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TOXICITY OF CARBON NANOTUBES IN THE LUNGS OF MICE 7 AND 90 DAYS AFTER INTRATRACHEAL INSTILLATION

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ABSTRACT

Single-walled carbon nanotubes have many potential applications in the electronic, computer, and aerospace industries. Because unprocessed nanotubes could become airborne and potentially reach the lungs, their pulmonary toxicity was investigated. The three products studied were made by different methods, and contained different types and amounts of residual catalytic metals. Mice were each intratracheally instilled once with 0, 0.1 or 0.5 mg of nanotubes, a carbon black negative control, or a quartz positive control, and killed for histopathological study 7 d or 90 d after the treatment. All nanotube products induced epithelioid granulomas and, in some cases, interstitial inflammation in the animals of the 7-d groups. These lesions persisted and were worse in the 90-d groups. We found that, if nanotubes reach the lung, they can be more toxic than quartz, which is considered a serious occupational health hazard in chronic inhalation exposures.

Carbon nanotubes structurally resemble rolled-up graphite sheets with one end capped. These tiny tubes can have single or multiple walls. Because single-walled nanotubes (NTs) possess highly desirable electrical, mechanical and thermal properties (Arepalli et al., 2001, Ball, 2001), enormous research efforts have been channeled to discover applications of this novel material; NASA has joined the search for its uses in aerospace.

The anticipated high demand has driven the production of NTs from laboratories to factories. Rice University (Houston, TX) has licensed its HiPco™ technology to Carbon Nanotechnology Incorporated (CNI, Houston, TX) for mass production, and MITSUI Corporation of Japan just built a manufacturing facility that is projected to have an annual output of 120 tons NTs (Mitsui, 2002). Richard Smalley of Rice has predicted that hundreds or thousands of tons of NTs could be produced in 5 to 10 years and "in time, millions of tonnes of nanotubes will be produced worldwide every year " (Ball, 2001, ICI, 2002). As the production and applications of NTs expand, potential human exposures will also increase.

NTs are commonly produced by deposition of graphite vaporized by an electric arc or by laser, or by chemical-vapor deposition using high-pressure CO conversion (HiPco™) (Bronikowski et al. 2001). All these products contain residual catalytic metals. An individual NT molecule is about 1 nm in diameter and several microns long (Ajayan and Ebbesen, 1997). NTs are both strong and stiff, yet flexible; in fact, they are the strongest of all synthetic fibers (Ball, 1999). Microscopically, individual NT fibers aggregate into bundles or ropes, which in turn agglomerate loosely into small clumps. A study by the National Institute of Occupational Safety and Health (Baron et al. 2002) on a raw HiPco™ NT sample (RNT, same batch of product used in our study [Fig. 1B] and similar to the product shown in Fig. 1A) showed that upon gentle agitation, large airborne clumps were visible to the naked eye, but very low numbers of smaller particles were present. However, at high agitation levels, more airborne NT particles were generated. They were mostly below 10 μm in sizes (respirable size) with some being in the ultrafine range (below 0.1 μm) (Baron et al. 2002).

Because no toxicity information about NTs is available and because the carbon atoms in NTs and in graphite configure in the same molecular hexagonal/honey-comb pattern, CNI, a major NT manufacturer, classifies this new form of carbon as synthetic graphite (CNI, 2002). Its material safety data sheet (MSDS) references the permissible exposure limit (PEL) set by the Occupational Safety and Health Administration (OSHA) for synthetic graphite at 15 mg/m³ of total dust and 5 mg/m³ for the respirable fraction. The absence of toxicity data for such an important commodity has concerned many (Gorman, 2002).

Concern about the potential for its workers to be exposed to materials of unknown toxicity prompted NASA to sponsor the present pulmonary toxicity study. The study was conducted on three NTs products (generously provided by Rice University or CarboLex Inc. [Lexington, KY]) made by different methods, and containing different types or amounts of residual metals. Metal analysis in our laboratory showed that the Rice HiPco-

NTs contained 27% (w/w) iron in the raw form (RNT), and 2% iron after purification (PNT); the CarboLex electric-arc product (CNT), sold by Aldrich, contained 26% nickel and 5% yttrium.

