeing great potential applications of the two closely related families (fullerenes and CNTs) of nanomaterials and other manufactured nanomaterials, more than a dozen government agencies under the leadership of the National Science and Technology Council (2003) jointly established the Interagency Working Group on Nanotechnology, shortly after the awarding of the Nobel Prize for fullerene research. In 2000, this federal effort was raised by President Clinton to the level of a federal initiative, which was known as the National Nanotechnology Initiative (NNI) (White House Press Secretary, 2000). One of the major objectives of the NNI is "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." The materials implicated in the initiative are CNTs.

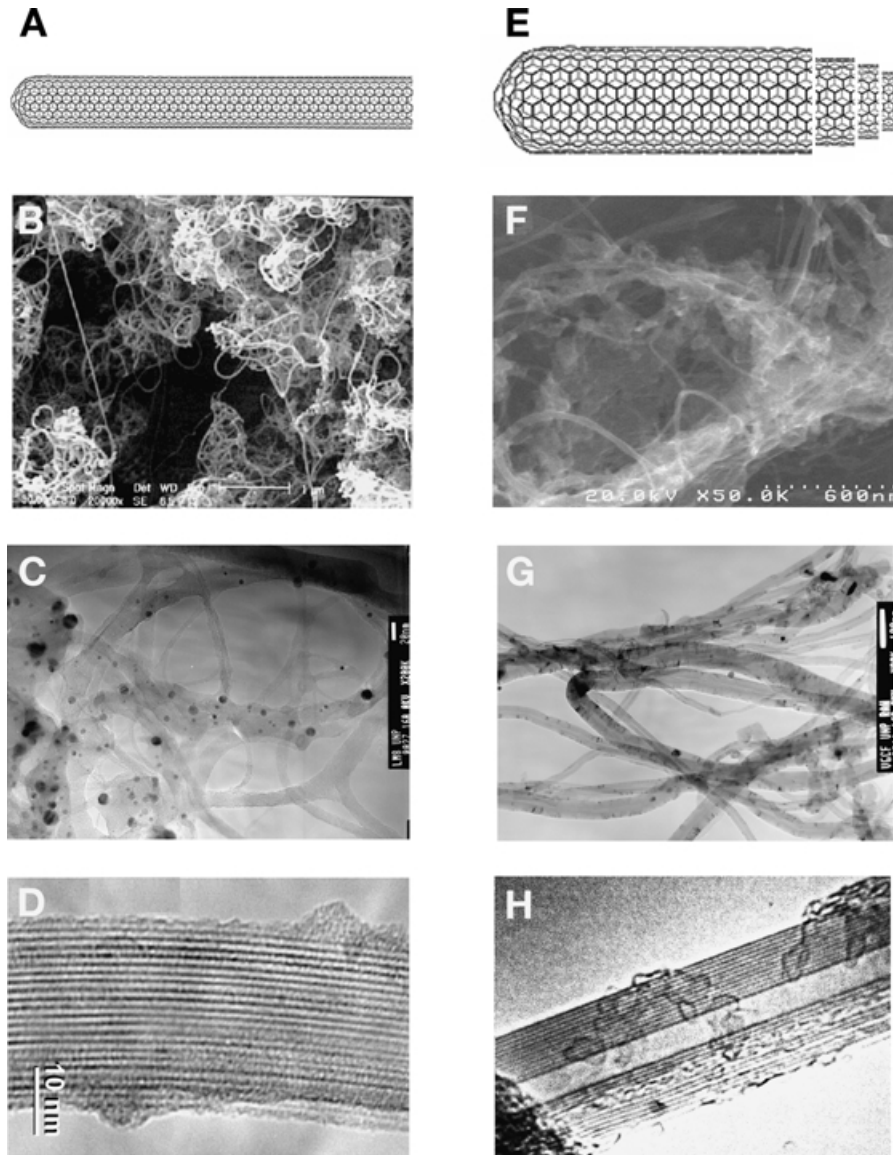


FIG. 1. (A) to (D): SWCNTs; (E) to (H): MWCNTs. Scanning electron microscope (SEM) images show SWCNT (B) and MWCNT (F) aggregates; transmission electron microscope (TEM) images show raw SWCNT bundles (ropes) with metal nanoparticles (C), and individual multiwall tubes (G). High-resolution TEM images show a cross-section of a SWCNT bundle (D) consisting of >25 tubes and some amorphous carbon on the edges, and a longitudinal cross-section of a MWCNT (H) with an empty central cavity and ~20 walls on each side and some amorphous carbon. (C), (G), and (H) are courtesy of P. Nikolaev of the JSC Nanomaterials Group, and (F) is courtesy of J. Rodriguez of Universitat de Barcelona, Spain.

2. Potential Wide Applications of Carbon Nanotubes

CNTs are light in weight and have the strongest tensile strength of any synthetic fiber. According to Smalley (1999), “They are expected to produce fibers 100 times stronger than steel at only 1/6th the weight—almost certainly the strongest fibers that will ever be made out of anything.” Even composite materials containing CNTs may have incredible strength, potentially sufficient to allow the building of such things as spacecraft structures, space elevators, artificial muscles, com-

bat jackets, and land and sea vehicles (Dresselhaus et al., 2000; Files, 2000; Wikipedia, 2005; Yowell et al., 2002).

In addition to their strength and light weight, some forms of CNTs can conduct electricity better than copper (Smalley, 1999). To harness these properties, the National Aeronautics and Space Administration (NASA) just awarded an \$11-million contract to the Carbon Nanotechnology Laboratory of Rice University to produce a prototype power cable made of CNTs (Johnson Space Center, 2005). Smalley remarked that, in addition to

their applications in space exploration, CNT cables will someday “rewire the world, replacing aluminum and copper in virtually every application and permitting a vast increase in the capacity of the nation’s electrical grid” (Boyd, 2005). Because CNTs can have conducting or semiconducting properties, depending on their nanostructures, the CNT nanofibers would have potential applications in the electronics and computer industries. These highly desirable electrical properties may allow CNT nanostructures to be formed by joining nanotubes of two different diameters end to end to form a diode, suggesting the possibility of constructing electronic computer circuits entirely out of nanotubes (Wikipedia, 2005). According to Smalley (1999), “Several decades from now we may see our current silicon-based microelectronics supplanted by a carbon-based nanoelectronics of vastly greater power and scope.” Nanotubes have been shown to be superconducting at low temperature. They also have unusual thermal and optical properties. As Ajayan et al. (1999) have stated, “It is rare to come across a material that has such a range of remarkable properties.”

3. *Synthesis of Carbon Nanotubes and Impurities in Synthetic Products*

Commercially, CNTs are produced from carbon atoms or clusters vaporized from graphite by arc discharge or by pulsed laser vaporization (Ando et al., 2004), or by a chemical-vapor deposition (CVD) process in which thermally and catalytically generated carbon atoms from hydrocarbon gaseous precursors are converted to solid structured materials (Leonhardt, 2004). Nikolaev in Smalley’s laboratory developed a gas-phase catalytic growth of SWCNTs from carbon atoms generated from a continuous high-pressure stream of carbon monoxide (Nikolaev et al., 1999). This patented synthetic method is a variation of CVD and is referred to by Smalley’s group as the HiPco process.

All of these synthetic processes involve thermal vaporization of carbon and metal catalysts. Carbon sources are graphite, or gaseous carbon-bearing compounds such as CO, methane, ethylene, or other hydrocarbons. The vaporized carbon atoms (or clusters) and hot metal catalysts co-condense into gas-phase molten nanoparticles where nanotubes grow (Moisala et al., 2003; Scott et al., 2001). MWCNTs can be produced without metals; however, the presence of a small amount of metal catalyst helps to align the nanotubes. An increase in the metal nanoparticles-to-carbon ratio favors the formation of SWCNTs (Iijima and Ichihashi, 1993). Synthesis of CNTs is generally carried out in an inert atmosphere within a temperature range of 600 to 1200°C. At the temperature of CNT synthesis, the metal(s) needs to be catalytically active and remain in the molten state, allowing dissolution of carbon atoms in the metal(s); of the metals that meet these requirements, those most commonly used are iron, nickel, cobalt, and molybdenum (Wikipedia, 2005). All of the SWCNT and MWCNT products produced by these methods contain residual metals (Arepalli et al., 2004b). Depending on the manufacturing process, an unprocessed SWCNT product may contain up to 50% of metal by weight; the metal content in

a MWCNT product is much less. The metal impurities are generally undesirable; some commercial products (Figure 2) are sold in purified forms after the removal of metals (Figure 3). CNT products also contain non-NT carbon impurities, such as soot, fullerene, and graphite; the amount and type of impurities depend on the synthetic methods and manufacturers. Raw and purified SWCNT products currently sold by SES (Houston, TX) contain nanotubes at only 20–40% and $\leq 75\%$, respectively (SES Research, 1999). The HiPco process of Rice University produces CNTs with very little non-nanotube carbon (Figure 3A); a test result of a purified HiPco CNT sample (Figure 3B) showed that $>99\%$ of the carbon content was in nanotube morphology and iron accounted for 0.23% of the sample weight (Shvedova et al., 2005).

4. *Physical Characteristics, Properties, and Appearance of Carbon Nanotube Products*

A SWCNT is a nanosize tube or fiber formed by bonded carbon atoms arranged in a hexagonal pattern; the tube is generally capped at one end (Figure 1A). Structurally, it resembles a rolled-up single layer of graphene or graphite sheet. Both SWCNTs and C₆₀ fullerene have diameters of about 1 nanometer ($\sim 1/50,000$ of a human hair). Fullerene has a soccer ball-like structure while a SWCNT appears like a single layer of rolled-up chicken wire. An individual SWCNT is a very thin fiber at least several micrometers long, with an aspect ratio (length/diameter) greater than 1000 (Ajayan and Ebbesen, 1997). MWCNTs have two or more concentric layers with various diameters and lengths (Figures 1G and 1H). Since the time CNTs were discovered, enormous research efforts have been devoted to making these fibrous materials longer.

SWCNTs do not naturally exist as individual tubes; van der Waals forces between the molecules cause them to aggregate into microscopic bundles or ropes, which in turn agglomerate loosely into small clumps. For example, SWCNT products made by the laser ablation process at the NASA Johnson Space Center (JSC) nanotechnology laboratory consist of aggregates of tiny ropes (Figure 1B). Each is generally formed by bundling 20 to 50 tubes (with a collective diameter of ~ 20 nm, Figure 1D) into a fiber several thousand nanometers long (Arepalli et al., 2004a). The ropes then aggregate (Figure 1B) into loose clumps or flakes; this unprocessed ash-like material (Figure 2A), if undisturbed, contains very little fine dust. The appearance of the bulk material depends on the processes by which it was synthesized or manufactured. Currently, SWCNTs made by the HiPco or laser process have fewest wall defects. Defects (like kinks in a straw) can diminish the effectiveness of the attraction force and subsequently reduce the formation of parallel bundles and ropes. In comparison, the arc-discharge SWCNT product (Figure 2B) made by CarboLex Inc. (Lexington, KY) and sold by Aldrich Chemical Company Inc. (Milwaukee, WI) looks like a black powder, similar to a carbon-black toner. The raw CNTs made by Rice’s HiPco process are loose black clumps (Figures 2D and 2F). This unprocessed HiPco product, although it contains 30%

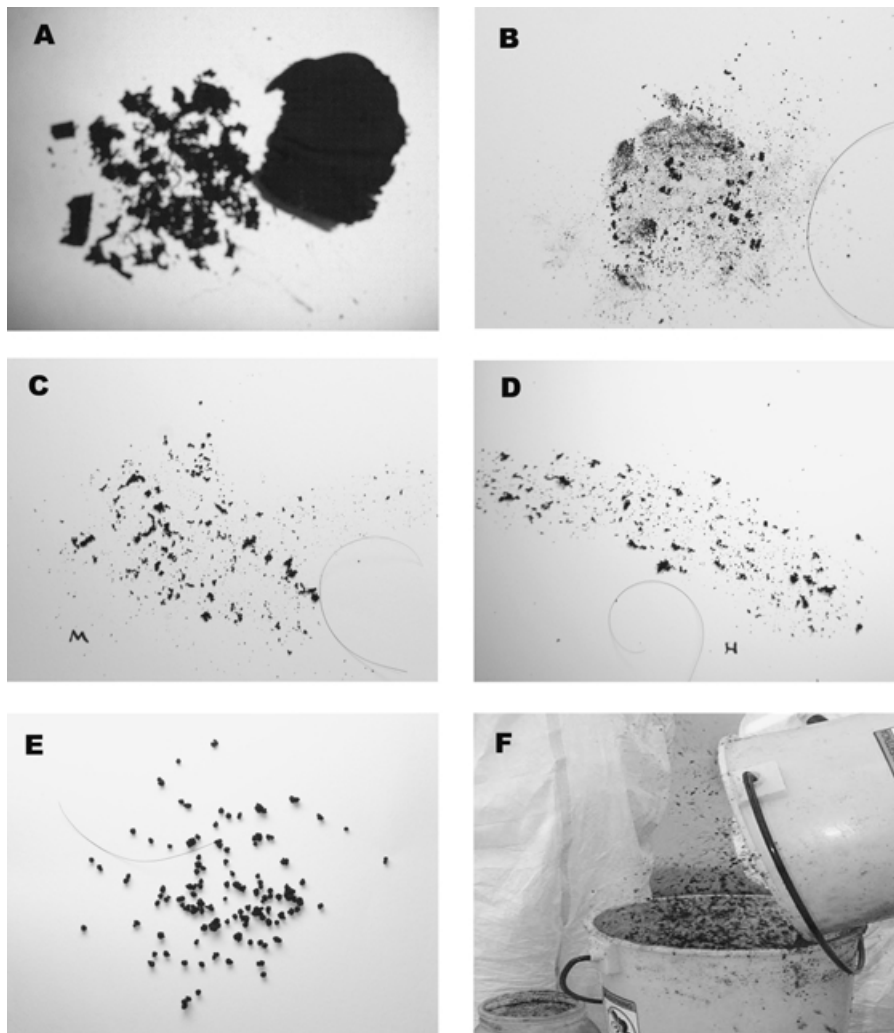


FIG. 2. (A) A sample of SWCNTs produced by the pulsed laser-vaporization process at NASA JSC Nanotube Arc Discharge Facility; this product contains very few fine particles. (B) SWCNTs produced by CarboLex's electric-arc process; the product is a fine powder. (C) MWCNTs produced by CarboLex electric arc; the product contains fine particles. (D) SWCNTs made by the Rice HiPco process; the product contains fine particles. (E) SWCNTs made by the HiPco process, purified, and produced as tiny pearl-like granules; the product contains essentially no fine particles. (F) The transfer of HiPco SWCNTs between containers shows that the product is light-weight and easily becomes airborne. (Figure F is courtesy of A. Maynard of NIOSH; Baron et al., 2003). A human hair is shown in some of the pictures for size comparison.

iron by weight, is very light (Figure 2F) and the bulk density is very low ($\sim 1 \text{ mg/cm}^3$) (Baron et al., 2003). MWCNTs also have van der Waals forces (Yu et al., 2000), but they are less effective than those of SWCNTs. Because of their weaker van der Waals forces and structural heterogeneity, MWCNTs generally exist in single tubes (like spaghetti) and form very few bundles. The tubes or bundles look like microscopic ropes (Figures 1F and 1G). However, the appearance of bulk MWCNT product (Figure 2C) is similar to that of SWCNTs (Figure 2B) made by the same company (CarboLex).

