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Respiratory toxicity of multi-wall carbon nanotubes

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Abstract

Carbon nanotubes focus the attention of many scientists because of their huge potential of industrial applications, but there is a paucity of information on the toxicological properties of this material. The aim of this experimental study was to characterize the biological reactivity of purified multi-wall carbon nanotubes in the rat lung and in vitro.

Multi-wall carbon nanotubes (CNT) or ground CNT were administered intratracheally (0.5, 2 or 5 mg) to Sprague–Dawley rats and we estimated lung persistence, inflammation and fibrosis biochemically and histologically. CNT and ground CNT were still present in the lung after 60 days (80% and 40% of the lowest dose) and both induced inflammatory and fibrotic reactions. At 2 months, pulmonary lesions induced by CNT were characterized by the formation of collagen-rich granulomas protruding in the bronchial lumen, in association with alveolitis in the surrounding tissues. These lesions were caused by the accumulation of large CNT agglomerates in the airways. Ground CNT were better dispersed in the lung parenchyma and also induced inflammatory and fibrotic responses. Both CNT and ground CNT stimulated the production of TNF-α in the lung of treated animals. In vitro, ground CNT induced the overproduction of TNF-α by macrophages. These results suggest that carbon nanotubes are potentially toxic to humans and that strict industrial hygiene measures should to be taken to limit exposure during their manipulation.
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Keywords: Carbon nanotubes; Lung toxicity; Inflammation; Fibrosis

Introduction

Carbon nanotubes are a new form of crystalline carbon currently attracting intense research efforts because of their unique properties that make them suitable, as such or after modification, for many industrial developments such as in high strength materials, electronics or biomedical applications (Martin and Kohli, 2003). The global market for carbon nanotubes was estimated at $12 million for 2002 and is expected to grow up to $700 million by 2005 (Carbon Nanotubes—Worldwide Status and Outlook: Applications, Applied Industries, Production, R&D and Commercial Implications, 2002). There is, however, a disquieting lack of information about the possible human health and environmental impacts of manufactured carbon nanotubes and other nanomaterials. The disaster associated with the use of asbestos fibers in the past highlights the importance of identifying rapidly the potential hazards of new materials (Mossman et al., 1990). The extreme aspect ratio of individual carbon nanotubes suggests toxic properties similar to those observed with other fibrous particles (Maynard et al., 2004). Additional concerns come from studies revealing that particles with nanoscopic dimension are markedly more toxic than larger sized particles (Oberdörster, 2001).

The existing information on the lung toxicity of carbon nanotubes is limited and remains inconclusive. In a short report, it has been found that non-purified carbon nanotubes did not induce significant signs of lung toxicity 4 weeks after intratracheal (i.t.) administration in guinea pigs

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(Huczko et al., 2001). Lam and colleagues observed in mice that a single i.t. treatment with single-wall carbon nanotubes induced persistent epithelioid granulomas and interstitial inflammation; the severity of the lesions was dose dependent (Lam et al., 2004). In another study, single-wall carbon nanotubes soot administered in rats produced transient inflammation and cell injury and resulted in the formation of multifocal granulomas centered around nanotubes, similar to a foreign body reaction. The toxicological relevance of these observations was, however, questioned because of the absence of a dose response and because the formation of granulomas was suspected to be the consequence of instilling a bolus of agglomerated nanotubes (nanoropes) (Warheit et al., 2004). A major difficulty reported by these investigators was that carbon nanotubes, because of their electrostatic nature, tend to form large agglomerates that are far beyond the range of respirable particles and therefore difficult to manipulate and administer to experimental animals. In order to circumvent these difficulties, we compared the lung response to multi-wall carbon nanotubes that had been ground or not.

The particles were administered intratracheally (0.5, 2 or 5 mg) to Sprague–Dawley rats. First, we determined whether carbon nanotubes are biopersistent in the lung. We characterized the lung inflammatory and fibrotic responses, and finally we examined the expression of TNF-α, a pro-inflammatory and pro-fibrotic cytokine, in vivo and in vitro. The response to nanotubes was calibrated with two reference particles (carbon black or asbestos).

Materials and methods

Animals. Female Sprague–Dawley rats weighing 200–250 g were purchased from Charles River Laboratories (St Germain-sur-l'Arbresle, France). The animals were kept in a conventional animal facility and housed in positive-pressure air-conditioned units (25 °C, 50% relative humidity) on a 12:12-h light/dark cycle. The experimental protocol has been approved by the local ethical committee for animals in research.

Particle preparation and characterization. All carbon nanotubes were provided by the laboratory of Nuclear Magnetic Resonance at the Facultés universitaires Notre-Dame de la Paix in Namur. Multi-wall carbon nanotubes (15 carbon layers on average) were synthesized by the decomposition of ethylene on an alumina support doped with a cobalt–iron catalyst mixture (Willems et al., 2000) and purified by subsequent treatment with NaOH. A fraction of multi-wall carbon nanotubes was ground in an oscillatory agate ball mill (Pulverisette 0, Fritsch), with a vertical vibration of 1 mm applied during 6 h. Asbestos (Rhodesian Chrysotile "A"; Asb) and ultrafine carbon black (ENSACO 250; CB) were obtained, respectively, from the Union Internationale contre le Cancer (Geneva, Switzerland) and ERACHEM Comilog S.A. (Willebroek, Belgium). CB had a specific surface area of 66.8 m^2/g, an ash content of 0.01%, volatile matter 0.11% and a density of 174 kg/m^3. The fiber length and width of UICC Rhodesian chrysotile A is, respectively, 2.4; 2.3 and 0.17; 1.8 μm (geometrical mean; standard deviation) (Kohyama et al., 1996).

Nanotube characterization. The morphological characterization of nanotubes (length, diameter) was done by low-resolution transmission electron microscopy (TEM), using a TECNAI 10 (Philips) microscope.

The carbon content of the samples was determined by thermal analysis on a STA-409 PC analyzer (Netzsch), under dry air flow (50 ml/min). Temperature range was from 30 to 1400 °C, at a heating rate of 10 °C/min. The residual mass was recorded at 1400 °C. The Co content of the nanotubes was determined by proton-induced X-ray emission (PIXE) with a proton beam of 2 MeV from a Tandetron accelerator.