From bulk preparations, it would be difficult to isolate and collect enough NT particles of respirable sizes for a prolonged inhalation study (Baron et al., 2002). Intratracheal instillation, an accepted route of exposure commonly used to screen dusts for potential pulmonary toxicity (Leong et al., 1999; Driscoll et al., 2000), was used for the present study. Intratracheal instillation studies also allow comparative toxicity investigation of several dusts simultaneously (Lam et al., 2002a; 2002b). We investigated the pulmonary effects of these three NT products (RNT, PNT, and CNT) in mice, using carbon black (CB) and quartz as reference dusts. CB (Printex 90®, a gift from Degussa Corp. [Germany]) is a very low toxicity dust, whereas quartz (Mil-U-Sil-5®, a gift from US Silica [Berkeley Spring, WV]) is a fibrogenic dust. The study followed a histopathological protocol similar to that of the National Toxicology Program for the subchronic study of dusts (NTP, 1995).

The bulk physical state of a NT product depends on the method of synthesis and post-synthetic treatment. The RNT sample consisted mainly of small loose clumps, with a small amount of tiny particulates. The RNT particles (Fig. 1B) tended to stick to each other or container walls. The purified NT (PNT; Fig. 1C) sample consisted of small solid lumps and some fine particles. Both HiPco-NT samples can easily be broken down to smaller particles. The CNT sample (Fig. 1D) is a fine powder with an appearance like carbon toner. The large particles of these unprocessed materials would need to be prepared in respirable-size range for a valid study in examining the potential effects of the dusts in the lungs. Because NTs are neither water soluble nor wetable, a dust intratracheal instillation study would require preparation of a fine particle suspension using a nontoxic dispersion vehicle (Leong et al., 1999; Driscoll et al., 2000). In a study, the Rice group used "aggressive sonication of purified NT samples in surfactants such as Triton-X or highly polar solvents, like dimethyl formamide" to make fine-particle suspensions (10 mg/L) containing mostly individual fibers and a few small bundles (Walters et al. 2001). In our study, NT suspensions (2 mg/ml or 10 mg/ml), which were 200 or 1000 times more concentrated than those prepared by the Rice group, were best obtained by briefly shearing (2 min in a glass homogenizing tube) and subsequently sonicating (0.5 min) NT samples in heat-inactivated mouse serum (Leong et al., 1999). The high concentration and brief sonication minimized deaggregation of a NT sample to individual fibers. A Rice NT scientist advised us that brief sonication would not shorten or change the fundamental nature of NTs (Hauge, 2001, personal communication). All samples (including serum controls) were processed similarly.

Male mice (C6B3F$_1$, ~ 30g, 4 or 5 per group) each were instilled with a 50-µl aliquot containing a relatively small amount of a test material (0.1 mg [low dose, LD] or 0.5 mg [high dose, HD]), following the procedures described in Lam et al. (2002a); the mice were killed 7 or 90 d after the single treatment for the lung histopathological study. A larger instillation volume would give a better lung distribution but could suffocate a mouse. Fig. 2 shows the distribution of black particles in lungs 90 d after they were

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instilled with 0.5 mg of CB or NTs. Some of the lungs had a relatively uniform particle distribution while others did not.

Using data from the International Commission of Respiratory Protection Task Group on pulmonary deposition of respirable dusts (ICRP, 1966), one can roughly estimate the equivalent inhalation exposure dose from an intratracheal dose. The fractional deposition of particles deep into the lung is about 30% for $3\text{-}\mu m$ particles and increases to 55% for $0.05\text{-}\mu m$ particles. If we assume that 40% of the inhaled respirable NT particles deposit in the pulmonary region, and that a 30-g mouse breathes in 30 ml of air per min (Parent 1991), then a mouse breathing respirable NT dust at 5 mg/m^3 for 8 h daily would accumulate 0.3 mg/day. If the 5 mg/m^3 PEL, which OSHA set for graphite respirable dust, were used as the exposure concentration of NTs for the mice, the amount we used as a low dose (0.1 mg) would be attained in the lung of a mouse in about 3 days and the high dose (0.5 mg) in about 17 days, assuming no elimination.