Because CNTs can be made by several methods and by different manufacturers who use different catalytic metals, carbon sources, and processing conditions (such as pressure and

temperature), the length of individual CNTs and the purity of the raw products can vary, even from batch to batch (Cui et al., 2000). In addition to the difference in synthetic processes, differences in postmanufacture processing affect the physical characteristics of a commercial CNT product. For example, the commercial purified HiPco product of Carbon Nanotechnologies Incorporated (CNI, Houston, TX) is prepared and sold as tiny pearl-like granules, which contain few or no fine particles that would pose an inhalation risk (Figure 2E). Compression of loose clumps into a powder or preparation of granules from purified CNTs increases the compactness of the bulk products for ease of handling and shipment and would also affect the physical appearance of the CNT particles. Thus, the physical

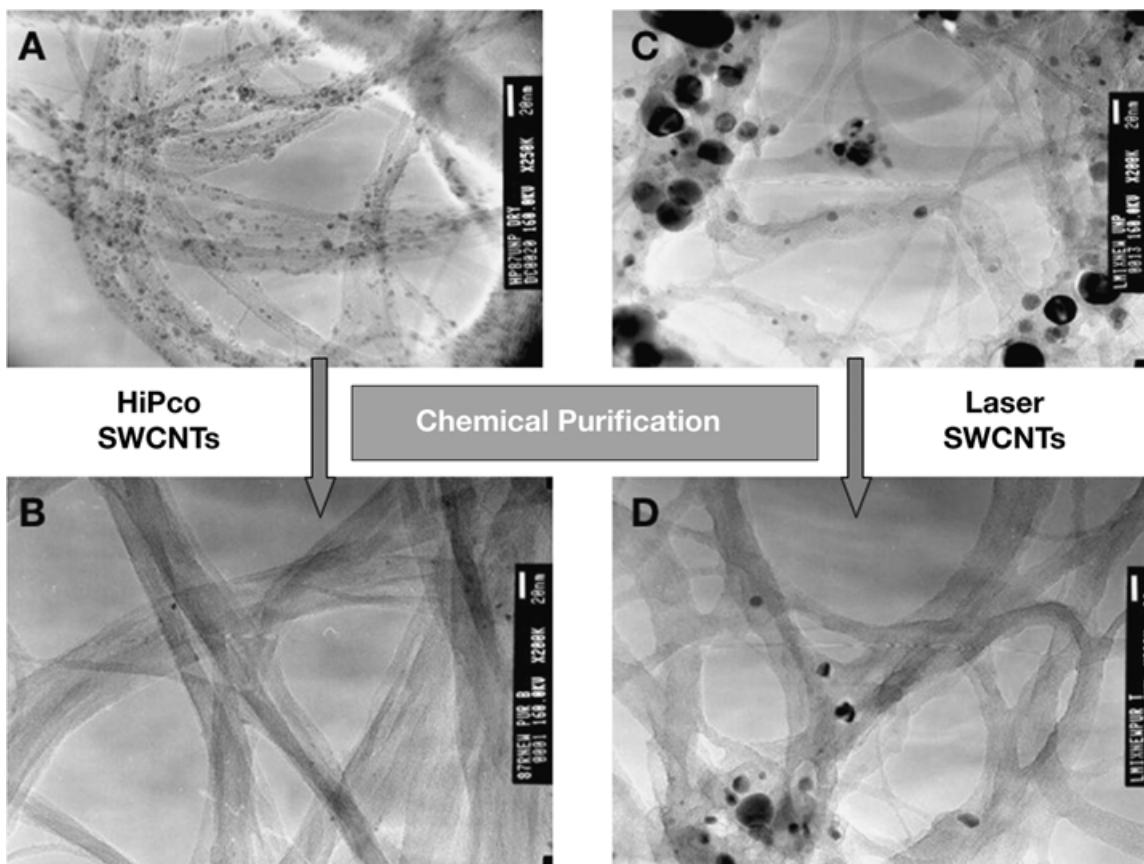


FIG. 3. TEM pictures on the top show the presence of impurities (carbon-coated metal nanoparticles, etc) in raw HiPco- and laser-produced CNT samples. The bottom pictures show the samples after purification. Notice that the purified HiPco sample contains essentially all carbon nanotubes; the purity of a final product depends on type of raw CNT product and extent of purification.

characteristics of a CNT product depend on its purity, the method by which it was synthesized, and postsynthetic processing. The difference in physical characteristics of CNTs, which depend on manufacturing processes, will affect the health risks of exposure to the product. The knowledge of these processes is important information for an industrial hygienist, toxicologist, or risk assessor who is assessing the toxicological risk of workers being exposed to a particular CNT product.

5. Potential Occupational Exposures to Manufactured Carbon Nanotubes

At present, the production volume of CNTs is still small and the products remain expensive. The price of SWCNTs was hundreds of thousands of dollars per pound in 2003, according to Richard Smalley (Bearden, 2003). Raw SWCNTs and MWCNTs are sold currently by BuckyUSA (Houston) at >\$100 per gram (or >\$50,000/lb) (BuckyUSA, 2005). In 2003, Baron et al. (2003) of the aerosol group of the National Institute of Occupational Safety and Health (NIOSH) visited CNT synthesis laboratories at Rice University and NASA's Johnson Space Center and the CNT manufacturing facility at Carbon Nanotechnologies, Inc. (CNI, Houston), where SWCNTs are produced by the HiPco or laser processes. The laboratories were making

only several grams per day at that time (Shvedova et al., 2005). These NIOSH scientists observed the recovery of CNTs from synthetic ovens and reported that handling of the collected samples was gentle, and losses of this expensive material were minimized. As expected, they found very few CNTs in the working environments of these facilities; the airborne concentrations of CNTs were less than $53 \mu\text{g}/\text{m}^3$ (Maynard et al., 2004). These facilities had implemented excellent industrial hygiene measures (Figure 4).

However, Smalley predicted that hundreds or thousands of tons of CNTs could be produced in 5 to 10 years (Ball, 2001), and "in time, millions of tonnes of nanotubes will be produced worldwide every year" (Taubes, 2002). The Department of Energy's 2010 target goal for the price of CNTs is \$8/kg (or <\$20/lb). The extent of industrial and commercial applications would depend on the price of CNT raw materials. If the CNT industry achieved the goal of "a couple dollars a pound" (Bearden, 2003), then millions of tons of CNTs could be produced annually. Such a large production volume would involve a very large workforce, and many workers would likely be exposed to these lightweight materials during CNT synthesis and postsynthetic material collection, handling, purification, and packaging (Figure 4). The purity of a CNT product and the manufacturing



FIG. 4. Photograph showing skin could be contaminated with very fine CNT dust if gloves were not worn. (Courtesy of A. Maynard of NIOSH; Baron et al., 2003).

process and postsynthetic processing it undergoes determine its physical characteristics (section I.B.4). These physical characteristics will determine its ability to enter the environment. Thus, the occupational risk of exposure of a worker will depend on what product is being handled and at which phase.

CNTs are strong and light; these very desirable properties would make CNTs very useful in new material development and structural engineering. Progress has been made in incorporating CNTs into fabrics, plastic, rubbers, reinforced structures, composite materials, and household commodities (Laxminarayana and Jalili, 2005; Nanocyl, 2005; Smalley, 2000; Sreekumar et al., 2004; Ward et al., 2005; Wikipedia, 2005). If CNT production increases and prices drop, uses in these areas will be greatly expanded. Occupational exposures to airborne CNT dust could occur in the processes of making these products. During incorporation of CNTs into some of these types of materials, the CNT raw materials (in such forms as the clumps shown in Figure 2A

and the purified granules shown in Figure 2E) will need to be pulverized and homogenized; working with pulverized CNTs, or resins or mixtures containing fine CNT particles, would pose a risk of inhalation exposure. It is important to note that some of the factories would be dustier because they would probably not have industrial hygiene standards of the same caliber as the first-class facilities that were visited by the NIOSH aerosol group, who found very little airborne CNT dust.

A study by the NIOSH aerosol group on a raw HiPco sample and a laser-synthesized CNT sample produced by Rice University revealed that gentle agitation (blowing air over the material shaken in a vortex) produced large airborne clumps (Baron et al., 2003; Maynard et al., 2004). Very few small particles were present. At high agitation levels, more airborne NT particles were generated; the particles were $10\ \mu\text{m}$ or less, which are mostly respirable sizes, with some being in the ultrafine range (Figure 5) (Maynard et al., 2004). Thus, if CNTs are subjected to vigorous mechanical processes (such as agitation, grinding, and pulverizing), dust of respirable sizes will be produced, and occupational inhalation exposures could occur. During manufacturing and handling, CNT particulate matter (PM) could also land on the skin of workers if it is not protected (Figure 4).

6. Environmental Health Issues of Manufactured Carbon Nanotubes

The synthesis of CNTs does not require organic solvents. It is considered a “green” industrial process. Currently, it is expected that very little of these very expensive materials will find its way to contaminate the outdoor environment as industrial waste. However, if the price drops substantially and CNTs are incorporated into industrial products and household commodities that are used world-wide, the general public could also be exposed to low levels of CNTs. In the United States we have

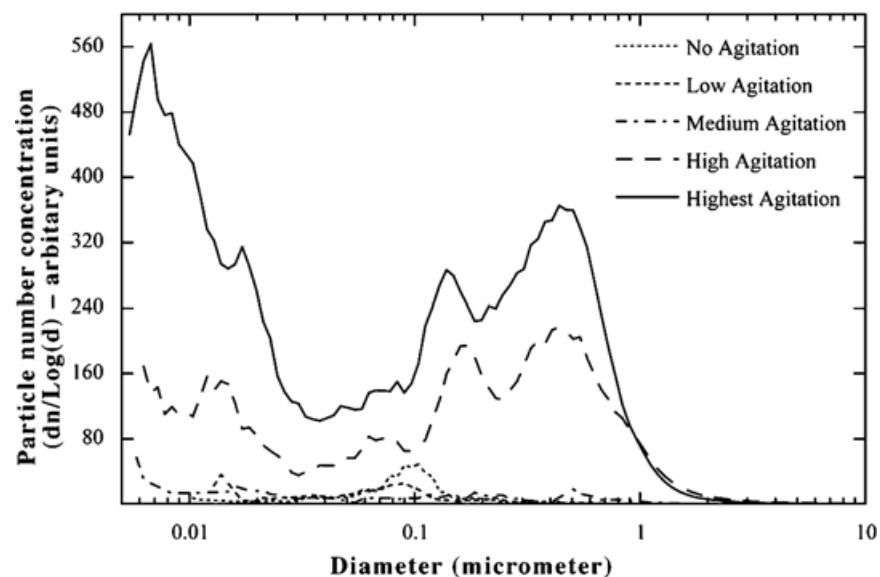


FIG. 5. Size distribution of particles generated from a raw HiPco carbon nanotube sample using a single-component vortex-shaker fluidized bed; data were smoothed for clarity by the original authors. (Courtesy of A. Maynard of NIOSH; Baron et al., 2003).

witnessed the use of asbestos in automotive brake shoes until it was found that asbestos particles generated from abrasion contribute to environmental pollution, and the U.S. Environmental Protection Agency banned use of this carcinogenic material in automobiles (Federal Register, 1989). CNTs are light and strong, and if the price of CNTs drops to a few dollars a pound, applications of CNTs will expand to automobiles and other products that could be subject to deterioration. Wear and tear on products containing CNTs could generate fine particulate matter that may contribute to environmental pollution. Incineration of discarded articles or wastes that contain CNTs may release some CNTs into the environment. Before the use of CNTs become more widespread, it is important that the chronic toxicity of CNT particulate matter be studied and known, and appropriate safeguards against environmental contamination be implemented.

C. Multiwall Carbon Nanotubes Generated by Fuel Combustion

As discussed in Section I.B.3, CNTs could be produced from carbon atoms thermally generated from hydrocarbons (such as methane and acetylene) (Kong et al., 1998; Ren et al., 1998). The synthesis of CNTs is generally carried out with the addition of catalytic metals. In fact, it is known that the synthesis of MWCNTs can occur without metal catalysts (Cumings and Zettl, 2004; Nanotech Co. Ltd., 2003; Tomanek, 1999). MWCNTs were first reported to have been produced from carbon thermally vaporized from graphite without the presence of a metal (Iijima, 1991). Che et al. (1998) observed formation of MWCNTs (diameters of about 20 nm) when ethylene/pyrene was pyrolyzed at 545°C in the presence of Ni; without catalysts, MWCNT formation occurred at 900°C. Many daily heat production or energy-generating activities involve burning of hydrocarbons at temperatures greater than 1000°C. One would suspect such activities could produce MWCNTs.

1. Formation of Multiwall Carbon Nanotubes from Fuel-Gas Burning Activities Indoors

In fact, MWCNTs and other fullerene-related multi-layer shell structures were observed in samples collected from the effluent stream of co-flowed flames of methane and air (Figures 6A and 6B) (Murr et al., 2004b). Bang et al. (2004) also found MWCNTs and carbonaceous nanoparticles in aggregates of particulate matter collected from propane or natural gas (containing 96% methane) flames generated by typical kitchen stoves; the aggregates had sizes ranging from 0.4 to 2 μm , putting them in the respirable range. These aggregates were essentially pure carbon or graphene and contained several hundred to several thousand MWCNTs or other related nanocrystal forms with an average diameter of 20 nm. The individual MWCNTs ranged approximately from 3 to 30 nm in diameter (Murr et al., 2004a). These findings indicate that MWCNTs and other carbonaceous nanoparticles are produced also by water heaters, furnaces, and household appliances powered by natural gas.