Specific surface areas were measured at −196 °C by nitrogen adsorption–desorption (Brunauer Emmet Teller method or BET) on an ASAP 2000 (Micrometrics). Prior to measurements, the samples were outgassed under vacuum at 320 °C. Oxidized forms of carbon on the surface of nanotubes were probed by X-ray photoelectron spectroscopy (SSX-100, Surface Science Instrument) (K_α = 1.486 keV).

Particle instillation. After heat inactivation of any possible trace of endotoxin (200 °C, 2 h), the preparations were suspended and sonicated in sterile 0.9% saline containing 1% of Tween 80. The suspensions were then injected directly into the lungs by i.t. instillation (Lasfargues et al., 1992). All instillations (500 μl/rat) were performed after surgical opening of the neck on animals anesthetized with a mix of Ketalar, 6 mg/rat (Warner-Lambert, Zaventem, Belgium), and Rompun, 0.8 mg/rat (Bayer A6, Leverkusen, Germany), given intraperitoneally.

General experimental design. Intact multi-wall carbon nanotubes (CNT) and ground CNT were administered intratracheally (0.5, 2 or 5 mg/animal) to Sprague–Dawley rats and we estimated pulmonary persistence, inflammation and fibrosis at different time points (Table 1). The lung response to nanotubes was calibrated with a single dose (2 mg/rat) of two reference particles sharing with carbon nanotubes nature (CB) or aspect properties (Asb). The inflammatory response was assessed at days 3 and 15 by measuring several parameters in bronchoalveolar lavage (Table 1). The fibrotic response was assessed biochemically (soluble collagen and hydroxyproline) and histopathologically at day 60. To assess the biopersistence of carbon nanotubes the lowest dose of CNT and ground CNT (0.5 mg) was selected because (1) doses of 0.5–2 mg have been recommended in the literature to perform biopersistence

Table 1
General experimental design (in vivo studies)

	Day 0	1 h	Days 3 and 15	Day 28	Day 60
Biopersistence	IT administration of NaCl 0.9%; 0.5 mg CNT; 0.5 mg ground CNT	Co-measurement	–	Co-measurement	Co-measurement
Inflammation	IT administration of NaCl 0.9%; 2 mg Asb; 2 mg CB; 0.5 or 2 mg CNT; 0.5 or 2 mg ground CNT	–	LDH activity; total proteins; cellular parameters; TNF-α protein	–	–
Fibrosis	IT administration of NaCl 0.9%; 2 mg Asb; 2 mg CB; 0.5, 2 or 5 mg CNT; 0.5, 2 or 5 mg ground CNT	–	–	–	Hydroxyproline; type I soluble collagen; histology; TNF-α protein

studies (Muhle and Belmann, 1995) and (2) to minimize the possible influence of inflammation. The biopersistence was assessed up to 2 months after administration to allow a direct comparison with the evaluation of the fibrotic response (2 months). The expression of TNF-α was measured in vivo during the inflammatory (day 3) and fibrotic (day 60) phases of the lung response.

To further characterize the biological response to CNT, we performed in vitro assays on peritoneal macrophages which have been intensively used to estimate and rank the toxicity of inhaled (Lison and Lauwerys, 1990; Miller, 1978). Macrophages were exposed to CB, Asb, intact and ground CNT (20, 50 and 100 μg/ml) and cytotoxicity was assessed by measuring LDH release. TNF-α production was assessed at the transcript and protein levels at 6 and 24 h, respectively.

Determination of nanotube biopersistence. At selected time intervals after nanotube administration (Table 1), whole lungs were excised and mineralized in acid (14N HNO_3: 12N HCl 65:35; both Merck, Darmstad, Germany) in a high pressure microwave (Multiwave, Anton Paar GmbH, Graz, Austria). The amount of nanotubes was then determined by measuring the cobalt content on an atomic absorption spectrometer (Spectra AA 300, Varian Zeeman Inc., Palo Alto, CA, USA) equipped with a graphite furnace atomizer and a Zeeman system for correction of non-specific absorbance.

Bronchoalveolar lavage (BAL) and whole lung homogenates. At the indicated time intervals after particle treatment, animals were sacrificed with sodium pentobarbital (60 mg/rat ip) and a BAL was performed by cannulating the trachea and perfusing the lungs with a volume of 10 ml saline. The recovered BAL fluid (BALF) was centrifuged (250 × *g*, 10 min, 4 °C) and the cell-free supernatant used for biochemical measurements. The cell pellets were resuspended in saline and then used to determine total and differential cell numbers. Leukocyte differentials were performed on cytocentrifuge preparations fixed in methanol and stained with Diff Quick (Dave NV/SA, Brussels, Belgium).

The perfused lungs were then excised and placed into a Falcon tube (Becton-Dickinson) chilled on ice to which 18 ml of phosphate-buffered saline (PBS) were added. The content of each tube was then homogenized using an Ultra-Turrax T25 homogenizer (Janke and Kunkel, Brussels, Belgium) for 1 min. The tubes were then centrifuged at 4 °C, 1000 × *g* for 10 min, and supernatants were kept frozen at −80 °C until use.

Biochemical assays. TNF-α concentrations in BALF were measured by ELISA (detection limit 7 pg/ml) according to the manufacturer's instructions (Pharmingen, BD Biosciences, San Diego, CA, USA). LDH activity was measured by following the reduction of NAD^+ at 340 nm (Technicon RA systems, Bayer Diagnostics Domont, France). Total protein concentration was estimated spectrophotometrically at 600 nm after complexation with molybdate pyrogallol red (Watanabe et al., 1986).

Collagen assays. Collagen deposition was estimated by measuring hydroxyproline and soluble type I collagen contents in lung homogenates. Hydroxyproline was assessed by high-performance liquid chromatography analysis in lung homogenates hydrolyzed in 6N HCl overnight at 110 °C (Biondi et al., 1997). Soluble type I collagen contents were measured in the supernatant of the homogenates with a standardized direct ELISA. Samples and standards were diluted in PBS and coated directly in Nunc-immuno ELISA plates (MaxiSorp) for overnight at 4 °C. After blocking with BSA, polyclonal anti-mouse type I collagen Ab (1:200 times; Biodesign, Saco, ME) were then added and incubated 2 h at room temperature. Polyclonal HRP-conjugated goat anti-rabbit Ig Ab (1:1000; BD Biosciences) was used to measure the fixation of primary Abs. Purified mouse type I collagen obtained from Novotec (Saint-Martin-la-Garenne, France) was used as standard to calibrate each assay. The detection limit of this ELISA is 40 ng/ml.