At an intratracheal dosage of 0.1 mg, all 9 mice treated with CNT (containing Ni and Y), showed no abnormal clinical signs. However, 5 of the 9 mice of the 0.5-mg groups died (2/4 in the 7-d group and 3/5 in the 90-d group). The deaths were generally preceded by lethargy and by losses in body weight. All deaths occurred 4 to 6 days after instillation of the CNT. The lungs of dead animals showed congestion and postmortem histopathological changes. The lungs of the 4 surviving HD mice had large aggregates of particles in macrophages in the alveolar space; some of these aggregates were also found in the interstitium, forming granulomas (Figs. 3D and 4C); some interstitial inflammation was apparent. Granulomas were not detected in the LD groups.

The iron-containing NTs (RNT and PNT) did not cause any animal deaths. Mild signs of inactivity, hypothermia, piloerection, and occasionally uncontrolled shivering were most noticeable 8-12 h after treatment with the HD RNT; these symptoms disappeared soon afterward. These clinical signs were not observed in the mice treated with PNT. Microscopically, the lungs of mice in the HD-7 d study that were treated with either RNT PNT showed prominent granulomas (Figs. 3E and 3F). These microscopic nodules were located primarily beneath the bronchial epithelium and were present throughout most of the lung fields. Some appeared to extend into the bronchi as polyps. The granulomas consisted of macrophages laden with black particles, but had very few lymphocytes, neutrophils, eosinophils, or other inflammatory cells. The macrophages showed abundant granular cytoplasm with indistinct borders. The black particles were almost entirely contained within these granulomas. Some of the lungs from HD-90 d NT-treated groups appeared grossly abnormal (Figs 2C and 2F). The microscopic pictures of the lungs in these groups showed the persistence of granulomas that contained particle-laden macrophages and NT particles (Figs. 4C-E). The lung lesions were generally worse than those of the HD-7 d groups (Figs. 3D-F); some also had necrosis, interstitial inflammation that had extended into the alveolar septa in the surrounding tissues, and peribronchial lymphocytes (Figs. 4E and 4F). In the LD HiPco-NT groups, granulomas and other pulmonary lesions were also seen in some of the treated mice (Table 1), but to a mild degree.

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None of the mice of the negative (CB) or positive (quartz) dust control groups had any clinical signs that could be attributed to treatment. Besides the presence of black particles that appeared essentially in alveoli, the lungs of CB-treated mice of the 7-d groups were unremarkable (Fig. 3B). The HD-90 d group had some aggregates of peribronchiolar lymphocytes, probably caused by prolonged antigenic stimulation, but no signs of inflammation (Fig. 4A). The lungs of the quartz LD groups were also unremarkable. Quartz at HD induced an increase in the number of alveolar macrophages in the lungs. It also produced a mild to moderate alveolar and interstitial inflammation. One of the mice in the 7-d group had a low-grade granulomatous reaction (Fig. 3C). The results for the 90-d quartz group were similar to those for the 7-d group,

DISCUSSION

The present study shows that all the three NT products, regardless of the type and amount of metal impurities, induced dose-dependent lung lesions characterized chiefly by interstitial granulomas (Table 1). The fact that PNT, which contained only a small amount of iron (2 % by weight), produced prominent granulomas and that insoluble iron and iron compounds have not been shown to produce these lung lesions (Warheit et al, 1992), strongly indicates that NTs themselves induced a granulomatous reaction in the lung.