2. Multiwall Carbon Nanotubes in the Outdoor Environment

MWCNTs and carbonaceous nanoparticles (Figures 6C–6F) were also observed in outdoor airborne particulate matter collected in El Paso (Texas) and Houston (Murr et al., 2004b). The aggregates were similar in structure to those collected indoors except that the outdoor particulate matter also included agglomerates with other common atmospheric mineral nanocrystals, such as silica. According to Murr et al. (2004a), about 15% of the El Paso particulate samples were carbonaceous aggregates consisting of MWCNTs and other nanoforms (shells, spheres, and other structures). Diesel-related aggregates accounted for 5% of the sample (Murr et al., 2004a). Murr's group (Bang et al., 2004) concluded that MWCNTs and carbonaceous nanoparticles are ubiquitous in the environment; they further speculated that MWCNTs are a major component of indoor and outdoor airborne particulate matter.

3. Multiwall Carbon Nanotubes Were Combustion Products of Ancient Anthropogenic or Natural Activities

MWCNTs and fullerene-like nanocrystal forms were also observed in numerous particle aggregates in a sample of ancient ice obtained from an ice core at the Greenland ice cap (Esquivel and Murr, 2004; Murr et al., 2004b). The ice sample, from a core drilled 1646 feet deep, was dated to roughly 10,000 years old (placing it in the Neolithic Stone Age). According to Murr et al. (2004b), the average diameter of the aggregates was <1 μm . These findings show that MWCNTs and other carbonaceous nanoparticles were present in airborne respirable dust aggregates in the prehistoric environment.

4. Multiwall Carbon Nanotubes in Fine Particulate Matter and the Implications for Public Health

The MWCNTs generated from fuel-gas combustion and found in indoor and outdoor environments (Bang et al., 2004; Murr et al., 2004a) are components of airborne particulate aggregates less than 2.5 μm (PM_{2.5}) in size. It has been well established that the sources of PM_{2.5} include fuel combustion from automobiles, power plants, wood burning, industrial processes, and diesel-powered vehicles such as buses and trucks (U.S. Environmental Protection Agency, 2003b). Combustion processes typically generate very fine particles of sizes from 0.01 to 2.5 μm (Huggins et al., 2004), and combustion of fossil fuels is the greatest contributor to fine particulates in the air. The particulate matter contains elemental carbon, organic carbon, trace elements, and common ions (U.S. Environmental Protection Agency, 2003a).

Natural gas is considered an environmentally clean fuel and produces less (7 lbs/billion Btu) particulate matter than oil (84 lb) or coal (2774 lb). However, because global fuel-gas consumption is very large, the contribution of MWCNTs to air pollution is very substantial. According to the National Energy Foundation (National Energy Foundation, 2002), the United States

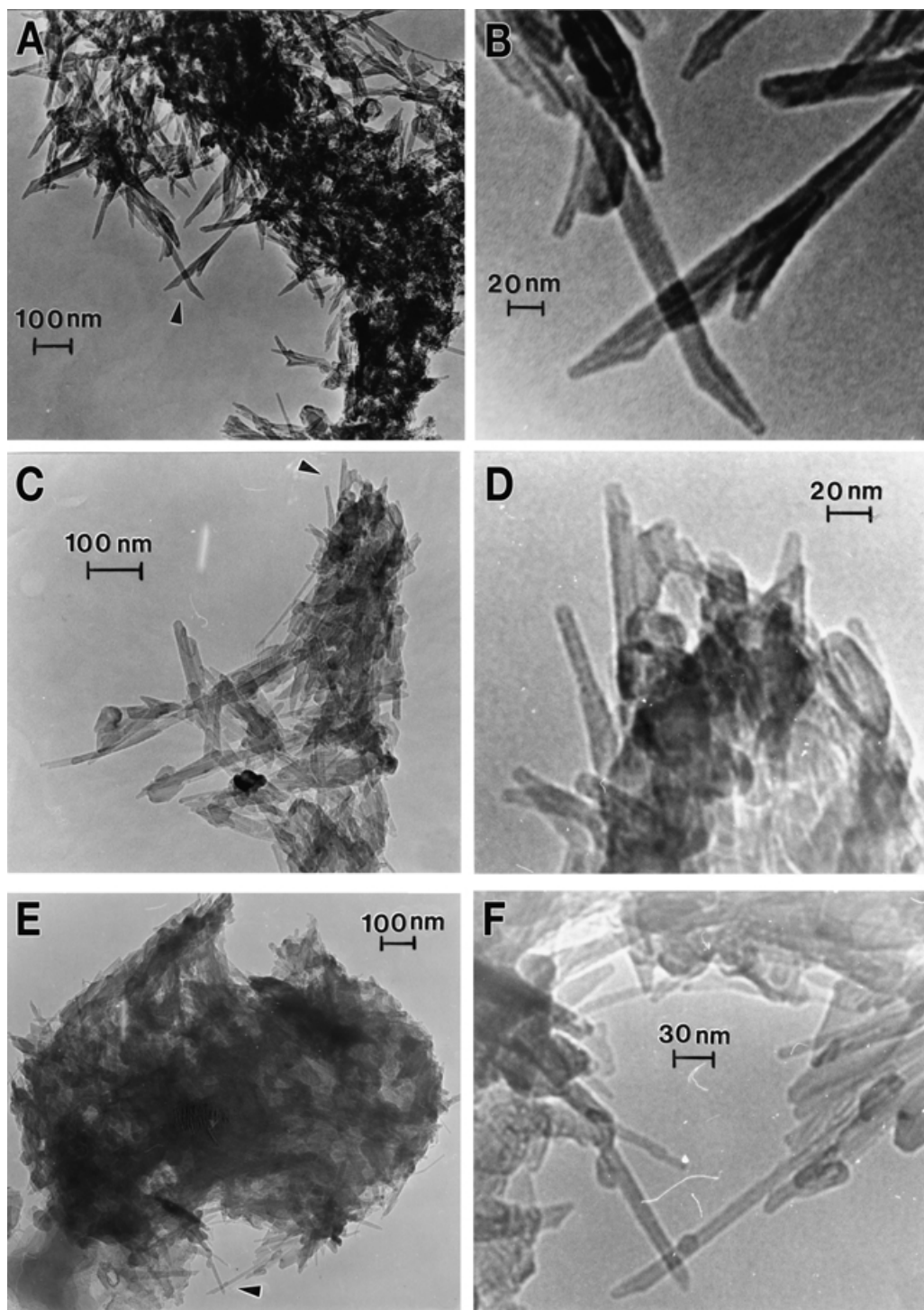


FIG. 6. (A) A transmission electron microscope (TEM) image of a particulate matter (PM) sample collected from a combustion stream of methane in the air. (B) High magnification of the area marked by the arrow in (A). (C) TEM image of an environmental PM sample collected from El Paso (TX). (D) High magnification of the area marked by the arrow in (C). (E) TEM image of an outdoor PM sample collected in Houston (TX). (F) High magnification of the area marked by the arrow in (E). Courtesy of L. E. Murr of University of Texas at El Paso, TX, and A. Holian of University of Montana at Missoula, with permission for reprint from Springer Publisher, Heidelberg, Germany (Murr et al., 2004a).

consumed 22,096 billion Btu of natural gas in 1999; this accounted for 27% of the global consumption. The consumption of this “environmentally clean” fuel is expected to increase, and the contribution of MWCNTs to air pollution will be even greater than before.

A study conducted by Harvard University (Boston) of mortality rates in six U.S. cities showed an association between fine particulate air pollution and excess mortality (Dockery et al., 1993). In an American Cancer Society-sponsored epidemiological study of 1.2 million adults, Pope et al. (2002, 2004) reported that fine particulate matter in ambient air is a risk factor associated with cardiopulmonary mortality and cardiovascular and pulmonary diseases. Gauderman et al. (2004) investigated the effect of air pollution on the growth of lung function of children during the period of rapid lung development that occurs between the ages of 10 and 18. They found that adverse effects on the lungs were associated with exposures to NO₂, acid vapor, fine particulate matter (PM_{2.5}), and elemental carbon; elemental carbon was shown to have the highest correlation ($p = .007$). Concerns about health effects from exposure to fine particulate matter of size $<2.5 \mu\text{m}$ (PM_{2.5}) prompted the U.S. Environmental Protection Agency to revise the National Ambient Air Quality Standards for particulate matter to include a standard for PM_{2.5} (U.S. Environmental Protection Agency, 2003b). Because MWCNTs are present in airborne particulate aggregates of respirable sizes generated from daily human activities and they are likely to be present in significant levels in our environment (Murr et al., 2004a), it is reasonable to postulate that all humans are exposed to CNTs.

It is true that much of the urban ambient PM_{2.5} is derived from combustion of diesel fuel and gasoline, and not from combustion of natural gas. However, if carbon atoms can be stripped off from ethylene/pyrene during pyrolysis that produced MWCNTs (Che et al., 1998), and from methane and propane during natural-gas combustion that generated MWCNTs, fullerenes, and other carbon nanoparticles (Bang et al., 2004; Murr et al., 2004a, 2004b), one could speculate that some of the carbon atoms formed in the automotive combustion chamber from gasoline (which contains mainly octane and other hydrocarbons) could form MWCNTs. One may speculate that MWCNTs are present in the soot of automotive exhaust. It is noteworthy that the fine ($<2.5 \mu\text{m}$) PM samples that contained MWCNTs collected in Houston were from an area in close proximity to a road with heavy traffic (Murr et al., 2004b).

D. Comparison of Manufactured Carbon Nanotubes and Fuel Combustion-Generated Carbon Nanotubes

In production ovens, CNTs are made in large amounts, allowing van der Waals forces to be effective in driving the aggregations of nanotubes into bundles, ropes, and clumps, most of them probably larger than respirable sizes. The synthesis conditions are also optimized to produce CNTs of a high degree of uniformity and long fibers for practical applications (such as spinning into threads or ropes). However, in the environment

outside the laboratory, where fuels are heterogeneous, combustion conditions are various, and the reaction environments are not confined, fuel-generated CNTs are expected to be highly irregular in size and quality (Wikipedia, 2005). These factors would reduce the effectiveness of van der Waals forces; the MWCNTs produced would be less orderly, shorter in length, fewer in numbers, and intermingled with other nanoparticles. The physical differences between MWCNTs that are manufactured and those that are combustion-generated (or found in the ambient environment) are illustrated by comparing Figure 1F with Figure 6. Figure 1F is an image of a laboratory sample showing manufactured MWCNTs appearing like microscopic ropes of length greater than a micrometer. Figure 6 shows environmental MWCNTs appearing like nanorods with lengths varying within several hundred nanometers, and intermingling with other nanoparticles. Murr's group showed that sizes of particulate matter containing environmental MWCNTs were in the respirable range (Murr et al., 2004a).

II. TOXICOLOGICAL STUDIES AND TOXICITY OF MANUFACTURED CARBON NANOTUBES

A. Methodology to Assess the Potential Toxicity of Carbon Nanotubes in the Lungs

When CNT synthesis moved from laboratories to factories and the products became commercial commodities, there was great concern about the toxicity of these light-weight fibrous materials and their potential effects on workers (Gorman, 2002). As pointed out earlier (section I.B.4), van der Waals forces cause SWCNTs to have a strong tendency to bundle into microscopic ropes, which may contain up to a few hundred parallel tubes (Salvetat et al., 1999). These secondary structures, in turn, form loose clumps, which can become airborne but are mostly larger than respirable size (Figure 2F). These larger particulates are not suitable for toxicology studies. To assess the potential toxicity of a dust in the lungs, particles of respirable size have to be isolated from the bulk material, or test materials have to be mechanically processed into particles in the respirable range.

Aside from direct observations of the effects of a dust on exposed human subjects, animal inhalation exposures are the most appropriate means to assess toxicity of a dust in the lung. As shown by the NIOSH aerosol scientists, it would be difficult to isolate and collect enough fine CNT particles or clumps of respirable size for an inhalation study (Baron et al., 2003). It is also difficult to generate a controlled CNT concentration in a chamber and monitor particle size and the actual exposure level. Even with more workable dusts or powders of other compounds, the technical difficulty and cost associated with inhalation toxicity experimentation have led investigators to assess the effects of these aerosols in the lungs by intratracheal instillation (ITI) (Driscoll et al., 2000; Leong et al., 1998; Sabaitis et al., 1999).

If an animal is exposed to a test dust by inhalation, histopathological changes in the upper respirable tract can be examined, in addition to assessing the effects in the pulmonary region. When

dust is administered by ITI, a bolus dose of a fine dust suspension is generally instilled into the trachea of a small animal and the dust is assumed to be drawn deeper into the lung during breathing. Besides having the disadvantage that this unnatural route of administration could not be used to assess the effects of a test dust in the upper respiratory tract, ITI also produces an artificial and less even distribution of dust in the lung. Often, in an ITI study, dust aggregates in aqueous suspensions need to be dissociated or broken down to respirable sizes by ultrasonication with or without a nontoxic dispersion agent. Moreover, the instilled bolus dose may overwhelm the dust clearance mechanism, causing the results to be exaggerated. However, an ITI study, designed appropriately by including carefully chosen doses and reference compounds of known inhalation toxicities, does allow the assessment of the relative toxicity of the test material in respirable sizes (Lam et al., 2002a, 2002b). ITI is an acceptable method of screening dusts for potential pulmonary toxicity, provided that the investigators or risk assessors recognize certain limitations associated with this unnatural way of exposing animals to dust (Driscoll et al., 2000). Dusts of comparable amounts (that reached the lung parenchyma) given by inhalation or intratracheal instillation were shown to produce similar toxicity in the lungs (Henderson et al., 1995). All the animal studies conducted so far to examine CNT pulmonary toxicity have been performed by ITI or similar techniques.

B. Toxicity Studies of Carbon Nanotubes in the Lungs

The first study of SWCNT toxicity, in which lung histopathology in exposed mice was examined, was conducted to address NASA's concern that workers in occupational settings could be exposed to the airborne dust of this novel material of unknown toxicity (Lam et al., 2004). The study was also supported by the Center of Nanoscale Science and Technology of Rice University; both organizations have facilities that make SWCNTs. Parallel to the NASA study, Warheit et al. (2004) of Du Pont Company (Wilmington, DE) conducted a toxicity study in rats of a SWCNT product made by their company. Although the two studies yielded some similar histopathological findings, the two groups reached different conclusions about the toxicological risk of exposures to SWCNTs; these have been the subject of wide debate (Shvedova et al., 2005). Shvedova et al. of NIOSH subsequently conducted a very comprehensive study in mice "to resolve this conflict" (Shvedova et al., 2005). The toxicity of MWCNTs had drawn little attention until very recently when a group led by J. Muller of Facultés Universitaires Notre-Dame de la Paix in Namur, Belgium (Muller et al., 2005), published its study. All of these toxicological studies are outlined in Table 1 and are the subjects of this review.