Histology. In a separate set of animals, the lungs were excised 2 months after administration and fixed in Bouin solution (Merck-Belgolabo, Belgium). Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome for light microscopic examination.

Peritoneal macrophage culture. Peritoneal macrophages were harvested from rats injected intraperitoneally 3 days

before with 5 ml of casein 6% in saline. After hypertonic lysis of the erythrocytes, the cells were pooled, counted and dispersed in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM) and antibiotics (penicillin 50 U/ml and streptomycin 50 μg/ml). Cells were plated in 24-well culture plates at a density of 10^6 macrophages/well. Cultures were incubated overnight at 37 °C in a humidified incubator in an atmosphere of 5% CO_2. On the second day of culture, the cells were washed with 1 ml PBS and exposed for 6 and 24 h in DMEM supplemented with FBS (0.5%), L-glutamine (2 mM) and antibiotics (penicillin 50 U/ml and streptomycin 50 μg/ml) containing different dilutions of the tested compound.

RNA extraction and quantification. Total RNA from adherent cells was isolated with the RNeasy mini-kits (Qiagen, Hilden, Germany) following the manufacturer's protocols. RNA (1 μg) was reverse-transcribed using Superscript RNase H^- reverse transcriptase (Invitrogen) with 350 pM random hexamers (Eurogentec, Seraing, Belgium) in a final volume of 25 μl. Resulting cDNA was then diluted 25-fold in sterile distilled water and used as a template in subsequent real-time polymerase chain reactions (PCR). Sequences of interest were amplified using the following forward primers (Invitrogen): 5′-GAGTACAA-CCTTCTTGCAGCTCC-3′ (β-actin), 5′-ATGGGCTCCCT-CTCATCAGT-3′ (TNF-α); and reverse primers: 5′-TTGTC-GACGACGAGCGC-3′ (β-actin), 5′-ACTCCAGCTGCTC-CTCTGCT-3′ (TNF-α). β-actin was used as endogenous reference housekeeping gene. The quantification of mRNA expression was performed on an ABI 7000 (Applied Biosystems, Foster City, CA) in the following conditions: 2 min 50 °C, 10 min 95 °C, (15 s 95 °C, 1 min 60 °C for) × 40 cycles. Six serial 1:10 dilutions of a standard of cDNA (asbestos-stimulated macrophages) were used to calibrate the assay in each reaction. Standard and samples (5 μl) were amplified with 300 nM primers using SYBR green PCR master mix (Applied Biosystems) in a total volume of 25 μl. The results were expressed as a ratio of product copies per sample to copies per sample of the housekeeping gene β-actin from the same RNA (respective cDNA) preparation.

Statistics. Treatment-related differences were evaluated using *t* tests or one-way analysis of variance followed by pairwise comparisons using the Student–Newman–Keuls test, as appropriate. Statistical significance was considered at $P < 0.05$.

Results

Effect of grinding on nanotubes

The morphology of nanotubes before and after grinding is illustrated on Fig. 1 and the main characteristics of both materials are presented in Table 2. While the length of individual nanotubes was significantly reduced by grinding, this treatment did not affect the other characteristics of the material. Specific surface area was slightly reduced by grinding.

We then tested the influence of grinding on the dispersion of nanotubes in the lungs after intratracheal instillation. As illustrated on Fig. 2, we found that while agglomerates of intact CNT remained entrapped in the largest airways, ground CNT were much better dispersed in the lung tissue.

Biopersistence of carbon nanotubes in the lung

We first assessed the capacity of the lung to eliminate nanotubes (0.5 mg/rat) because the biopersistence of an inhaled particle often determines its toxicity. To quantify nanotubes, we took advantage of the small amount of cobalt catalyst (0.95%) that remains present in CNT and ground CNT after purification. As this cobalt is tightly bound to CNT and ground CNT (maximum 5% release after 60 days of incubation in saline at 37 °C), it could be used to follow

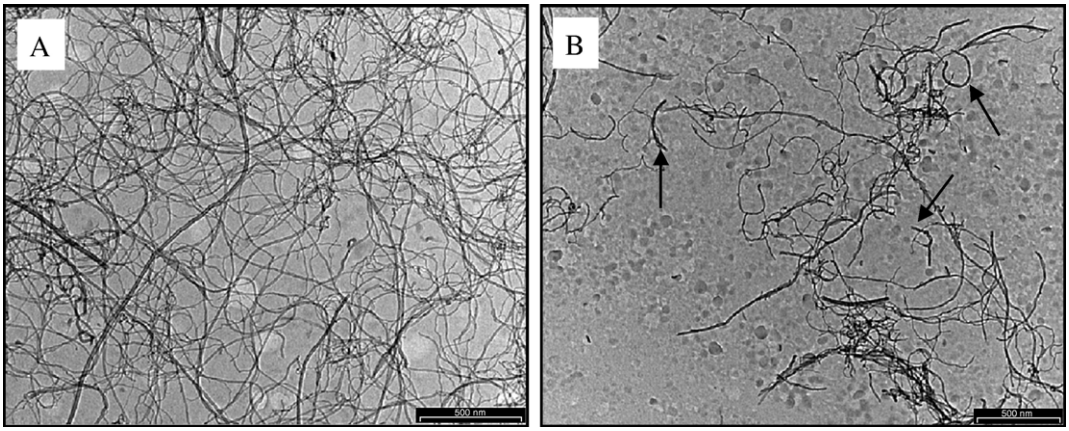

Fig. 1. Transmission electron microscopic (TEM) images of multi-wall carbon nanotubes (A) and of typical ground multi-wall carbon nanotubes indicated by the arrows (B). Scale bars represent 500 nm.