Certainly the residual catalytic metals in NT products could also contribute to the overall toxicity of a NT product. The present study showed that the nickel-containing NTs (CNT), but not the iron-containing NTs (RNT & PNT), induced acute lethality in the exposed mice. While insoluble iron and iron compounds are very low in toxicity (Warheit et al., 1992), nickel and its compounds are highly toxic. Benson et al. (1987) reported that the lung burden of nickel in mice exposed to Ni_3S_2 at 2.5 mg/m^3 for 12 days (6 h/d) was 4 µg/lung; similar information was not available for the group exposed to 10 mg/m^3 because all 10 mice died before the end of the 12-day study. Treatment with a CNT dose of 0.5 mg/mouse would load 130 µg of nickel and 25 µg of yttrium into the lung. We could not rule out the possibility that some of the nickel and yttrium, surrounded by graphite or NTs, were freed by ultrasonication and subsequently contributed to the acute toxicity. If sonication-freed nickel contributed to the acute toxicity, then deaths should not occur in inhalation-exposed animals. It appeared that CNTs induced milder granulomatous reaction in the lung than PNTs or RNTs, but the HD CNTs predisposed the animals to pneumonia (90-d but not the 7-d group).

In the lungs, while both types of carbon particles (CB and NTs) were taken up by alveolar macrophages, their fate and reactions in the tissue were very different. CB-laden macrophages scattered in the alveolar space, whereas NT-laden macrophages moved rapidly to centrilobular locations, where they entered alveolar septae and clustered to form epithelioid granulomas. If the lung is not dust-overloaded, dust-laden macrophages in the alveolar space are generally removed from the lung by the escalator/mucociliary system up to the trachea and eventually cleared into the esophagus. However, when dusts enter the interstitial/subepithelial space, they are very difficult to clear from the lung. Thus, if a biopersistent dust is irritating or toxic, the lesions resulting from the persistent interaction between the cells and the dust trapped in the interstitium would generally worsen with time, as is the case with NT. These findings indicate that NT fibers and amorphous CB have very different intrinsic toxicity in the lungs.

Formation of interstitial granulomas, induction of tissue inflammation, and necrosis are not unique to NTs. Graphite pneumoconiosis, a lung disease that has long been recognized in workers involved in mining and processing graphite, is characterized by granulomas, interstitial fibrosis, perifocal emphysema, necrosis, and severe vascular sclerosis (Jaaffe, 1951, Gaensler et al. 1966, NRC, 1999). At least 600 cases of graphite-pneumoconiosis have been documented (Hanoa, 1983). The observations that natural graphite and coal contain some crystalline silica (quartz), and the fact that the pathologies

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of coal and graphite pneumoconiosis are similar, led most investigators to believe that the diseases associated with graphite were produced by quartz impurities (ACGIH, 2001; NRC, 1999). Following the same line of thought, OSHA established a PEL of 3 mg/m^3 for natural graphite, allowing respirable dust to contain not more than 1% quartz, and a PEL of 15 mg/m^3 (5 mg/m^3 for the respirable fraction) for synthetic graphite, which generally contains less quartz.

However, at a carbon electrode manufacturing plant, Okutani et al. (1964) found 112 cases of pneumoconiosis among 256 workers who had been exposed to an average 60 mg/m^3 of graphite that contained little (<0.1%) quartz. It can be calculated that on average the workers were exposed to <0.1 mg/m^3 of quartz. OSHA set a PEL for quartz of 0.1 mg/m^3 for occupational exposure, judging that quartz at this level will not cause lung disease. Therefore, the quartz impurities in graphite used in these carbon electrode plants must not be the causative agent responsible for pneumoconiosis. Furthermore, graphite-containing granulomas in lung lymphoid tissue were seen in rats exposed to pure synthetic graphite at 100 mg/m^3 for 13 weeks (Aranyi et al. 1992). These studies strongly suggest that pure graphite at high concentrations could also induce granulomas.

One may question that whether graphite as an impurity in NTs was the culprit that induced granulomas in NT-treated mice. Even though CNTs contain a substantial amount graphite, HiPco-NTs (RNT and PNT), which were synthesized from carbon monoxide, have very little or no graphite (Bronikowski et al. 2001). The fact that the RNT or PNT sample contained more carbon nanotubes than the CNT product on an equal weight basis, and our findings that the RNT or PNT produced granulomas in some animals of the LD-groups while no animals in the CNT LD group had granulomas would argue against graphite inducing granulomas in our study (Table 1).