1. Study of a Carbon Nanotube Product in Guinea Pigs by Huczko et al.

The first published report on CNT toxicity was based on a study conducted by Huczko et al. (2001) of Warsaw University

on two groups of five guinea pigs. Each animal was intratracheally instilled once with 0 or 25 mg of CNT-containing soot in 0.5 ml saline (with Tween as a dispersing agent). After 4 weeks, tidal volume, breathing frequency, and pulmonary resistance were assessed. Bronchoalveolar lavage fluid (BALF) was obtained from these animals for measurement of cell differentials and total protein concentration. The investigators found no differences between groups in the measured parameters, and concluded that "the soot with a high content of CNTs does not induce any abnormalities of pulmonary function or measurable inflammation in guinea pigs treated with carbon nanotubes." The authors of this short communication, which was published in a nontoxicology journal, further concluded that "working with soot containing carbon nanotubes is unlikely to be associated with any health risk." However, examination of lung pathology, which is the most critical toxicological endpoint of any pulmonary toxicity study with dust, was not included in the study. Lung pathology was examined in the other animal studies reviewed later.

2. Study of Several Single-Wall Carbon Nanotube Products in Mice by Lam et al.

For several years, NASA has had a facility that makes SWCNTs using the laser process developed by Rice University. At the time (2000) this study was initiated, no toxicity data on this material existed and NASA was concerned about its workers being exposed to a novel material of unknown toxicity. Richard Smalley's group at Rice, also concerned about potential CNT toxicity (Gorman, 2002), joined NASA in support of our toxicity study on SWCNTs.

To assess the pulmonary toxicity of CNT particles by ITI, investigators must prepare the particles in respirable sizes. In our study, the CNTs were prepared in mouse serum by brief homogenization (shearing) and ultrasonication (Lam et al., 2004). Figure 7 shows light micrographs of CNT particles in serum suspensions and shows that the predominate size of the particles was several micrometers; smaller particles were present but could not be captured in light-microscope photographs. Particles of size about 1 μm were abundant under the microscope; sizes smaller than 0.5 μm could not be detected or resolved by light microscopy. Characterization of the CNT particles in serum suspensions by scanning electron microscopy (SEM) was unsuccessful because serum used as a dispersion agent for CNTs interfered with SEM detection of CNT particles. Removal of serum would change the particle dynamics and sizes in the suspension. Using a transmission electron microscope, Shvedova et al. (2005) reported that the dried images of nebulized CNT suspension droplets (mass median particle diameter of $\sim 5 \mu\text{m}$) showed continuous mats of intertwined carbon nanoropes with varying diameters. The actual sizes of these respirable CNT particles that reached the lung remained unknown. Because we were interested only in finding out the toxicity of respirable-sized particles of SWCNTs in the lungs and not in investigating nanoparticle toxicity, no efforts were carried out to make suspensions

TABLE 1
Pulmonary toxicity studies of CNTs in animals and characteristics of these materials

Test materials	Maker of test materials	Synthetic process	Metal content ^a	Animal species	Method of administration	References
Soot containing CNTs	Toyo Tanso Co. Ltd., Japan	Electric arc	Co/Ni No info on %	Guinea pig	ITI	Huczko et al., 2001
SWCNTs	Rice University, Houston, TX	Laser ^b	Ni: 10% ^c	Mouse	ITI	Lam and McCluskey, 2000 (unpublished report)
SWCNTs	Rice University, Houston, TX	HiPco ^b	Fe: 26.9% Mo: 0.95% Ni: 0.8%	Mouse	ITFI ^d	Lam et al., 2004
SWCNTs, purified	Rice University, Houston, TX	HiPco ^b	Fe: 2.1%	Mouse	ITFI	Lam et al., 2004
SWCNTs	CarboLex Inc. Lexington, KY	Electric arc	Ni: 26.0% Y: 5.0% Fe: 0.5%	Mouse	ITFI	Lam et al., 2004
SWCNTs	DuPont Co. Wilmington, DE (or Rice University, TX)	Laser ^b	Ni: 5% Co: 5%	Rat	ITI	Warheit et al., 2004
SWCNTs, purified	Carbon Nano-Technologies Inc., Houston, TX	HiPco ^b	Fe: 0.23%	Mouse	IPA ^e	Shvedova et al., 2005
MWCNTs	Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium	CVD	Co: 0.95% Fe: ~ 1%	Rat	ITI	Muller et al., 2005

^aPercent by weight in final products.

^bProcess developed by Rice University or originally developed by Rice University.

^cInformation provided by D. Colbert of R. Smalley's group at Rice University.

^dIntratracheal fast instillation.

^eIntrapharyngeal aspiration.

containing CNT particles predominately in nanosizes. Moreover, CNTs are very unlikely to exist in nanosizes in the air, and the doses prepared in our animal study, by briefly (≤ 1 min) ultrasonicated CNTs and suspending them in mouse serum at

concentrations of 0.1 mg/50 μ l (equivalent to 2000 mg/L) and 0.5 mg/50 μ l, were too concentrated for the particles to exist in nanosize (Figure 7). As is routinely done when preparing CNTs for physiochemical investigations, Smalley's group at Rice used

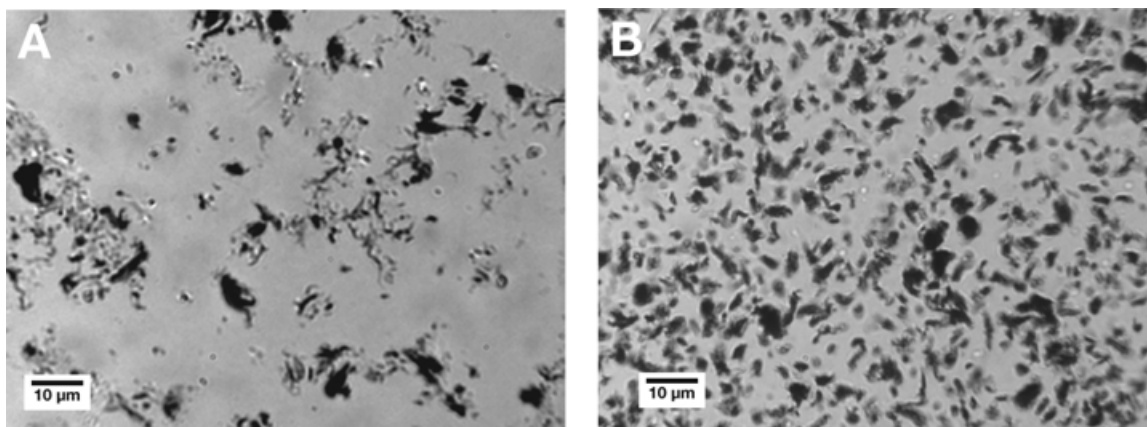


FIG. 7. Light micrographs of sonicated suspended samples of (A) raw HiPco CNTs (0.1 mg/50 μ l), and (B) purified HiPco CNTs (0.5 mg/50 μ l) showing the particles were predominately in respirable sizes (several micrometers or less).

“aggressive sonication of purified NT samples in surfactants such as Triton X or highly polar solvents like dimethyl formamide” to make nanoparticle suspensions of 10 mg/L containing mostly individual fibers and a few small bundles (Walters et al., 2001).

Pilot Study (Lam and McCluskey, 2000 [unpublished report]) A pilot study was conducted on an early experimental SWCNT sample made at Rice University by the laser ablation process and containing about 10% nickel (by weight) and some iron (information provided by D. Colbert of Rice University). In this study, anesthetized mice (C57/BL/6J) were each intratracheally instilled with 100 μ l of mouse serum containing 0, 0.1, or 1 mg of a CNT sample. Microscopic examination of the lungs in the lower-dose group 1 week after the treatment showed minimal tissue reactions. However, the lungs of 5 mice treated with the high CNT dose had focally severe foreign-body reactions characterized by widespread prominent foci of particle-laden macrophages and giant cells, and prominent peribronchial and perivascular lymphocytic infiltrates (Figure 8B).

This high CNT dose killed some mice immediately after the instillation; their respiratory distress apparently indicated that mechanical blockage of the airways occurred, apparently from the large volume (100 μ l/mouse) of a bolus instillant. Because a few animals died of suffocation in our pilot study, in our core study we reduced the volume of the dose to 50 μ l/mouse and modified the instillation technique (Lam et al., 2004) by adopting the intratracheal fast instillation of Sabaitis et al. (1999). We also replaced parenteral ketamine/xylazine anesthetic with isoflurane inhalation anesthetic to achieve quick anesthesia induction and reduce mucus production. No more deaths from airway blockage occurred. Because the low dose in our pilot study produced only minimal effects in the lung, we halved the high dose and used these two doses (0.1 and 0.5 mg/50 μ l/mouse) for our core study.

Core Study (Lam et al., 2004) Because the CNT sample used in the pilot study contained a substantial amount of nickel, and nickel is highly toxic, we could not with certainty ascribe the lung lesions to CNT itself. To more definitively characterize the intrinsic toxicity of CNTs and the influence of metals in the toxicological manifestation of the test compounds, we followed the pilot study with a core study of three CNT products made by different manufacturing processes and containing different types and/or amounts of residual metals (Lam et al., 2004). When this study was initiated, Rice had shifted from the laser process to the HiPco process for synthesizing CNTs. The study was conducted on (1) unprocessed iron-containing SWCNTs made by the new HiPco process, (2) a purified HiPco product that had been vigorously treated with concentrated acid to remove metal residues by the method published by Rinzler et al. (1998), and (3) a CarboLex SWCNT sample, made by an arc-discharge process, that contained nickel and yttrium (Table 1). Included in the core study were two standard reference materials: ultra-fine carbon black (Printex 90, a dust with relatively low toxicity in mice) and quartz (Min-U-Sil-5, a fibrogenic

dust). Groups of B6C3F₁ mice (4/group for 7 days, 5/group for 90 days) were intratracheally instilled with a test-dust suspension (0, 0.1, or 0.5 mg/50 μ l/mouse, respectively equal to about 0, 3.3, or 17 mg/kg) and were euthanized 7 or 90 days after the single treatment. The lungs were excised, fixed, and stained for histopathological study (Lam et al., 2004).

All CNT samples tested, regardless of the type and amount of metal impurities they contained, induced dose-dependent lesions characterized chiefly by interstitial granulomas (microscopic nodules) in the lungs of mice in the 7-day (Figures 8C and 8D) and 90-day groups (Figures 8E and 8F) (Lam et al., 2004). The granulomas were located beneath the bronchial epithelium and were present throughout most of the microscopic fields of lung tissue. The lungs of all the mice that received high doses of CNTs had prominent granulomas. Granulomas were less prominent but were still observed in the mice treated with the low dose of HiPco-synthesized CNTs; they were not seen in the groups treated with the low dose of graphite arc-derived CNTs that contain lower content of SWCNTs. The findings were similar to those observed in the mice treated with a laser-synthesized CNT product produced in our pilot study; the pathology team of the pilot study described lung lesions as focally severe foreign-body reactions characterized by widespread prominent foci of particle-laden macrophages and giant cells (Figure 8B). The lung lesions in the 90-day high-dose groups were generally more pronounced than those in the 7-day high-dose groups (compare Figures 8C and 8D with Figures 8E and 8F). In the 90-day high-dose groups, many more lymphocytes were seen and fibrosis was more evident (Figures 8E and 8F); the lungs of some of these animals showed severe peribronchial and interstitial inflammation, fibrosis, and necrosis that had extended into the alveolar septa.

As expected, the lungs of mice in the serum control groups were normal. The mice treated with the high dose of quartz showed mild to moderate pulmonary inflammation. This manifestation of quartz-induced toxicity was considered less severe than lesions induced by CNTs. Mice in the group treated with carbon black had black particles in alveolar regions, with minimal tissue reactions (Figure 8A).

3. Study of a Single-Wall Carbon Nanotube Product in Rats by Warheit et al.

Using a different rodent species, Warheit et al. (2004) of Du Pont Company also showed that CNTs produced granulomas in the lungs. This ITI study in Sprague-Dawley rats was conducted on a laser-synthesized SWCNT product that contained 5% nickel and 5% cobalt. The sample consisted of SWCNT ropes, each ~30 nm in diameter. According to Warheit, this CNT sample was produced by the Rice laser ablation process (Rinzler et al., 1998). In 2002, DuPont obtained this manufacturing method from Carbon Nanotechnologies, Inc., which was cofounded by Richard Smalley of Rice University (Cui et al., 2000). This same process was used to make the laser CNT sample that was provided by Rice to NASA for its pilot study (Lam