Table 2
Characteristics of individual carbon nanotubes (CNT) ground during 6 h or not

	CNT	Ground CNT
Length (μm)	5.9 ± 0.05	0.7 ± 0.07
Average inner diameter (nm)	5.2 ± 1.5	5.1 ± 2.1
Average outer diameter (nm)	9.7 ± 2.1	11.3 ± 3.9
Specific surface area (m^2/g)	378 ± 20	307 ± 15
Oxidized forms (atomic %)	13.7 ± 0.7	13.1 ± 0.7
Carbon content (%)	97.8 ± 0.2	98.0 ± 0.2

the persistence of carbon nanotubes in the lungs. Rats were sacrificed at day 0, 28 or 60 after administration of CNT or ground CNT and cobalt contents measured in whole lung homogenates as an index of nanotube biopersistence. The data presented in Table 3 indicate that CNT were not or slowly eliminated from the lung (81.2% of CNT recovered after 60 days) whereas ground CNT were cleared more rapidly, especially during the second month (36% after 60 days). Thus, we conclude that CNT persist in the lung and their length appears to modulate clearance kinetics.

Pulmonary inflammation in response to carbon nanotubes

We then examined whether nanotubes could cause pulmonary inflammation. Three days after administration, bronchoalveolar lavage (BAL) fluid was obtained from rats administered with CNT (0.5 or 2 mg/rat), ground CNT (0.5 or 2 mg/rat), asbestos (Asb, 2 mg/rat), carbon black (CB, 2 mg/rat) or a saline solution (NaCl, 0.9%, controls). BAL fluid LDH activity, a marker of cell toxicity, was significantly increased after Asb, but not after CB. Administration of ground CNT and CNT induced a dose-dependent increase in LDH release which was more marked with the ground material (Fig. 3A). The protein concentration in BAL fluid, which reflects alveolo–capillar permeability and/or alveolitis, was also increased after administration of ground CNT and CNT (Fig. 3B). Accumulation of granulocytes in the lung was also evident after treatment with the different particles. CNT and ground CNT induced the accumulation of both neutrophils and eosinophils (Figs. 3C and D). Similar data were observed 15 days after particle administration (data not shown).

Altogether, we concluded that nanotubes induced an inflammatory response which was to some extent more marked with ground CNT.

Pulmonary fibrosis in response to carbon nanotubes

Next, we examined whether these nanotubes could induce lung fibrosis. Collagen deposition was first assessed quantitatively by measuring lung hydroxyproline (OH-proline) and soluble collagen I contents 60 days after particle administration. While OH-proline levels were significantly and dose-dependently increased after CNT, only the highest dose of ground CNT (5 mg/rat) induced a significant elevation of OH-proline levels (Fig. 4A). As expected, Asb treatment was also accompanied by exaggerated OH-proline accumulation and no similar increase was noted after CB (Fig. 4A). Asb, CNT as well as ground CNT induced a significant increase of the type I collagen lung levels in comparison with the control rats. No similar increase was noted after administration of CB (Fig. 4B).

Both measurements therefore indicated that the fibrotic response to nanotubes was dose dependent. Of note, the intensity of the fibrotic response induced by 5 mg of ground CNT was equivalent to that induced by 2 mg of CNT.

Sixty days after particles treatment, the morphological characteristics of the lesions induced by CNT, ground CNT, Asb or CB (2 mg/rats) were also evaluated by standard hematoxylin–eosin and Masson trichrome staining (Figs. 5A and B). This histopathological study revealed also the presence of collagen-rich granulomas in the bronchi of animals instilled with CNT, which blocked partially or completely the bronchial lumen. These granulomas were re-epithelialized, organized around CNT material and formed of multinuclear giant cells as well as macrophages and other mononuclear inflammatory cells. Histological analysis clearly showed that ground CNT were better dispersed in the parenchyma and induced granulomas in the interstitial tissue. These granulomas were localized in the alveolar spaces or the interstitium and consisted of macrophages laden with particles, multinuclear giant cells and some inflammatory cells. As expected, the administration of Asb induced a parenchymal thickening with fibrosis and alveolitis. Occasionally, obstructive lesions were localized in bronchioles. Those injuries consisted of lymphocytes, fibroblasts, collagen deposition and sometimes of multinuclear giant cells. In CB-treated rats, we observed a simple accumulation of particles in alveolar macrophages without significant change of the alveolar architecture. Altogether, we concluded that nanotubes were able to induce a fibrotic response.

TNF-α in response to carbon nanotubes (in vivo)

Because TNF-α is a key mediator in inflammation and fibrosis, we then assessed TNF-α production in the lung of experimental rats. During the acute inflammatory reaction (day 3), the BAL levels of TNF-α were significantly increased after instillation of Asb, CB, CNT (2 mg) or ground CNT (0.5 and 2 mg) (Fig. 6A). At the fibrotic stage (day 60), this increased expression persisted only in the groups of rats treated with Asb or ground CNT (0.5 and 2 mg) (Fig. 6B).

In vitro studies

Finally, we examined the effects induced by carbon nanotubes on peritoneal macrophages used as models to study the biological reactivity of mineral dust exposure (Miller, 1978).