Granulomas, along with other lung lesions, have long been observed in animals exposed to quartz at relatively high concentrations or doses. A granulomatous reaction in the lungs was observed in mice exposed to 1.5-2.1 mg/m^3 for 150 or 300 d (Wilson et al., 1986), in rats exposed to 25 mg/m^3 for 100 d (Eden and von Seebach, 1976), and in rats each intratracheally instilled once with 50 mg quartz and examined 1 to 12 months after treatment (Reiser et al, 1982). In our study in which mice were exposed to less quartz than the animals in the above studies, one mouse had low-grade granulomas (Fig. 3C); the rest revealed only mild to moderate inflammation in the lungs. The present study showed, for the test conditions described here and on an equal weight basis, that if NTs reach the lungs, they are much more toxic than CB and could be more toxic than quartz, which is considered a serious occupational health hazard in chronic inhalation exposures. It can be concluded that in lung toxicity, NTs > quartz > graphite.

It is relevant to note that in 1966, the American Conference of Industrial Hygienists (ACGIH) treated graphite as nuisance dust and set a Threshold Limit Value (TLV®) of 15 mg/m^3 (total dust), which was adopted by OSHA as the PEL. Unable to rule out that graphite itself did contribute to the pathogenesis of pneumoconiosis, ACGIH in 1989 reduced the TLV graphite (both nature and synthetic) to 2 mg/m^3. It also explicitly excluded graphite fibers from this setting (ACGIH, 2001). The ACGIH experts were certainly aware that fibers are toxicologically different from other classes of dusts.

The toxicological implication of the fibrous structure of NT products must be considered. Microscopically, the thin NT fibers pack tightly and in parallel (like bundles of long drinking straws) to form ropes or rods (ISI, 2002; Unrau, 1996a). According to McClellan (1997), an authoritative particulate toxicologist, "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." Therefore, toxicologically, individual NT molecules, assembled NT ropes, rods, or bundles are fibers. Some of the NT particles were clearly seen in the lungs as fiber/rope structures (Figs. 5A –C). This physical structure would make NTs toxicologically different from synthetic graphite. NTs are totally insoluble and probably one of the most biologically nondegradable man-made materials. It is well established that the pathogenesis of a fiber in the lungs directly correlates with its biopersistency (Oberdorster, 2002). Granulomas, consisting of particles, live and dead cells, and debris, would impair cellular and physiological (gas exchange) lung functions, could give rise to fibrosis, more defined nodules, and other lesions. Determining how the NT-induced granulomas will progress would require a longer-duration study with this biopersistent material.

It is noteworthy that even though CB (an amorphous carbon) and amorphous silica (or sand) are nuisance dusts, neither have toxic activity in the lungs; however, their respective crystalline forms, quartz (a crystalline silica) and graphite (a soft crystalline carbon, ACGIH, 2001; NRC, 1997) can produce a spectrum of lung lesions including granulomas. Furthermore, when carbon or silica is processed or fabricated into NTs or fiberglass, respectively, both of these fibers can induce severe lung lesions. Strikingly similar to the results of our NT study, respirable fiberglass dust intratracheally instilled to mice (1 mg/mouse) produced granulomas and fibrosis when examined 2 or 4 weeks after the treatment (Adamson et al., 1995). The toxicity of both silica and carbon follows the pattern: fibers>crystalline>amorphous.

The increased use of NTs will lead to an increase in potential human exposures. Toxicity information on these novel products is needed for assessing toxicological risk of exposures and could be useful in establishing permissible exposure limits. The data also will be useful for designing industrial hygienic procedures (Baron et al., 2003). Since NTs are water insoluble and are trapped in the lung, the lung burden of NTs received from a bolus intratracheal instillation could be attained by repeated exposures (concentration x time) to airborne respirable NT dust; comparing total lung burdens of a dust allows us to estimate an equivalent airborne exposure concentration and duration (Lam et al., 2002a). Using this information, exposures potentially harmful to humans can be estimated. As calculated above, a mouse inhaling 5 mg/m^3 of respirable NT particles 8 h daily for17 days would acquire a lung burden of 0.5 mg NTs, which was shown to be toxic to mice. OSHA has set a PEL of 5 mg/m^3 for synthetic graphite dust of respirable size (the PEL, which is a 40-h/wk time-weighted average concentration, was set for lifetime occupational exposures); workers chronically exposed to respirable NT dust even at a fraction of this concentration would be very likely to have serious lung lesions. Therefore the PEL for synthetic graphite must not be used for NTs. The results of the present study should be useful to NT manufacturers, who are required by law to provide