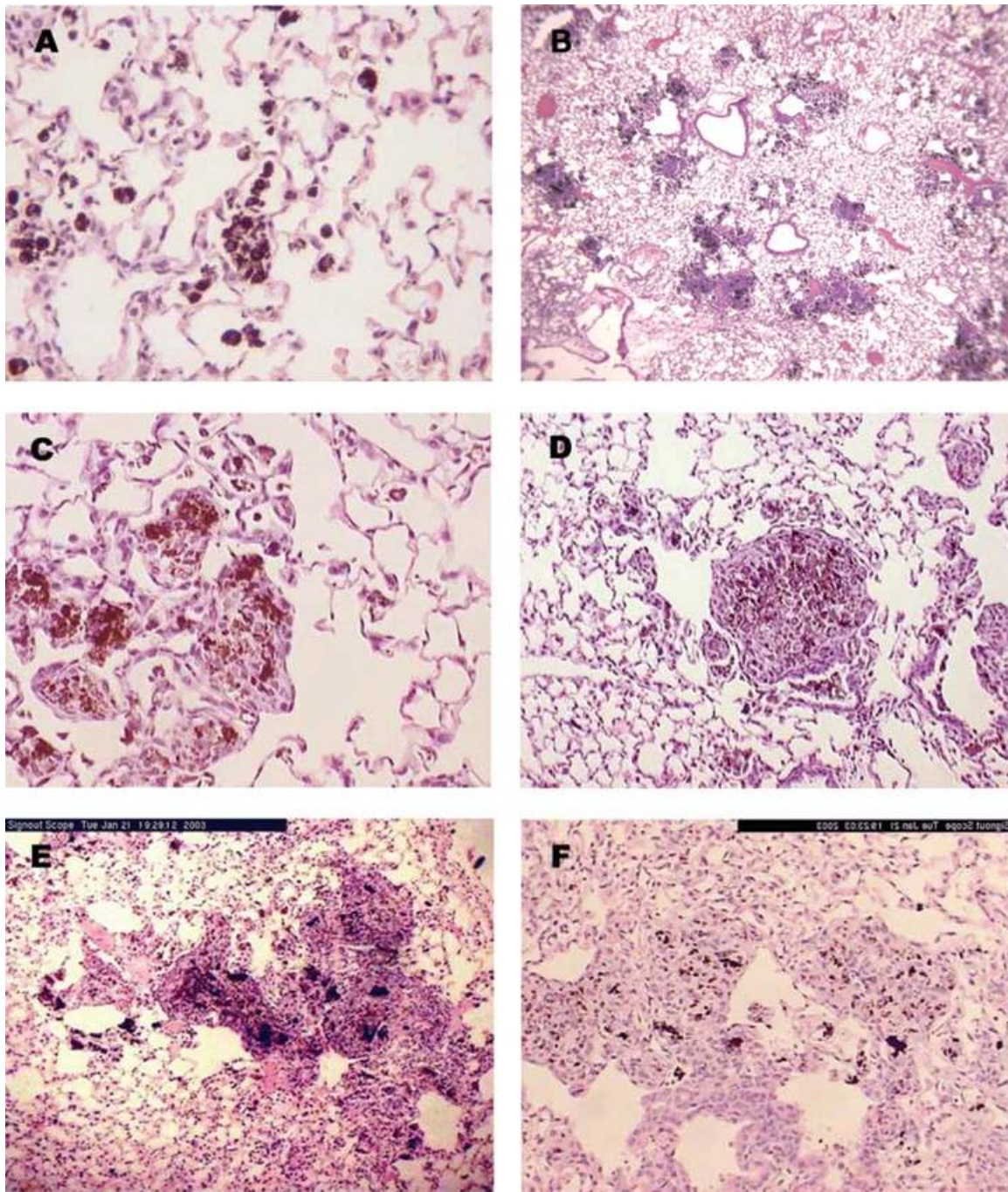


FIG. 8. Lung tissues from mice intratracheally instilled with 0.5 mg/mouse [1 mg/mouse for (B)] of a test material and euthanized 7 or 90 days after the single treatment. (A) Carbon black, 7 days. Particles were scattered in alveoli, and no tissue reaction was observed. (B) Rice University laser-produced carbon nanotubes (CNTs), 7 days. Widespread prominent foci (focal granulomas) contain CNT-laden macrophages. (C) CarboLex arc-produced CNTs, 7 days. Focal granulomas were present. (D) Unprocessed HiPco CNTs, 7 days. The figure shows a well-defined granuloma with some fibrosis (thickening) of alveolar walls. (E) Unprocessed HiPco CNTs, 90 days. A picture of a large nodular lesion shows fibrosis, necrosis, and surrounding tissue, all of which represent a severe reaction. (F) Purified HiPco CNTs, 90 days. Nodular tissue shows fibrosis and necrosis; tissue in this area had very little normal parenchymal structure. Magnifications 40 to 200 \times (Lam et al., 2004).

and McCluskey, 2000) (unpublished report). Warheit's laboratory examined histopathology in the lungs and biomarkers of toxicity in BALF. Graphite powder (particle sizes $<1 \mu\text{m}$ and having the same amount of incorporated metals as CNTs) and quartz were used as negative and positive control dusts. The animals were given the test material (suspended in phosphate-buffered saline with 1% Tween 80) at 0, 1, or 5 mg/kg (0, 0.25, or 1.25 mg/rat). The animals were euthanized for histopathological examination of the lungs at 1, 7, 30, or 90 days after the single treatment. Multifocal granulomas became evident 1 month after exposure (Figures 9C and 9D). The authors stated that granulomatous lesions in the lungs of the CNT-treated rats were non-dose-dependent, nonuniform, and nonprogressive. On the other hand, quartz was observed to produce cytotoxicity, inflammation, and fibrosis in a dose-dependent fashion. Graphite that contained amounts of nickel and cobalt equal to those of the CNT sample produced no signs of toxicity in the lungs.

The turnover of lung parenchymal cells was assessed in groups of rats euthanized at 1, 7, 30, or 90 days after the single-dose dust treatment. Six hours before they were killed, the animals were injected with bromodeoxyuridine (a modified DNA base) to examine the extent of DNA synthesis in lung cells. The results showed no statistically significant change in cell-labeling indices of any CNT-treated group. Significant increases in cell proliferation indices were detected in rats treated with high doses of quartz and euthanized at 24 h and 1 month.

Study of the BALF showed that quartz at a high dose produced an increase in lactate dehydrogenase (LDH) and protein concentrations at all time points; SWCNTs induced only a transient increase in the 1-day group. Quartz at a high dose produced an increase in LDH and protein concentrations at all time points.

The findings of no dose-dependent and no time-dependent granulomatous response, and absence of prolonged inflammation in the lungs, led Warheit et al. to conclude that the granulomatous reaction was a nonspecific response to instilled aggregates of SWCNTs and that the results "may not have physiological relevance, and may be related to the instillation of a bolus of agglomerated nanotubes" (Warheit et al., 2004).

4. *Study of a Single-Wall Carbon Nanotube Product in Mice by Shvedova et al.*

Both Lam et al. (2004) and Warheit et al. (2004) found that CNTs produced lung lesions, including granulomas, but the two groups reached different conclusions regarding the potential hazard of exposures to SWCNTs. To address this difference in toxicological assessments, Shvedova et al. (2005) of NIOSH carried out a pulmonary toxicity study in mice (C57CL/6). The study was conducted on a purified HiPco CNT product ($>99\%$ SWCNTs) that was exhaustively purified by the NASA JSC nanomaterials group to remove metals (final iron content 0.23% by weight) (Figure 3B). The animals were given a single treatment of CNTs, carbon black, or quartz at a dose of 0, 10, 20, or 40 $\mu\text{g}/\text{mouse}$ (about 0, 0.5, 1, or 2 mg/kg). Aqueous suspensions of test dusts were aspirated at the pharyngeal area of

mice, allowing droplets to be pulled into the lung during inspiration. The mice were then killed 1, 3, 7, 28, or 60 days after the treatment. Histopathological examination of the lungs showed an acute inflammation, early onset of formation of granulomas, and progressive fibrosis. The histopathology was characterized by SWCNT-induced granulomas, mainly associated with hypertrophied epithelial cells surrounding the dust aggregates, and diffusive interstitial fibrosis and alveolar wall thickening likely associated with dispersed SWCNTs. The total mass of granulomas in the lungs of mice in the 60-d group increased with an increase in the CNT dose (Figures 10A and B). In general, lung lesions were dose-dependent and progressive, like those reported by Lam et al. (2004). Judging by the published micrographs of lung histopathology, the granulomatous lesions in the mice treated with purified HiPco CNTs in Lam's study (Lam et al., 2004) were more prominent than those reported by Shvedova et al., who also used a purified HiPco CNT sample but with a dose range about an order of magnitude lower (Shvedova et al., 2005). Shvedova et al. further reported that there was significant damage to pulmonary cells. An increase of alveolar type II (AT-II) cells, the progenitor cells that replicate following AT-I cells' death, was detected. Pulmonary function tests showed increases in functional respiratory deficiencies with increased concentrations of CNTs, a finding consistent with fibrosis. Compared with saline-treated controls, CNT-treated mice showed slower bacterial clearance assessed 7 days after bacterial inoculation. At these test doses, quartz and carbon black did not induce granulomas or fibrosis.

To confirm Warheit's BALF findings, Shvedova et al. (2005) also examined the biomarkers of toxicity in the lavaged fluid from CNT-treated mice. The results mostly disagreed with Warheit's findings, showing increases in total protein concentration, cell counts, concentration of transforming growth factor beta (TGF- β), lactate dehydrogenase (LDH), and γ -glutamyltranspeptidase activities, and glutathione depletion; these biomarkers of inflammation, oxidative stress, or cytotoxicity in the lungs were dose-dependent. Shvedova et al. concluded that crystalline silica caused less cytotoxicity than CNTs (compared on an equal-weight basis) and recruited fewer polymorphonuclear leucocytes into the lungs. Like Lam et al. (2004), Shvedova et al. (2005) demonstrated that CNTs were intrinsically toxic and cautioned that exposure of workers to respirable SWCNT particulate matter may pose a risk of developing lung lesions.

5. *Study of a Multiwall Carbon Nanotube Product in Rats by Muller et al.*

MWCNTs have been shown to produce lung lesions similar to those observed in studies with SWCNTs. Muller et al. (2005) tested two forms of MWCNTs, unprocessed MWCNTs and MWCNTs that had been ground. They reported that 60 days after rats (Sprague-Dawley) were each given a single ITI dose of 0.5, 2, or 5 mg MWCNTs (sonicated and suspended in a normal saline solution containing a dispersing agent, Tween 80),

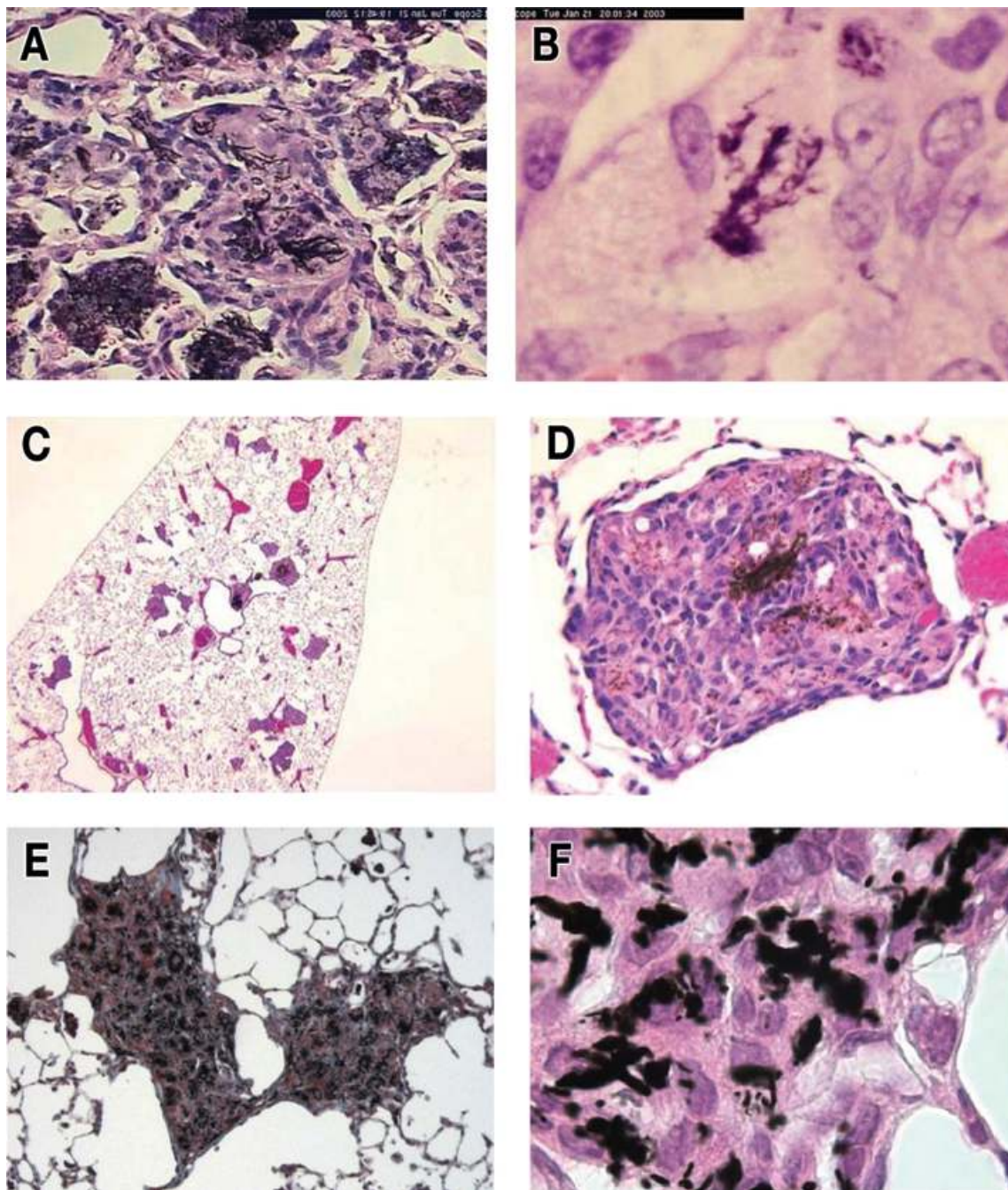


FIG. 9. (A, B) A granuloma in a mouse lung 90 days after instillation with 0.5 mg HiPco SWCNT/mouse showing CNT fibers (Lam et al., 2004). (C) Multifocal granulomas in lung tissue from a rat instilled with 1 mg/kg of laser-produced SWCNTs and microscopically examined 1 month after the treatment. (D) High magnification of a granuloma in (C); the CNTs in the granuloma show fibrous structure. (E) Granulomas that appeared in lung of a rat 60 days after it was instilled with 2 mg/rat of an MWCNT suspension. (F) A granuloma at high magnification. (C) and (D) are courtesy of Warheit et al. (2004) with reprint permission from Oxford University Press (Oxford, UK); (E) and (F) are courtesy of Muller et al. (2005), with permission for reprint from Elsevier Publishing Co. (Philadelphia, PA).

their lungs showed inflammation, granulomas, and fibrosis (Figures 9E and 9F). The unground CNTs remained in the bronchial lumen and produced collagen-rich granulomas. The bronchial lumen was partly or completely blocked, likely an effect similar

to that observed by Warheit et al. (2004); very few CNT particles were seen in the parenchymal (alveolar) region. The ground CNTs were “better dispersed” in the parenchyma, and in the interstitium they induced granulomas consisting of macrophages