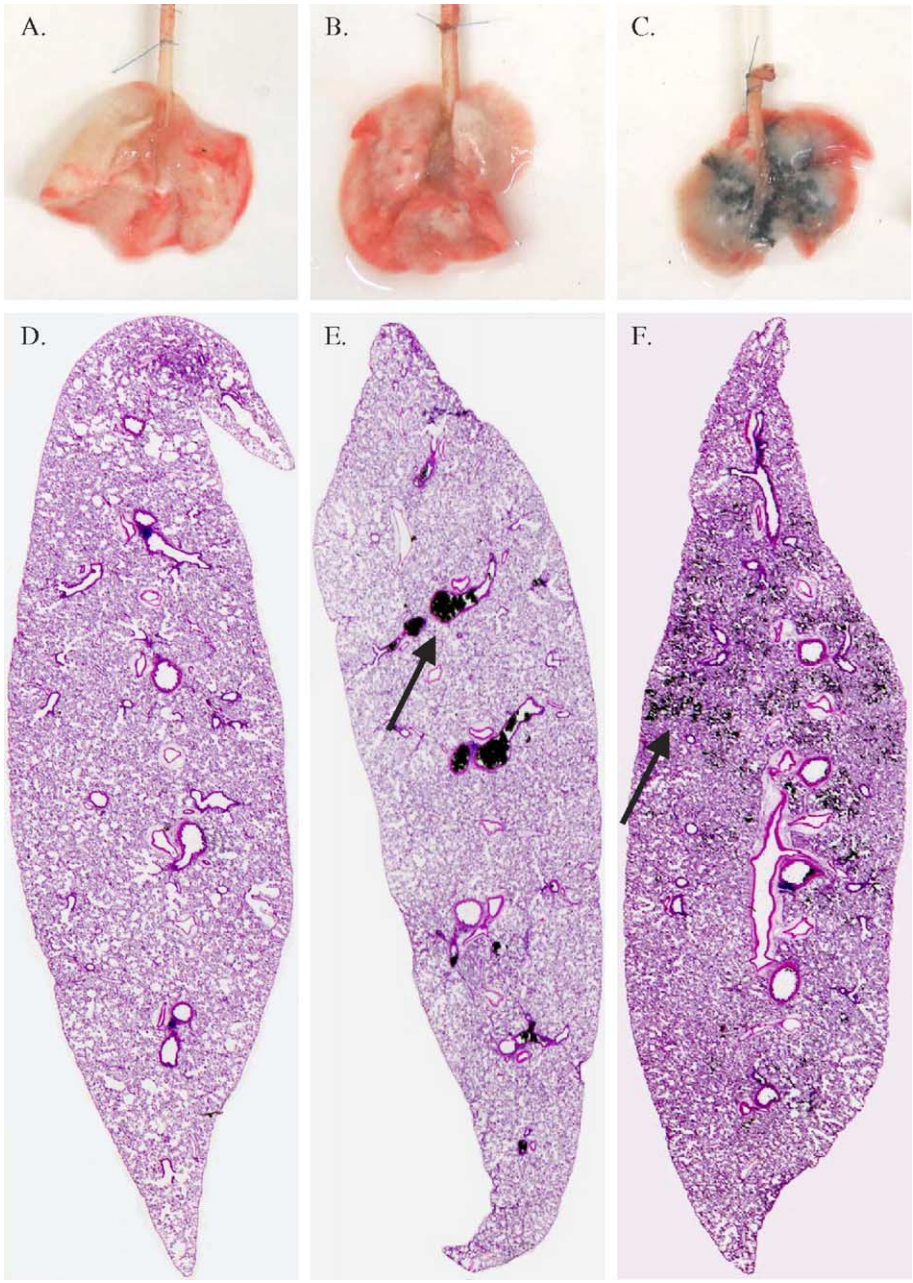

Fig. 2. Effect of grinding on the dispersion of carbon nanotubes (CNT) in the lungs after intratracheal administration. Animals were sacrificed immediately after administration and the lungs were perfused with a saline solution by cannulating the right ventricle. The panels present macroscopic views and hematoxylin/eosin-stained lung sections from saline (A, D), CNT (B, E; 2 mg/rat) or ground CNT (C, F; 2 mg/rat) rats after i.t. instillation. Intact CNT remained mainly entrapped in the large airways and ground CNT were better dispersed in the lung tissue (arrows). Original magnifications were ×2 (D–F).

While ground CNT were relatively well dispersed in the culture medium and sedimented on the cell layer, we observed that CNT formed large aggregates that remained floating on the medium and were not in contact with the cultured cells (Fig. 7A). Asb, CB and ground CNT but not CNT induced a significant and dose-dependent cytotoxicity as reflected by the amplitude of LDH release (Fig. 7B). In parallel, the levels of TNF-α in the supernatant of macrophage cultures were also significantly increased after 24 h of exposure to Asb, CB or ground CNT but not CNT (Fig. 7C). We could, however, not exclude that the increased TNF-α level measured in cell cultures treated with Asb, CB and ground CNT was not related to cell toxicity and/or membrane damage. Therefore, we measured TNF-α expression at the mRNA level in macrophage cultures, after 6 h of incubation with the particles. TNF-α mRNA was strongly upregulated after exposure to the highest dose of Asb, CB or ground CNT but not CNT (Fig. 8).

We concluded that, when they enter in contact with target cells, nanotubes have the capacity to upregulate the expression of the pro-inflammatory and pro-fibrotic medi-

Table 3
Pulmonary biopersistence of carbon nanotubes (CNT) ground during 6 h or not

Single i.t. dose	Time after particle administration		
	Day 0	Day 28	Day 60
NaCl 0.9%	ND[a]	ND[a]	ND[a]
	0.4 ± 0.1[b]	0.3 ± 0.1[b]	0.4 ± 0.1[b]
0.5 mg CNT		(78.4% ± 15.3)[c]	(81.2% ± 26.4)[c]
	0.5 ± 0.1[b]	0.4 ± 0.1[b]	0.2 ± 0.1[b]
0.5 mg ground CNT		(78.4% ± 12.4)[c]	(36.0% ± 13.2)[c]

[a] ND = not detected.
[b] Amount of CNT recovered in the lung of rats (mg, means ± SD, $n = 5$) based on AAS measurement of cobalt (see Materials and methods).
[c] Relative to day 0.

ator TNF-α. The apparent absence of effect of CNT on TNF-α expression in vitro is most probably related to their low availability to the test system.

Discussion

In this study, we show that when they reach the lungs multi-wall carbon nanotubes are not rapidly eliminated and have the potential to cause inflammatory and fibrotic reactions. The pro-inflammatory and pro-fibrotic mediator TNF-α is upregulated in response to carbon nanotubes in vivo and in vitro.