toxicity information to their workers and users of their products. The managers of CarboLex, another NT supplier in the U.S., have expressed the desire to provide our published results with their MSDS (William, 2002, personal communication). In conclusion, fine NT particles are rather toxic to the lungs. If they are present in a work environment, exposure protection strategies to minimize human exposures must be implemented.

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Table 1. Incidence of pulmonary lesions in mice 90 days after intratracheal instillation with nanotubes*

Dust dose (mg)*	Type of lung lesion	Carbon black	Quartz	RNT (27% Fe)	PNT (2% Fe)	CNT (26% Ni & 5% Y)
0.1	Inflammation	0**	1	3	2	0
0.1	Granulomas	0	0	5	2	0
0.5	Inflammation	0	4	3	5	0
0.5	Granulomas	0	0	5	5	5***

*Mice (5/group) were each instilled with 0.1 or 0.5 mg and killed 90 d after the single treatment. Lungs were microscopically examined by a pathologist who had no knowledge of the treatment of a particular animal.   **Number of animals showed the lesion. ***Including the 3 mice that died in the first week.
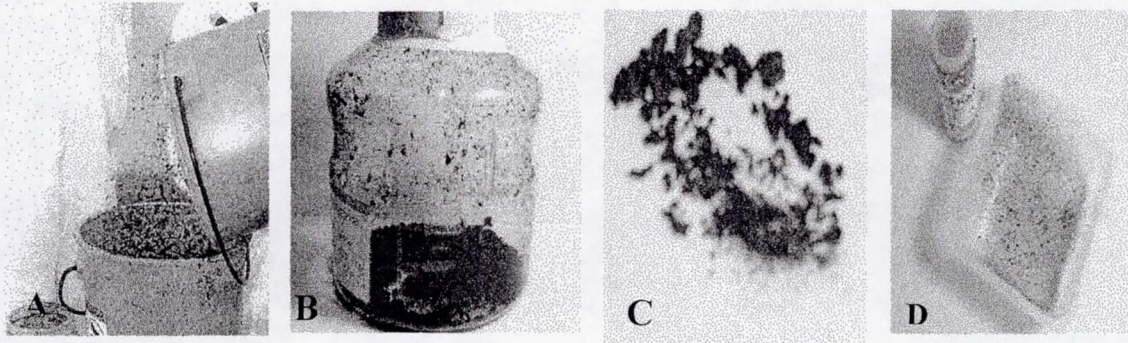
Fig. 1. **(A)** Some HiPco NT becomes airborne when sample (unrefined) is poured between containers (Courtesy of Baron et al., 2003). **(B)** raw HiPco NT, **(C)** purified HiPco NT, and **(D)** CarboLex NT, were the samples used in the present study. Note that these products all contain fine particles.
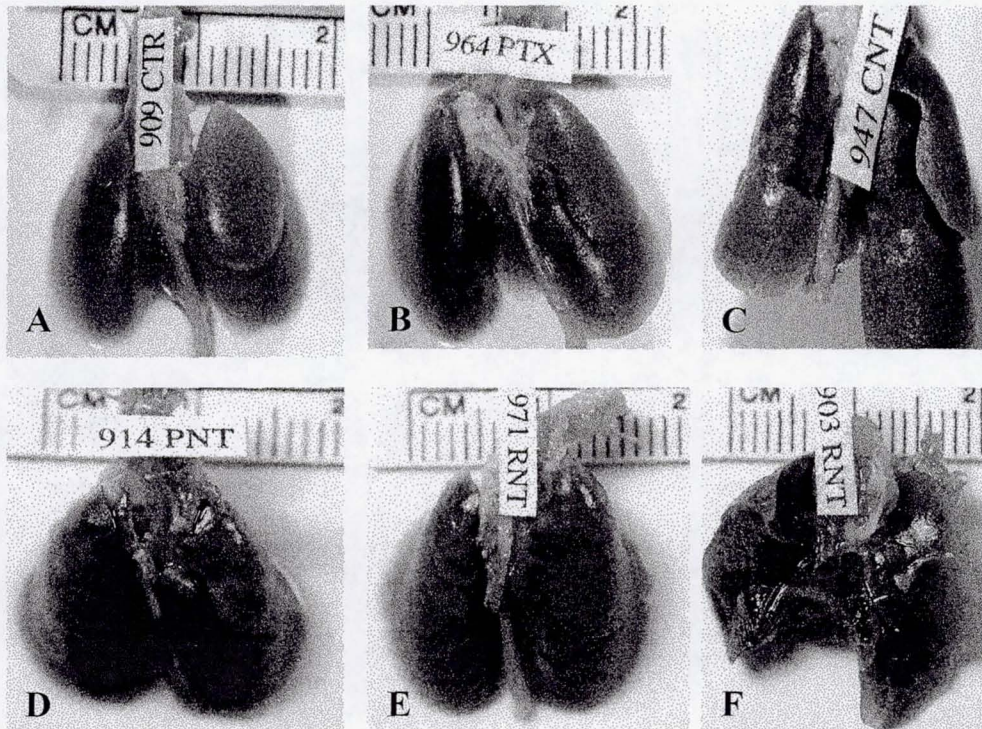
Fig. 2 Lungs from mice instilled with 0.5 mg of a test material per mouse and killed 90 d after the single treatment: (**A**) serum control, (**B**) carbon black (Printex): (**C**) CNT: the portions of the lung receiving NT had an abnormal appearance – shrunken and lumpy, (**D**) PNT: clusters of black pigment probably corresponding to granulomas, (**E**) RNT: clusters of black pigment probably corresponding to granulomas, and (**F**) RNT: dorsal view shows necrotic changes.
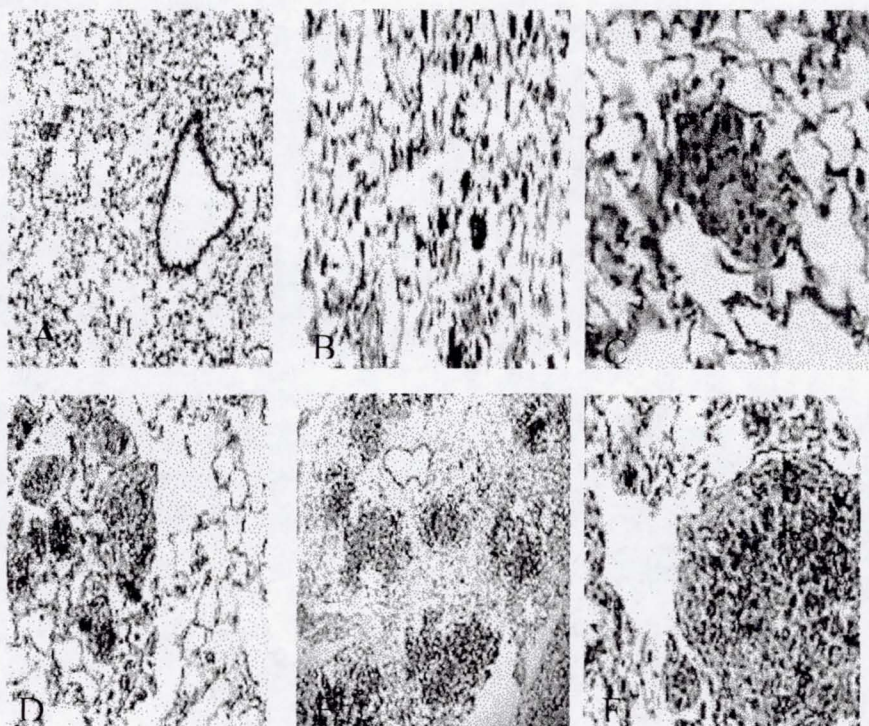
Fig. 3. Lung tissue from mice instilled with 0.5 mg of a test material per mouse and killed 7 d after the single treatment: (**A**) serum control, (**B**) carbon black; particles evenly