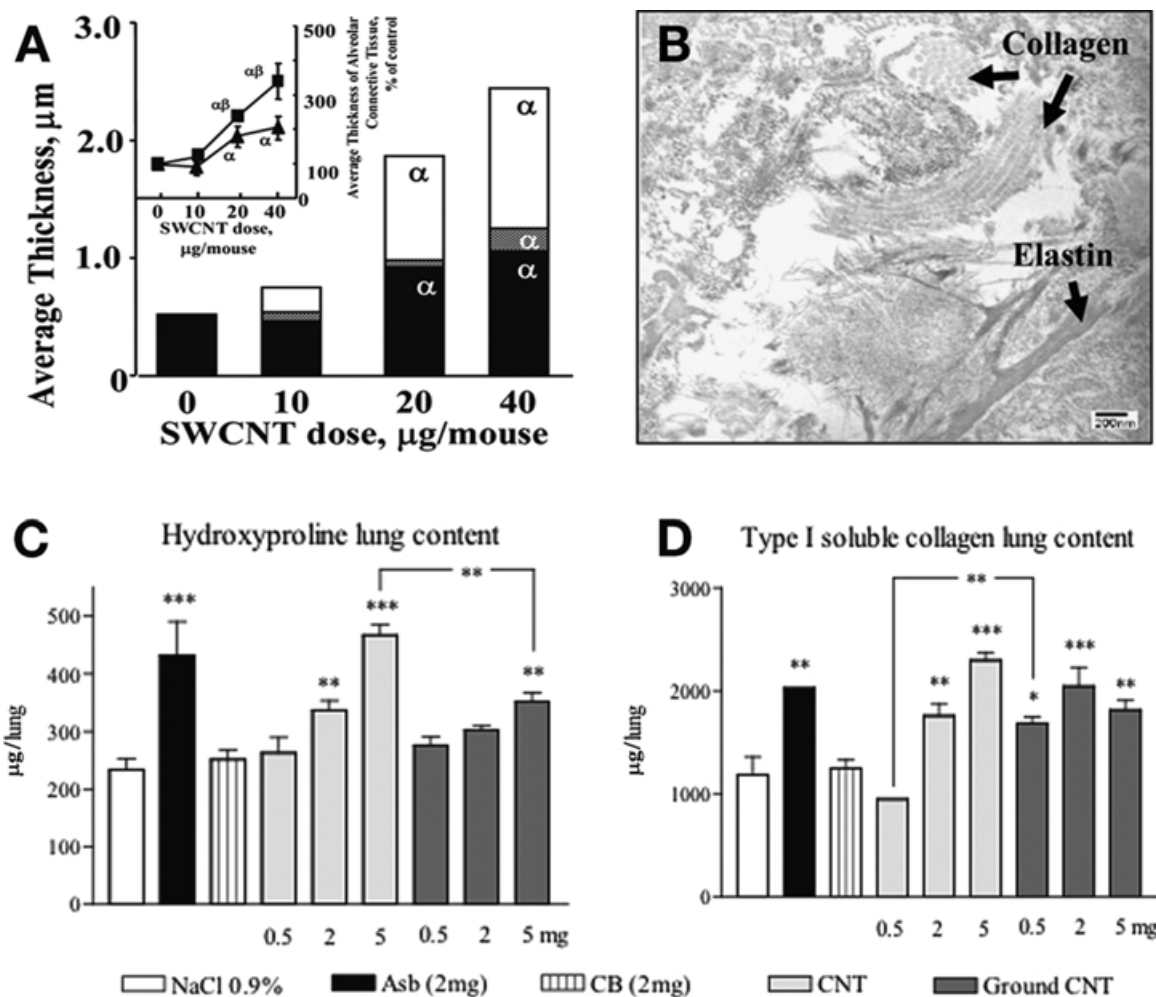


FIG. 10. (A) Morphometric measurement of connective and cellular tissues of mouse lung sections 28 days after postpharyngeal aspiration of 10–40 μg SWCNTs/mouse (open bar, granulomatous cellular tissue; gray bar, granulomatous connective tissue; black bar, alveolar connective tissue). *Inset*: morphometric changes in connective tissue of alveolar wall regions in response to SWCNT (10–40 $\mu\text{g}/\text{mouse}$) 28 and 60 days posttreatment (\blacktriangle , 28 days; \blacksquare , 60 days); means \pm SE ($n = 6$ mice/group). $^{\alpha} p < .05$ vs. PBS-treated control mice; $^{\beta} p < .05$ vs. mice treated with 10 $\mu\text{g}/\text{mouse}$ SWCNT. (B) TEM of lung interstitium shows deposition of collagen and elastin (Shevdova et al., 2005). (C) Hydroxyproline and (D) collagen content of rat lungs 60 days after they were instilled with MWCNTs (ground or unground; 0.5, 2, or 5 mg/rat), asbestos (Asb, 2 mg/rat), or carbon black (CB, 2 mg/rat). CNTs induced a dose-dependent increase of hydroxyproline and collagen production in the lung of treated animals (Muller et al., 2005). (A) and (B) are courtesy of A. Shvedova of NIOSH with reprint permission from the American Physiological Society (Bethesda, MD). (C) and (D) are courtesy of J. Muller, with permission for reprint from Elsevier Publishing Co. (Philadelphia, PA).

laden with particles, multinuclear giant cells, and some inflammatory cells, like those discussed earlier (Lam et al., 2004; Shvedova et al., 2005; Warheit et al., 2004) that were reported for SWCNTs. Muller et al. (2005) also showed that hydroxyproline and soluble collagen, two biomarkers of fibrosis, increased in the lung tissues in a dose-dependent fashion (Figures 10C and 10D), like that reported by Shvedova et al., 2005. BALF obtained from rats 3 days after the CNT treatment showed dose-dependent increases in LDH activity, total protein concentration, and neutrophil number. Muller et al. (2005) also concluded

that CNTs are potentially toxic and advocated strict industrial hygiene.

C. Effects of Single-Wall Carbon Nanotubes in the Mouse Heart

Shvedova's group at NIOSH also examined mitochondrial DNA in the aortas of animals that received CNTs by pharyngeal aspiration (Li et al., 2005); they reported dose-dependent damage to the DNA at 7, 28, and 60 days after the CNT treatment. They concluded that these oxidative changes were the result of

altered expression of inflammatory genes, including MCP-1 and VCAM-1, in the heart. To further examine the effects of CNTs in the heart, 10 hypercholesterolemic (ApoE^{-/-}) mice were each instilled pharyngeally with a total dose of 20 μ g SWCNTs for 8 weeks (once every other week). At the end of exposure, Li et al. (2006) observed that the percent of aortic area covered by plaque was significantly increased in the CNT-treated mice compared with the vehicle-treated controls. Morphometric analysis revealed a significant increase of atherosclerotic lesions in the brachiocephalic arteries of the CNT-treated mice. The authors concluded that the effects in the heart might be caused by cytokines released from the inflammation areas in the lungs and/or by CNTs that leave the lungs and enter the systemic circulation (Li et al., 2005, 2006).

D. Effects of Carbon Nanotubes on Skin Cells in In Vitro Culture Systems

1. Human Skin-Cell Study with a Single-Wall Carbon Nanotube Product

The effect of SWCNTs on skin cells in culture was investigated by Shvedova et al. (2003). Immortalized human epidermal keratinocytes (HEKs) were incubated (37°C) with raw HiPco CNTs [containing 30% iron, and similar to the raw HiPco product used by Lam et al. (2004)] at 0.06, 0.12, or 0.24 mg/ml for 18 h; cell homogenates were then prepared and assayed. In the cells treated with SWCNTs, the authors observed formation of free-radical species, accumulation of peroxidative products, reduction of total sulfhydryls, and a decrease in content of vitamin E. The observed effects were dose dependent. Because some iron compounds are known to induce oxidation or peroxidation in cells, the authors attributed these oxidative effects to the iron in the carbon nanotubes (Shvedova et al., 2003). This conclusion seems inconsistent with the findings on MWCNTs of Monteiro-Riviere et al. (2005), discussed next. In an animal study conducted later, Shvedova et al. (2005) found iron-free CNTs were capable of producing these cytotoxic effects (section II.B.4). The role of iron in CNT-induced cytotoxicity would need further investigation.

2. Human Skin-Cell Study with a Multiwall Carbon Nanotube Product

Monteiro-Riviere et al. (2005) tested a MWCNT product on HEKs; the doses were 0.1, 0.2, and 0.4 mg/ml, and incubation time was up to 48 h. Uptake of particles by the HEKs was demonstrated by transmission electron microscopy. The cells in the 0.4-mg/ml culture were found to release the proinflammatory cytokine interleukin 8 in a time-dependent fashion. The elemental analysis of cells showed that the particles did not contain iron (iron nanofilms were used to induce MWCNT formation). Monteiro-Riviere and colleagues concluded that the effects of MWCNTs on the cells were not caused by the catalytic metal.

III. DISCUSSION

A. Highlights of the Pulmonary Toxicity Studies—Agreements and Disagreements among the Investigator Groups on Potential Health Risk of Exposure to Manufactured Carbon Nanotubes

Lam et al. (2004) conducted a study in mice given three SWCNT products made by different methods and containing different types and amounts of residual catalytic metals. The authors concluded that collectively CNTs induced dose-dependent and time-dependent interstitial inflammation, and epithelioid granulomas. They also concluded that, if CNTs reached the lungs, they could be rather toxic, even more than quartz on an equal-weight basis, and cautioned that a human exposure risk exists. Warheit et al. conducted a similar study in rats and also found that CNTs induced granulomas, but they did not observe prolonged inflammation and fibrosis. The findings that the granulomatous lesions were non-dose-dependent and nonprogressive, and that there was no prolonged inflammation, led Warheit et al. to conclude that the granulomatous reaction was a non-specific response to instillation of a bolus dose of agglomerated SWCNT aggregates. The fact that CNTs have a strong tendency to aggregate, coupled with the finding that some instilled CNTs lodged in the major airways and the mechanical blockage suffocated 15% of the high-dose group, led Warheit to conclude that these toxicological results may not be relevant to human exposures.

As pointed out by Shvedova et al. (2005), the differences in results and interpretations of these two studies led to great debate on toxicological hazards of CNTs. Thus, Shvedova et al. conducted an extensive study “to resolve this conflict.” The results of this NIOSH study showed that CNTs produced an unusual acute inflammation, early onset of formation of granulomas, and progressive fibrosis in CNT-exposed mice. Besides being progressive, the lung lesions were also dose dependent, like those reported by Lam et al. (2004). In addition, these NIOSH investigators also showed that CNTs produced dose-dependent functional respiratory deficiencies and impaired bacterial clearance in the lungs. The test doses of quartz and carbon black did not induce granulomas or fibrosis, or impair lung functions. Shvedova et al. also examined the biomarkers of toxicity in the BALF from CNT-treated mice; the results mostly disagreed with Warheit’s findings in rats (see sections II.B.3 and II.B.4.). Like Lam et al. (2004), Shvedova et al. (2005) demonstrated that CNTs were intrinsically toxic and cautioned that exposure of workers to respirable SWCNT particles may pose a risk that they will develop lung lesions.

The findings of nonprogressive and non-dose-dependent granulomas coupled with the absence of fibrosis and associated biomarkers of cell injury and inflammation in BALF led Warheit et al. to conclude that CNTs did not follow the normal paradigm of other toxic dusts and the lesions were due to a nonspecific response to instillants. Commenting on these findings, Dreher (2004), on the other hand, speculated that a new

mechanism of pulmonary toxicity and injury was possible with CNTs. In testing Warheit's hypothesis, Shvedova et al. (2005) found progressive and dose-dependent fibrotic changes in CNT-treated mice; they also showed in the BALF that CNTs induced an "unusual" and "robust" acute inflammation (did not persist over 2 months after CNT treatment), oxidative stress, and release of toxic cytokines in a dose-dependent fashion.

One of the possible reasons why Warheit et al. (2004) failed to observe a progression and dose and time dependence of lung lesions may be that a substantial fraction of the instilled bolus dose did not reach the alveolar region where the lesions occur. The fractional loss of instillate to major airways was substantial because the mechanical blockage killed 15% of rats in the high-dose groups. For survivors, in some cases even after 3 months, some of the CNT instillate remained as large boluses and had not transited the airways into the alveolar region (Warheit et al., 2004).

We also observed that instillants mechanically blocked the airways in our pilot ITI study (Lam et al., 2004), resulting in animal deaths when the mice were each given a bolus dose of a 1 mg/100 μ l CNT suspension. However, after we reduced the dosing volume of the instillate and changed the dosing technique from ITI to intratracheal fast instillation (modified from the method initially developed by Sabaitis et al., 1999), we did not lose another mouse because of CNT administration and did not see CNTs remaining in the airways. Shvedova et al. (2005), who used the pharyngeal aspiration technique in their large-scale (~1000 animals) study, did not report animal deaths or CNTs not entering the alveolar region. In their rat study similar to the study of Warheit et al., Muller et al. (2005) also saw blockage of respirable airways when suspensions of unprocessed (unground) MWCNTs were instilled, but found instilled ground CNTs in the alveolar region. Thus, the ability of the administered material to reach the target area depends on dosing technique, sample preparation, and choice of dispersion agent. If air contains CNTs in small enough sizes, they will reach the pulmonary region.

B. Carbon Nanotubes Themselves Caused Lung Lesions in the Treated Rodents

Lam et al. used a Rice HiPco product (using iron as a catalyst) that was treated vigorously to remove iron, and showed that this purified CNT product (residual iron content 2.3%) produced inflammation, granulomas, and fibrosis in the lungs; they concluded that CNTs themselves produce these lung lesions (Lam et al., 2004). The conclusions were supported by the results of Shvedova et al. (2005), who also used a purified HiPco product that contained only 0.23% iron and <1% amorphous carbon (99.7% of the carbon in the product had nanotube morphology); this product was reported to produce "unusual inflammatory and fibrogenic pulmonary responses." Residual iron nanoparticles in HiPco products are generally covered by carbon shells. In the purification process, iron is removed by repeated processes of oxidation of carbon to expose iron followed by strong acid treatment to remove iron (Chiang et al., 2001). Using electron para-

magnetic resonance (EPR) spectroscopy, Shvedova et al. (2005) were able to detect iron signals from unpurified HiPco products but not from the purified sample used in their study. Warheit et al. (2004) found that CNTs but not graphite (both contained 5% nickel and 5% cobalt) produced granulomas in the lung of exposed rats.

C. Toxicity of Different Manufactured Carbon Nanotube Products—Methods of Synthesis and Impurities

1. Methods of Synthesis and Impurities in Carbon Nanotubes

The toxicity studies collectively showed that all of the CNT products tested, regardless of the method of synthesis, could induce inflammation, granulomas, and fibrosis in the lungs of treated mice or rats. As pointed out in the previous section, CNTs themselves produced these lung lesions. However, the presence of impurities might affect the severity of lesions (Lam et al., 2004). Depending on the manufacturing processes and postsynthetic purification, both the non-nanotube carbon and the metal residues in a CNT product can vary greatly (Table 1 and section I.B.3). For example, raw SWCNT products currently sold by SES Research (Houston, TX) contain only 20–40% nanotubes with the remaining material consisting of metal catalyst, carbon-coated metal nanoparticles, amorphous carbon, and other carbon nanoparticles (SES Research, 1999); the purified HiPco sample used in the study by Shvedova et al. (2005) contained < 1% non-nanotube carbon and 0.23% iron. Since 1 g of a purified HiPco product contains about 3 times more CNTs than the same weight of a raw SWCNT product of SES Research, an equal amount of these two products will be expected to produce different degrees of toxic insults to the lungs of treated animals.