Intratracheal instillation is a useful procedure for the evaluation of the respiratory toxicity of particles (hazard assessment), especially when, as in the case of carbon nanotubes, the amount of test material is too limited for the generation of an atmosphere at adequate concentrations and for a sufficient duration. It yields qualitatively similar results

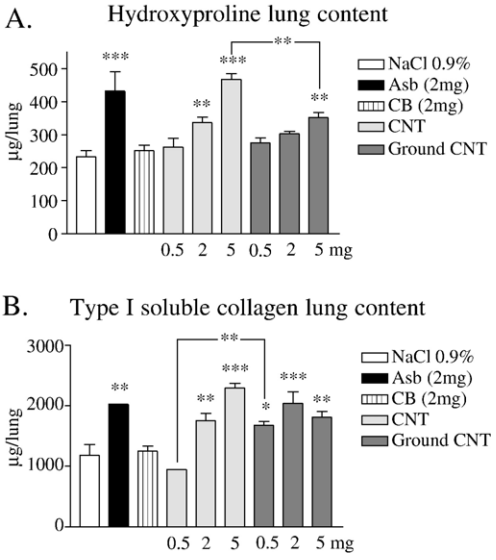

Fig. 4. Instillation of carbon nanotubes (CNT or ground CNT) induced a fibrotic response in the rat lung. The graphs represent hydroxyproline levels (A) and type I collagen content (B) in the lung tissue 60 days after intratracheal instillation of CNT (0.5, 2 or 5 mg/rat), ground CNT (0.5, 2 or 5 mg/rat), asbestos (Asb, 2 mg/rat), carbon black (CB, 2 mg/rat) or saline (NaCl, 0.9%). Each bar represents the mean ± SEM of 4–6 observations. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$ denote significant differences between mean values measured in the indicated group compared to saline, as analyzed by the Student–Newman–Student–Newman–Keuls multiple comparison test.

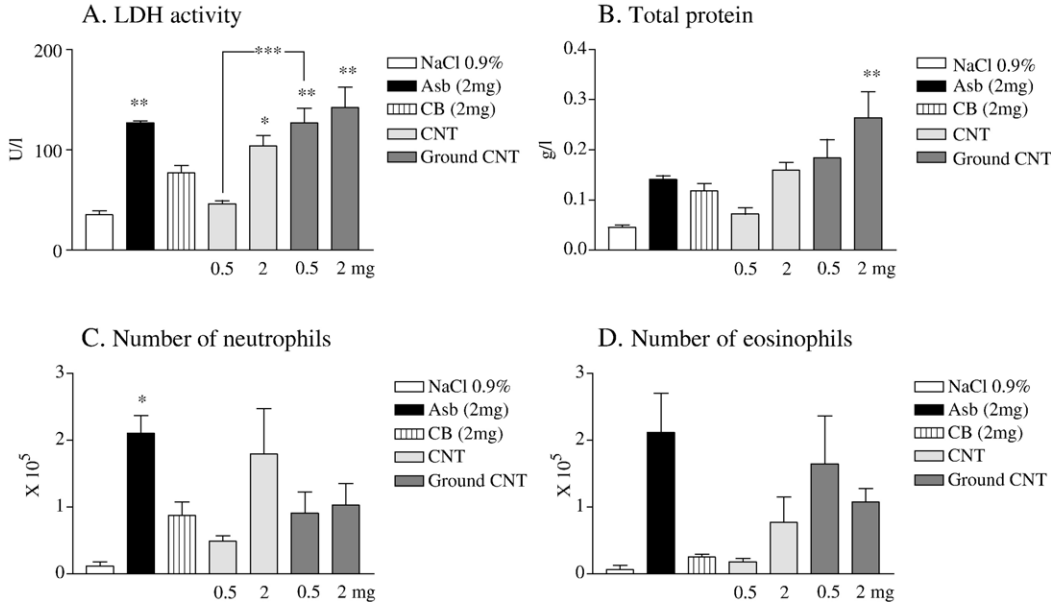

Fig. 3. Instillation of carbon nanotubes (CNT or ground CNT) induced an inflammatory response in the rat lung. Sprague–Dawley rats were administered by intratracheal instillation with CNT (0.5 or 2 mg/rat), ground CNT (0.5 or 2 mg/rat), asbestos (Asb, 2 mg/rat), carbon black (CB, 2 mg/rat) or saline (NaCl, 0.9%). Levels of lactate dehydrogenase (LDH, A), total proteins (B), number of neutrophils (C) as well as eosinophils (D) were determined in bronchoalveolar lavage fluid (BALF) 3 days after particle treatment. Each bar represents the mean ± SEM of 4–6 observations. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$ denote significant differences between mean values measured in the indicated group compared to saline, as analyzed by the Student–Newman–Keuls multiple comparison test.