scattered, (**C**) silica quartz: a low grade-granulomas, (**D**) CNT: granulomas (**E**) RNT: black particles predominately inside granulomas, and (**F**) PNT: close-up of granulomas. Magnification varied (40 – 400x) for illustration purpose.
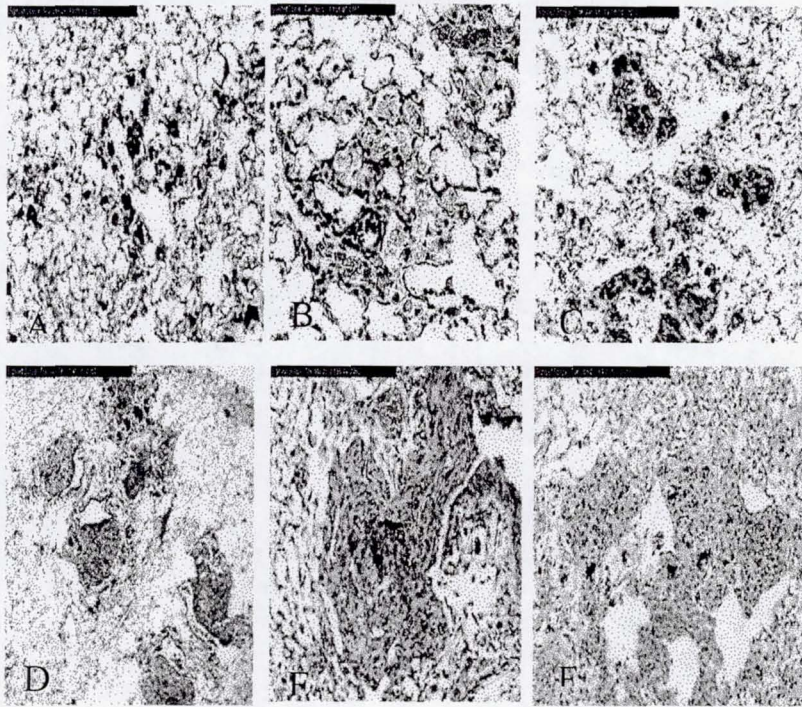
Fig. 4. Lung tissue from mice instilled with 0.5 mg of a test material per mouse and killed 90 d after the single treatment: (**A**) carbon black: particles scattered in alveoli, (**B**) quartz: inflammation cells (lymphocytes in blue) around the quartz particles, (**C**) CNT; granulomas containing black particles (**D**) RNT: granulomas at low magn., (**E**) RNT; shows granulomas at high magn., (**F**) PNT: a large granuloma underwent degeneration with necrosis. Magnification varied (40 – 200x) for illustration purpose.
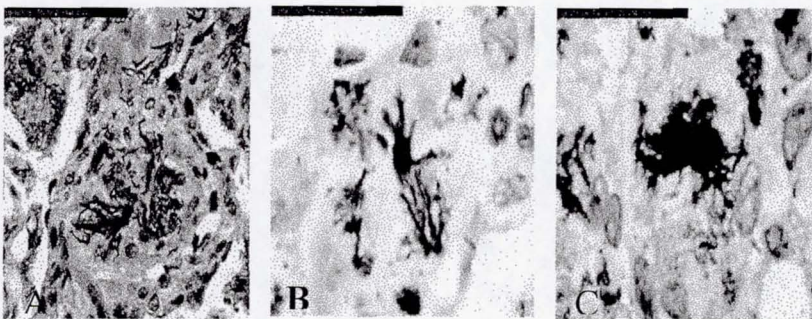

Fig. 5. Lung tissue from mice instilled with 0.5 mg of a test material per mouse and killed 90 d after the single treatment: (**A**) RNT: NT fibers in a granuloma, (**B**) PNT: NT fibers in a granuloma, and (C) PNT: NT clumps of in a granuloma. (Magnification 900x).