2. Metal Contents in Carbon Nanotubes and Cautions in Assessing Toxicity of Metals in the Products

The metal particles are the sites of SWCNT synthesis. The content of residual catalytic metals in a raw CNT product can be as high as 50% (Table 1). The metals themselves, such as nickel, could have toxic effects in the lungs, and one may wonder if some of the lesions could be attributed to the metals in CNT products. These metal nanoparticles, which are 3–5 nm in size, are "encased in thin carbon shell and distributed throughout the sample" (Figure 3) (Chiang et al., 2001). The encased metal would not be expected to interact with cells. However, Lam et al. (2004) observed that a dose of 0.5 mg/mouse of CNTs (sonicated suspension) containing 26% nickel and 5% yttrium killed about 50% of the animals within 7 days after dosing. The deaths were attributed to nickel that was released during ultrasonication. It has been suspected that ultrasonication of CNT suspensions may release the metals from carbon entrapment or encapsulation allowing them to come into contact with lung cells to elicit toxicity. In a study of cell cultures treated with a raw HiPco product (~25–30% iron), Shvedova et al. (2003) attributed the oxidative and peroxidative changes they observed to the iron in the product. In a subsequent animal study, Shvedova et al. (2005)

used a highly purified HiPco sample (0.23% iron) and showed that CNTs produced a dose-dependent increase in biomarkers associated with inflammation and oxidative stress in the lungs. The inflammatory and oxidative reactions in the lungs were thus attributed to the CNTs themselves. Therefore, caution should be exercised in assessing the effects of metals contained in CNT samples in intratracheal instillation and cell-culture studies that involve ultrasonication. Investigators who use residual metal in CNTs to measure the half-life of instilled CNTs in the lungs (Muller et al., 2005) also should be aware that the metal nanoparticles may be freed by ultrasonication from the carbon shells or CNT entrapment, leading to a difference in half-lives between the tracer metal nanoparticles and CNT fibers in the lungs.

D. Possible Mechanisms of Carbon Nanotube Pathogenesis in the Lungs and Toxicological Difference From Carbon Black

When instilled into the lungs, both carbon black (CB) and CNT particles were taken up by alveolar macrophages, but their fate and reaction with the lung tissue were very different (Lam et al., 2004). CB-laden macrophages remained scattered in the alveolar space. It is well known that if the lung is not overloaded with dust, dust-laden macrophages on the alveolar surface will migrate upward and be carried by the mucociliary escalator system up the trachea, and cleared into the esophagus (McClellan, 1997a). CNT-laden macrophages, however, moved rapidly to centrilobular locations, where they entered alveolar septa and clustered to form epithelioid granulomas. When a dust enters the interstitial or subepithelial space, clearing it from the lung is very difficult. 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 was observed for CNTs by Lam et al. (2004) and Shvedova et al. (2005).

1. Surface Chemistry, Electrical Properties, and Oxidative Potential of Carbon Nanotubes

CNTs are known to possess unique surface chemistry; we have pointed out that their unusual chemical and electrical properties could contribute to the toxicity of CNTs (Lam et al., 2004). According to Richard Smalley's group at Rice University, SWCNTs are among the best electrical conductors and can conduct electricity twice as well as copper (Rice University, 2005). SWCNTs have unique electron-transport properties; they may be either metallic (armchair configuration) or semiconducting (zigzag configuration), determined by the chiral vector of the tubes (Jensen et al., 2004). In a typical batch, one-third of the SWCNTs are metal conductors and two-thirds are semiconductors (Stahl, 2000; Watts et al., 2002). Carbon black is devoid of this chemical/electrical property. It is conceivable that CNTs may participate in oxidative and peroxidative reactions by removing electrons from or shifting electrons around in the cells. Shvedova et al. (2005), who used a highly purified HiPco prod-

uct containing only trace levels of iron, detected robust acute inflammation, which was characterized by biochemical toxicity changes including accumulation of 4-hydroxynonenal (an oxidative biomarker), elevation of proinflammatory cytokines, and depletion of glutathione. It is very likely that the unique electron transport property of CNTs is the culprit in inducing oxidative inflammation in the lungs. The nanotechnology field discovered a potentially very useful property in this tubular molecule made of a nonmetal element and able to conduct electricity. The unique property of being able to remove or provide electrons may also enable CNTs to participate in cellular oxidative processes that could result in unanticipated toxicity.

2. Fibrous Structure of Carbon Nanotubes

Carbon black is amorphous, but CNTs microscopically are thin fibers packed tightly and in parallel to form ropes or rods (Figures 1B and 1F) (Taubes, 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, 1997a). Therefore, toxicologically, individual nanotube molecules and assembled nanotube ropes, rods, and bundles are fibers. The fibrous structures of the CNT particles were clearly seen in the lungs in our study (Figures 9A and 9B) and Warheit's study (Figure 9D). It is reasonable to expect that it would be more difficult for macrophages to safely engulf relatively rigid and fibrous CNT structures, especially if some of them can conduct electrons (Smalley, 1999); rigid or semirigid electronically active fibers can potentially perforate cell membranes and could disturb cellular functions, resulting in toxicity and even cell death. We did observe necrosis in the lungs (Lam et al., 2004), and Shvedova et al. (2005) observed an indication of an increase of AT-1 cell death in the lungs of mice treated with CNTs. It is well known that the geometry of particulate matter plays an important role in pathogenesis caused by the particles. CNTs are totally 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 (McClellan and Hesterberg, 1994; Oberdorster, 2000).

E. Particle Size Issues of Manufactured Carbon Nanotubes Studied Toxicologically

It is well known that the particle size of a dust has great influence on its toxicity, not only on the location of its deposition in the respiratory tract, but also on how it interacts with the target cells (Oberdorster et al., 2005). Even for a respirable dust (dust that can reach the alveoli) on an equal-weight basis, toxicity generally is greater with smaller particle size (Oberdorster et al., 1994). One mg of a dust of $d = 0.1 \mu\text{m}$ has 1000 more particles than the same weight of a dust with $d = 1 \mu\text{m}$ [$m = v \times \rho = (1/6)d^3 \times \rho$, where m is mass, ρ is density, and volume (v) of a spherical particle = $((4/3)r^3) = (1/6)d^3$], and more particles

provide greater surface area for physical contact between cells and the dust.

Because of the van der Waals force attraction, CNTs bundle into ropes, which are further associated into loose aggregates. We prepared CNT suspensions by brief shearing (2 min) of the agglomerates in serum followed by brief (1 min) sonication. As shown in light micrographs in Figure 7, the bulk of the HiPco-CNTs in a suspended test sample existed predominantly in length of several micrometers. Lam et al. (2004) and Shvedova et al. (2005) both used ultrafine carbon black (Printex 90), which has a median particle size of 14.3 nm, as a reference dust in their studies, and showed that CNTs, but not ultrafine carbon black, produced lung lesions. Shvedova et al. also pointed out that “the interstitial fibrotic response to SWCNTs was not mimicked by an equivalent mass exposure to either ultrafine carbon black (a reference nanoparticle) or fine crystalline silica (a classic fibrogenic particle).” Therefore, the reported differences between these two carbon allotropes in lung toxicity were attributed to their difference in other physicochemical properties and not their size difference. That is to say, the observed toxicity of CNTs was not due to the fact that CNT particles were in nanosizes. Because none of the investigators of the animal studies discussed here showed that they had prepared CNT particles in nanosizes, it is noteworthy that none of the CNT toxicity studies reported so far were investigations of nanoparticle toxicity of CNTs, but rather of toxicity of respirable particles of materials of great importance to nanotechnology.

F. Toxicological Risk Assessment of Occupational Exposures to Manufactured Carbon Nanotubes

The risk assessment of human health effects from exposure to a chemical is a complicated and difficult process, and the process is continuously refined by filling data gaps with new research data (McClellan, 1999). When potential exposure is by the inhalation route, the target is the lung, and the chemical is a fibrous dust, the risk assessment takes an even more difficult path (McClellan, 1997b), as in the case of CNTs. The CNT manufacturing industry is still in its infancy and the production volume is still small. Data pertaining to CNT toxicity from human exposures will not be available for many years; results from inhalation studies will not be available for some time. The current lack of data from human exposures and animal inhalation toxicity studies makes the assessment here, which is based on toxicological data sets obtained from administering CNTs to animals intratracheally or by modified routes, preliminary. The discussion here of the existing toxicity data follows the National Research Council’s risk assessment scheme (National Research Council, 1994), including identification of data gaps for further research (McClellan, 1999).

1. Hazard Identification

The animal studies of CNT pulmonary toxicity collectively showed that CNTs are capable of inducing inflammation, epithe-

TABLE 2
Incidence of pulmonary lesions in mice 90 days after intratracheal instillation with carbon nanotubes^a

Dust dose (mg)	Type of lung lesion	Carbon black	Quartz	Raw CNTs	Purified CNTs	Carbolex CNTs
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 ^b

^aMice (5/group) were each instilled with 0.1 or 0.5 mg CNTs and euthanized 90 days after the single treatment. Lungs were microscopically examined by a pathologist who had no knowledge of the treatment of each animal. Table originally published in Lam et al. (2004).

^bIncludes three mice that died in the first week.

loid granulomas, fibrosis, and biochemical toxicity changes in the lungs that might impair pulmonary functions. Carbon black (ultrafine) or graphite, given to the animals in the same manner by these research groups, produced no lung lesions. Cell culture studies also showed that CNTs were cytotoxic. These results indicate that, if CNT particulates reach the lung in sufficient quantity, they will produce a toxic response.

2. Dose-Response and Exposure Duration-Response Assessment

In the NASA study in which lungs of exposed mice were examined 3 months after instillation, granulomas were observed in the high-dose group treated with a SWCNT product made by Carbolex, but not in the low-dose group (Lam et al., 2004); the Rice HiPco CNTs produced prominent granulomas in all animals exposed to the high dose, while in the low-dose groups, granulomas were less prominent or not detected (Table 2). A more quantitative and morphometric study by Shvedova et al. (2005) showed that the total mass of granulomas increased with the increase of treatment doses (Figures 10A and 10B). The effect of increased SWCNT dose in mice was assessed 60 days after the treatment. The extent of fibrosis and the biomarkers of toxicity in BALF were reported to be dose dependent, and the lesions in mouse lungs became worse with time. Results for MWCNTs reported by Muller et al. (2005) also showed a dose-dependent increase in hydroxyproline and collagen contents in the lungs of rats treated with the compound (Figures 10C and 10D); these two proteins are biomarkers for fibrosis. These data strongly indicate that, if CNT particles reach the lung, the effects will be dose dependent and time dependent.

3. Exposure Risk Assessment

Because CNTs tend to stick to each other, forming clumps, it is difficult to separate substantial numbers of particles of respirable size from bulk amounts of CNTs. Exposure risk will

depend on the proportion of particles of respirable size that can dissociate from the clumps. Particle deaggregation from unprocessed HiPco and laser-ablated products was investigated by aerosol scientists at NIOSH (Baron et al., 2003). They reported that relatively gentle agitation in a two-component fluidized bed did not lead to significant aerosol generation. More energetic effort by blowing air over a CNT sample (from a laser product) mixed with copper beads in a centrifuge tube, which had been agitated with a vortex shaker, led to the generation of particles smaller than 100 nm. Vigorous agitation of a HiPco sample produced more particles, most of which were in the respirable range, with some being in the ultrafine range (Figure 5). According to these NIOSH scientists, "generation rates of fine particles from the nanotube material were found to be approximately two orders of magnitude below that for fumed alumina for similar volumes of material."

In a field study, when the NIOSH investigators sampled airborne CNTs at the NASA JSC nanotechnology laboratory, the Rice University nanotechnology research laboratory, and the CNI manufacturing facility in 2002, the laboratories were making only several grams of CNTs per day (Shvedova et al., 2005). The researchers found very low concentrations (31 to 117 $\mu\text{g}/\text{m}^3$) of HiPco or laser-produced CNT dust. Coarse and nonrespirable particles contributed most of the mass. However, because of their low density, nanotube particles were observed to remain airborne for some time (duration not specified) (Baron et al., 2003). The recovery of CNTs from synthetic ovens and handling of the collected samples was gentle. Thus, losses of this expensive material ($> \$500/\text{g}$ at that time) were minimized. Warheit et al. (2004) used these NIOSH findings that very little amounts of airborne CNT dust were found in these laboratories and that majority of the particles were not in respirable range to support their argument that airborne CNT dust is unlikely to pose an exposure risk.

However, if Richard Smalley's prediction that hundreds or thousands of tons of nanotubes could be produced in 5 to 10 years and "in time, millions of tonnes of nanotubes will be produced worldwide every year" (Ball, 2001; Taubes, 2002) comes true, and if the CNT industry achieves the goal of pricing CNTs at "a couple dollars a pound" (Bearden, 2003), then some production facilities with less stringent industrial hygiene practices could have a much dustier environment than the laboratories sampled by the NIOSH aerosol group. Moreover, CNT particles in some products made by other manufacturers using different synthetic processes may have lesser tendency to aggregate like those investigated by the aerosol scientists (see Section I.B.4), who visited the facilities using only the processes (HiPco and laser ablation) that were developed by Smalley's group at Rice University. Further more, if CNTs are incorporated into reinforced structures, composite materials, and other products, airborne CNT dust found in manufacturing plants that make these products may have different particle size distribution that observed by Baron et al. A large work force will be exposed to CNTs (see section I.B.5).

4. Risk Characterization

The animal toxicity studies discussed here were conducted by ITI or modified processes, and all of the test materials were prepared as fine-particle suspensions by ultrasonication in an aqueous system with a dispersing agent or by boiling. These instillation or aspiration studies used unnatural routes of administration; thus the studies were considered preliminary assays to screen CNTs for potential pulmonary toxicity (Lam et al., 2004; Warheit et al., 2004). Even though these studies do not answer the important health risk question of whether airborne CNT particles can reach the lungs, they do reveal the intrinsic toxicity of CNTs. All rat or mouse studies showed that CNTs, but not carbon black or graphite administered similarly, produced severe lesions in the lungs. These findings convey an important message that, if a work environment contains respirable CNT particles in significant concentration, prolonged exposures would likely result in serious lung diseases. Henderson et al. (1995) reported that when the amount of particle deposition was comparable, pulmonary responses to a bolus instillation and an inhalation exposure were comparable. Therefore, it can be inferred that differences between the animal results reported here and the results from future inhalation studies are likely to be quantitative, but not qualitative. Certainly the risk assessment of CNTs needs to be substantiated with animal inhalation studies.

The results of the toxicity studies conducted by NASA and NIOSH demonstrate that, on an equal-weight basis and under the test conditions described here, CNTs are more toxic than quartz, which is considered a serious occupational health hazard in chronic inhalation exposures. CNT products are much lighter in weight than quartz, and a gram of CNT respirable dust of comparable sizes would have many more fine particles than a gram of quartz dust. The Occupational Safety and Health Administration (OSHA) current permissible exposure limit (PEL) on quartz for occupational exposures is 0.1 mg/m^3 . OSHA PELs are time-weighted concentrations (40 h/week) and are set for lifetime occupational exposures. OSHA has not established a PEL for CNTs. A major CNT manufacturer and supplier classified this new form of carbon as synthetic graphite; its material safety data sheet (Carbon Nanotechnologies Incorporated, 2002) references the OSHA PEL for synthetic graphite of 5 mg/m^3 for the respirable fraction (NIOSH/OSHA, 1988).