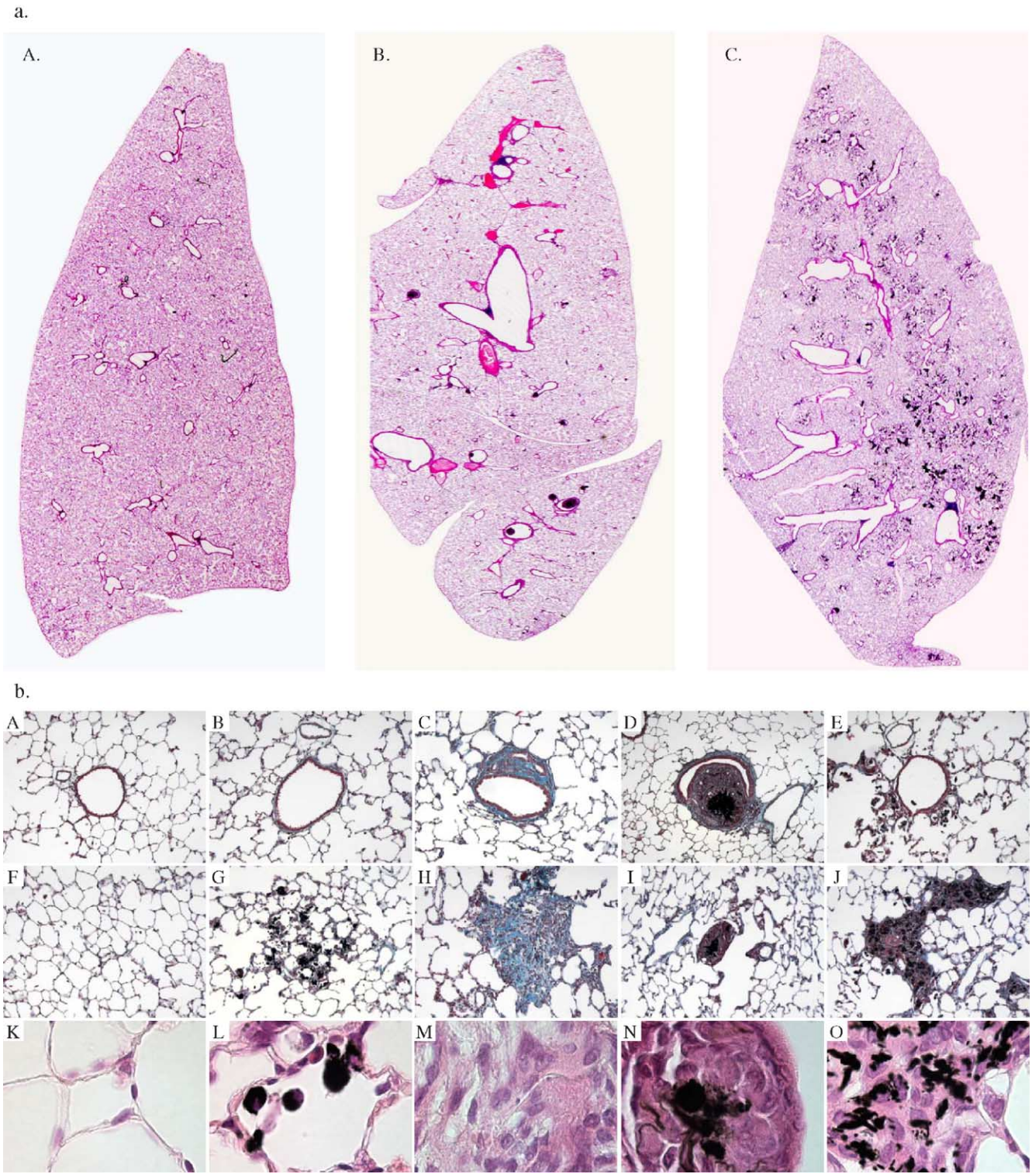

Fig. 5. (a) The lesions induced by ground carbon nanotubes were distributed more evenly in the lungs than those induced by intact nanotubes. The panels present hematoxylin- and eosin-stained lung sections from saline (A)-, CNT (B; 2 mg/rat)- or ground CNT (C; 2 mg/rat)-treated rats 60 days after i.t. instillation. Original magnifications were ×2 (A–C). (b) Pulmonary lesions induced by carbon nanotubes (CNT or ground CNT) are characterized by the formation of collagen-rich granulomas. The panels present Masson trichrome (A–J) or hematoxylin and eosin (K–O)-stained lung sections from saline (A, F, K)-, carbon black (B, G, L; 2 mg/rat)-, asbestos (C, H, M; 2 mg/rat)-, CNT (D, I, N; 2 mg/rat)- or ground CNT (E, J, O; 2 mg/rat)-treated rats after i.t. instillation (day 60). Different airway levels corresponding to bronchi (A–E), alveolar space (F–J) or to some typical characteristics of the administered particles (K–O) are shown. Original magnifications were ×10 (A–J) or ×100 (K–O).

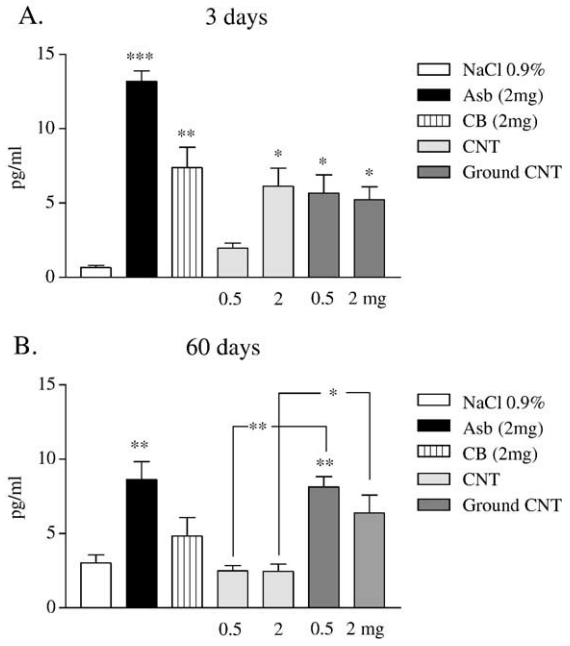

Fig. 6. Instillation of carbon nanotubes (CNT or ground CNT) induced an increase of TNF-α in BAL fluid. Sprague–Dawley rats were administered by intratracheal instillation with CNT (0.5 or 2 mg/rat), ground CNT (0.5 or 2 mg/rat), asbestos (Asb, 2 mg/rat), carbon black (CB, 2 mg/rat) or saline (NaCl, 0.9%). Level of TNF-α was measured in BAL fluid 3 days (A) or 60 days (B) after particle treatment. Each bar represents the mean ± SEM of 4–6 observations. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ denote significant differences between mean values measured in the indicated group compared to saline, as analyzed by the Student–Newman–Keuls multiple comparison test.

than those obtained by inhalation for a variety of biologic endpoints such as pulmonary inflammation and fibrosis in rats (Driscoll et al., 2000). However, intratracheal administration has several limitations, in particular because the administration of a single bolus of often entangled material may produce artifactual granulomatous lesions in the airways (Drew et al., 1987). Thus, previous investigators who examined the toxicity of carbon nanotubes faced difficulties to adequately deliver the test material to the lung, mainly because these particles tended to form aggregates that remained entrapped in the airways. They were therefore perplex about the significance of their observations (non-uniform distribution of the lesions, no dose response, absence of surrounding inflammation) and questioned the toxicological relevance of their findings (Warheit et al., 2004). In the present study, we also found that the administration of intact multi-wall carbon nanotubes (CNT) induces the formation of bronchial granulomas developing around focal aggregates of CNT (Fig. 5B); these granulomas were very similar to those reported previously in the airways of rats (Warheit et al., 2004) and in the airways and interstitium of mice (Lam et al., 2004). Ground CNT were much better dispersed in the vehicle, which allowed a better distribution of the particles in the lungs and avoided the formation of the large intraluminal granulomas observed with CNT (Fig. 5B). Ground CNT induced the formation of lung inflammation and fibrosis and the lesions had a more uniform distribution, followed a dose–effect relationship and persisted for at least 2 months. Since the nature of carbon nanotubes was not significantly affected by grinding, our data support the idea that carbon nanotubes are intrinsically toxic to the lung.