To estimate, from intratracheal doses that produced lung lesions, the risk associated with human inhalation exposure to a dust, it is necessary to extrapolate from ITI to inhalation and from animal to human. These processes are very complicated and difficult (U.S. EPA, 2004), and the results may be questionable. Pulmonary deposition of a test dust depends on the physiological condition and characteristics of the exposed animal/subject [respiratory tract anatomy, breathing patterns (oral-nasal or nose-only, fast or slow, shallow or deep)] and on the physical characteristics of the dust (density, morphology, geometry, and particle distribution). The fractional deposition with respect to the mass median aerodynamic diameter (MMAD) of

the particles in various regions of the respiratory system has been calculated and estimated mostly for humans. Aerodynamic diameters are dependent on the physical characteristic of the particles. CNTs are very light and are aggregates of ropes, each rope consisting of a bundle of parallel tubes. It would be more difficult to determine the MMAD of CNTs than it would be to determine the MMAD of common dusts studied by toxicologists. To perform an ITI route to inhalation route extrapolation and to calculate the amount of dust deposition in an inhalation-exposed animal, we previously assumed that 40% of the inhaled respirable CNT particles were deposited in the pulmonary region (Lam et al., 2004). The 40% was derived from data of the International Commission of Respiratory Protection Task Group (Bates et al., 1966), which show that the fractional deposition of particles deep in the human lung is about 30% for 3- μm particles and increases to 55% for 0.05- μm particles. These data were gathered many years ago; they have been replaced with recent data.

Recent data compiled by the U.S. EPA (2004) show the fractional deposition of particles (density 1 g/cc) with an MMAD of 5.7 μm in the tracheobronchiolar region and alveolar region of a human are 2.4% and 5.5% respectively; the corresponding values for particles of 2 μm in a rat are 4% and 5.5%. Raabe et al. (1988) reported that the alveolar deposition of 5- μm particles in mice was 3%–4%. It appears that our previously assumed 40% alveolar deposition was an overestimate (Lam et al., 2004), so that a reassessment of exposure risk here is warranted.

CNTs are considered by some manufacturers to be synthetic graphite; the MSDS from a major CNT supplier states the PEL value of 5 mg/m³ set by OSHA for respirable synthetic graphite dust (CNI, 2002). If we assume that a mouse is exposed to 5 mg/m³ of a respirable CNT dust and 4% of this dust will be deposited in the alveolar region, this would allow us to estimate the length of time required for the animal to acquire a lung burden equivalent to an ITI dose. CNT particles are neither soluble nor biodegradable, and the instilled particles were found predominately in the interstitium, where they could not be removed from the lung by the macrophage-mucociliary clearance mechanism and would remain in the lung for prolonged periods. Therefore, it is reasonable and for simplicity to assume no dust clearance in the lung. If we also assume that a 30-g mouse breathes in 30 ml (0.03 L or 0.00003 m³) of air per min or 0.0018 m³/h (Parent, 1992), then a mouse breathing respirable CNT dust at 5 mg/m³ for 8 h for one day would accumulate 0.0029 mg CNT/day (5 mg/m³ \times 4% \times 0.0018 m³/h \times 8 h = 0.00288 mg).

The above calculation shows that an amount of 0.0029 mg of CNTs would be deposited in the lung if a mouse were exposed for 8 hours to an airborne concentration of 5 mg/m³ of respirable CNT dust. Using this CNT exposure concentration and daily lung deposition of 0.0029 mg, it can be calculated that it will take 179 daily 8-h exposures for a mouse to accumulate 0.5 mg CNTs in its lungs (assuming no CNT elimination). In our study, when 0.5 mg (the high dose) of CNTs was instilled into the

lung of a mouse, we observed severe lung lesions (Lam et al., 2004). It is noteworthy that even the low dose of 0.1 mg of HiPco CNTs caused granulomas in 7 of the 10 treated mice (Table 2). Henderson et al. (1995) reported that pulmonary responses to an equal amount of dust deposited by an inhalation exposure or by a bolus instillation were comparable. This calculation shows that an animal breathing 5 mg/m³ for 179 days (8 h/d) would be expected to have severe lung lesions. It is reasonable to assume that if a person were to breathe respirable-size dust of CNTs at this concentration and duration, his/her lungs would have some lesions similar to those observed in the mice by Lam et al. Thus, OSHA's permissible exposure limit of 5 mg/m³ set for respirable synthetic graphite dust should not be recommended for CNTs. This warning is consistent with the findings of Warheit et al. (2004) showing that CNTs produced granulomas while graphite (used as a negative control) elicited no toxicity in the lungs. Toxicologically, CNTs are not equivalent to synthetic graphite.

Shvedova et al. (2005), using doses (0, 10, 20, or 40 μg /mouse; about 0, 0.5, 1, or 2 mg/kg) that were about an order of magnitude lower than those used by Lam et al. showed that CNT toxicity in the lungs of treated mice is also dose-dependent and progressive. Assuming a tidal volume of 500 ml and 18 breaths/min, they calculated the airway and alveolar deposition efficiencies of particles (size 5 μm) in a human lung to be 8.3% and 1.1% respectively. Using these data, Shvedova et al. further calculated that the total lung deposition in a worker working 20 days (8 h/d) and breathing 5 mg/m³ CNTs of particle size 5 μm would be about 40 mg. Of this amount, 4.7 mg would be in alveolar regions. The proximal alveolar deposition in the worker's lung after 20 days of CNT exposure would be 15.7 mg/m²; the alveolar deposition in a mouse aspirating 20 μg CNTs would be 16.6 mg/m². The dose of 20 μg /mouse was shown to produce a robust acute inflammation, rapid fibrogenic and granulomatous reactions, and impaired pulmonary functions. Thus, a worker exposed to 5 mg/m³ CNTs for 20 days could have lung lesions similar to those of the CNT-treated mice.

To compare the risk estimations arrived at by two groups using different approaches, one could use Shvedova's approach with Lam's assumption (4% alveolar deposition) and data. Lam et al. instilled 0.5 mg/mouse (or 500 μg /mouse), a dose that produced more severe lung lesions than those observed by Shvedova et al. Shvedova's calculation shows that the dust mass per unit alveolar surface area (mg/m²) of a mouse instilled with 20 μg is approximately equal to that of a worker breathing 5 mg/m³ CNTs for 20 days. Using the same approach, one can calculate that, if the instilled dose is 500 μg , a worker would take 500 days to achieve the same density of dust on his/her alveolar surface. If Shvedova et al. had assumed a fractional alveolar deposition of 4% (as we assumed above in our recalculation with more recent data) instead of 1.1%, they would have found that the worker would take 136 days instead of 500 days to achieve the same density of dust on the alveolar surface. Thus, using an instilled dose of 0.5 mg and a deposition of 4%, Shvedova would calculate a value of 136 days while Lam would calculate 170 days

(see above). These numbers are considered close given that they were obtained by different approaches. The difference may have resulted from the assumption of 4% alveolar deposition for both mice and humans; the 179 days were calculated for 4% deposition in mice and the 136 days were calculated for 4% deposition in humans.

Both Lam et al. (2004) and Shvedova et al. (2005) showed that an equal weight of CNTs was more toxic than quartz in the lungs of treated mice. In a cell culture study with alveolar macrophages, Jia et al. (2005) showed that both SWCNTs and MWCNTs were more cytotoxic than quartz; CNTs, especially SWCNTs, impaired phagocytosis and caused cell death. The OSHA PEL for quartz is 0.1 mg/m^3 . Using the NIOSH group's approach and data, it can be estimated that a worker breathing 0.1 mg/m^3 would take 1000 days (~3 years) to reach the alveolar burden of 4.7 mg, also assuming no pulmonary elimination occurred; this exposure regimen is expected to produce some lung lesions. Results of inhalation studies will be needed to substantiate the pathological findings in animals and to set an appropriate exposure limit. However, until such data are available, we recommend that the occupational exposure limit for respirable CNT dust be set at a value no greater than 0.1 mg/m^3 .

G. Assessment of the Role of Environmental Multi-Wall Carbon Nanotubes in Air Pollution-Related Cardiopulmonary Diseases in the General Public

As discussed in the Introduction (sections I.C and I.D), MWCNTs and fullerenes not only are produced by high-technology laboratories but also are found in particulate matter from ordinary combustion of fuel gases (Murr et al., 2004a). "They're also made in every candle flame and in forest fires," according to Richard Smalley (Amato, 2001; Wikipedia, 2005). Therefore, MWCNTs are probably ubiquitous in our environment. MWCNTs generated outside laboratories or factories would be expected to be shorter (Figure 6) than those produced in the synthetic ovens (Figure 1B); because the van der Waals force is less effective, they would also aggregate with themselves and with other nanoparticles more loosely than manufactured CNTs do (section I.D). In the lung, shorter nanofibers and loose aggregates would favor dissociation of MWCNTs from the inhaled and deposited aggregate matrix, potentially allowing some of them to leave the lung and be deposited in extrapulmonary organs. In a group of mice that received SWCNTs by pharyngeal aspiration, Li et al. (2005), of Shvedova's group at NIOSH, observed dose-dependent damage to the mitochondrial DNA in the aorta at 7, 28, and 60 days after the CNT treatment. They concluded that these oxidative changes were the result of altered expression of inflammatory genes, including MCP-1 and VCAM-1, in the heart. Li et al. (2006) further reported that the percent of plaque areas in the aortas was significantly increased in the SWCNT-instilled hypercholesterolemic mice compared with those instilled with the vehicle. The CNT-treated mice showed a significant increase of atherosclerotic lesions in the brachiocephalic arteries. The

authors speculated that these effects in the heart might be caused by CNTs that leave the lung or by cytokines released from inflammation areas in the lungs. If the toxicity of manufactured and combustion-generated MWCNTs is similar to that of SWCNTs, one would expect that the looser aggregates of shorter MWCNTs could have effects on the heart as well as the lung.

Fine particulate matter (PM 2.5) has been found to have a strong association with cardiopulmonary diseases (Dockery et al., 1993; Gauderman et al., 2004; Pope et al., 2002, 2004) (section I.C.4). However, the underlying mechanisms of pathogenesis of cardiopulmonary diseases associated with exposures to fine particulate matter are not known (Pope, 2000). A few mechanisms have been proposed including production of reactive and oxidative species resulting in lung inflammation and damage (Dreher, 2000; Pope et al., 1999), and release of potentially harmful cytokines from fine particles induced alveolar inflammation (Seaton et al., 1995).

Besides inducing histopathological changes, including granuloma formation and fibrosis, both SWCNTs and MWCNTs were found to release toxic cytokines and biomarkers of inflammation, oxidative stress, and cytotoxicity (Muller et al., 2005; Shvedova et al., 2005). As discussed above, Shvedova's group further found that SWCNTs produced functional respiratory deficiency, decreased bacterial clearance, and biochemical toxicity in the heart in treated mice. It is true that inorganic, metallic and organic constituents in the PM could contribute to the pathogenesis of lung diseases (Dreher, 2000). The fact that inorganic carbon, which includes MWCNTs, accounts for a significant fraction in the combustion-generated PM, and the findings that CNTs were rather toxic and capable of producing so many lung lesions and chemical and cellular changes commonly associated with air pollutants, make MWCNT particulates a prime suspect in the pathogenesis of cardiopulmonary diseases induced by fine PM.

IV. CONCLUSIONS AND RECOMMENDATIONS

A. Manufactured Carbon Nanotubes

The animal studies of CNT pulmonary toxicity show that CNTs are capable of inducing inflammation, epithelioid granulomas, fibrosis, and biochemical toxicity changes in the lungs that might impair pulmonary functions. However, the studies reviewed here were conducted using ITI or modified techniques to administer to rodents CNT suspensions that had been mechanically ultrasonicated. Even though ITI and modifications of this technique are common routes of administration used to assess the toxicity of dust in the lungs, it is imperative that inhalation toxicity studies be conducted to demonstrate whether CNT particles can reach the lung to produce the lung lesions seen in the ITI studies. Results from inhalation exposure studies would also allow assessment of the effects of CNTs on the upper respiratory tract and establishment of an exposure limit. With respect to occupational exposure limits and exposure hazard, CNTs should not be treated as synthetic graphite. Unless and until unequivocal data from inhalation studies can show

that CNTs are devoid of toxicity, it is prudent to presume that prolonged occupational exposure to airborne fine CNT particulate matter could produce serious lesions in the lungs similar to those seen in animals. Therefore, if airborne fine CNT particles are present in the workplace, strategies to minimize human exposures must be implemented, as recommended by Lam et al. (2004).

B. Multiwall Carbon Nanotubes Generated by Fuel Combustion

Fine environmental particulate matter (PM 2.5) has been found to have a strong association with cardiopulmonary diseases (Dockery et al., 1993; Gauderman et al., 2004; Pope et al., 2002, 2004), and MWCNTs were found in significant amounts in fine particulate matter in environmental samples collected in El Paso and Houston (Murr et al., 2004b). The results of pulmonary toxicity studies discussed here showed that manufactured SWCNTs and MWCNTs caused histopathological changes in rodent lungs, including granuloma formation and fibrosis; both were found to release toxic cytokines and biomarkers of inflammation, oxidative stress, and cytotoxicity (Muller et al., 2005; Shvedova et al., 2005). In addition to producing pulmonary lesions, SWCNTs were shown to produce cardiac toxicity (Li et al., 2005, 2006). These findings suggest that MWCNT particulates may be involved in the pathogenesis of fine PM-induced cardiopulmonary diseases. For this to be substantiated, however, a direct link between pollution-associated cardiopulmonary diseases and environmental MWCNTs would need to be established. The fine PM_{2.5} samples collected by Dockery and Pope's group (Dockery et al., 1993; Gauderman et al., 2004; Pope et al., 2002; Pope et al., 2004) or by others in environmental studies should be analyzed for their content of MWCNTs. Gauderman et al. (2004) showed that impairment of lung functions resulted from air pollutants that had the highest correlation between pathological effects and amounts of elemental carbon. Did this environmental elemental carbon contain substantial amounts of MWCNTs, a newly identified carbon allotrope? The discovery of CNTs not only has triggered an enormous amount of research on applications of these manufactured novel materials; it soon will trigger a vast amount of research on the effects of these long-existing, combustion-generated environmental pollutants on human health.

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