Industrial applications of carbon nanotubes include a number of instances in which this material is included in polymers or other matrices. For these applications, a perfect dispersion of the particles is essential and ground nanotubes are used to this end (Liu et al., 2004; Pierard et al., 2001). It is likely that in the near future, the industrial use of

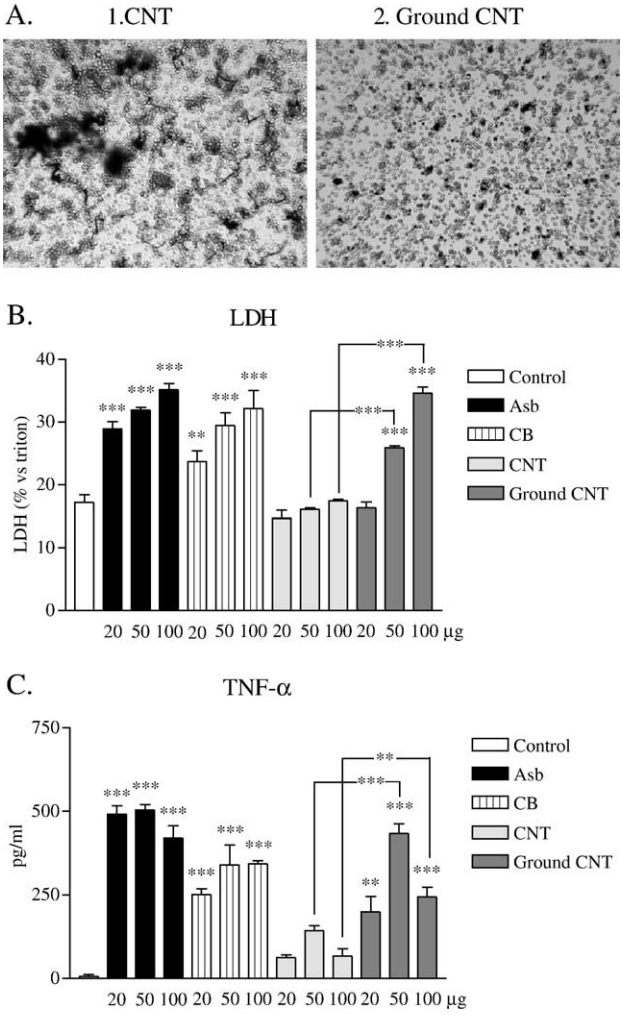

Fig. 7. Treatment with carbon nanotubes (CNT or ground CNT) induced different effects in vitro. Here we show the typical dispersion of CNT (A_1) or ground CNT (A_2) in peritoneal macrophages cultures. 24 h after CNT (20, 50 or 100 μg/well), ground CNT (20, 50 or 100 μg/well), asbestos (Asb; 20, 50 or 100 μg/well), carbon black (CB; 20, 50 or 100 μg/well) or culture medium (control), we assessed the level of TNF-α (B) and of lactate dehydrogenase (LDH, C) in the supernatant of macrophage cultures. Total LDH content was determined in parallel after cell disruption in 0.1% Triton X-100. Each bar represents the mean ± SEM of 3 observations. $**P < 0.01$, $***P < 0.001$ denote significant differences between mean values measured in the indicated group compared to saline, as analyzed by the Student–Newman–Keuls multiple comparison test.

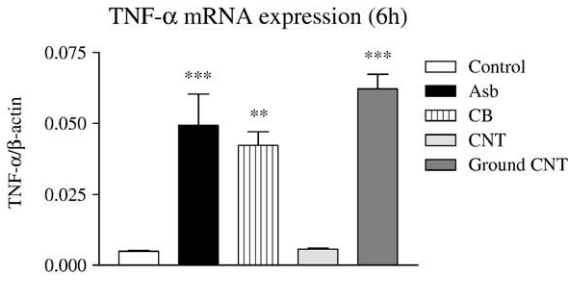

Fig. 8. Exposure to ground carbon nanotubes induced an increased mRNA expression of TNF-α. Peritoneal macrophages cultures were exposed 6 h to CNT (100 μg/well), ground CNT (100 μg/well), asbestos (Asb, 100 μg/well), carbon black (CB, 100 μg/well) or to culture medium only (control). All data were standardized with the housekeeping gene β-actin. Each bar represents the mean ± SEM of 3 observations. $^{**}P < 0.01$, $^{***}P < 0.001$ denote significant differences between mean values measured in the indicated group compared to saline, as analyzed by the Student–Newman–Keuls multiple comparison test.

ground carbon nanotubes will increase sharply and there is therefore also a relevance to examine the toxicity of ground material.

The pulmonary persistence (biopersistence) of an inhaled particle often determines its toxicity (Oberdörster, 2002). By measuring lung cobalt content, we clearly demonstrated that the multi-wall CNT instilled were not or slowly cleared from the lungs (Table 3). While the data obtained with CNT must be interpreted with caution because of the possible bias introduced by the artifactual retention of large agglomerates of material, the results indicate that ground CNT were cleared more rapidly, but a significant fraction of the administered dose (36%) still remained in the lung after 60 days. The relative biopersistence of CNT in the rat lung points therefore to a potential toxicity to the respiratory tract, and their length appears to modulate clearance kinetics.

The inflammatory response induced by inhaled particles is considered as a forerunner of lung fibrosis. In addition, in vivo data support the existence of a relation between inflammation and mutagenicity. Indeed, several studies have demonstrated that leukocytes accumulated in the lung after inhalation of toxic particles produce mediators such as cytokines and oxidants, respectively, responsible for the fibrotic response and the occurrence of mutations in epithelial cells (Piguet et al., 1990; Knaapen et al., 2002). Thus, we determined the amplitude and the characteristics of the inflammatory response after particle administration. CNT and ground CNT induced inflammation as demonstrated by high levels of LDH and proteins as well as a marked neutrophilic and eosinophilic accumulation. The apparently higher degree of inflammation induced by ground CNT compared to CNT (BALF LDH activity and protein content) is possibly the reflection of the better dispersion of these particles in the deep lung, causing greater alveolar cell toxicity and increased alveolo-capillar permeability.

In view of their potent inflammatory effects, we examined whether carbon nanotubes could in the longer term induce lung fibrosis. Histological and biochemical analyses clearly demonstrated the presence of a fibrotic reaction in the lung of rat treated with CNT and ground CNT (Figs. 4 and 5). The pulmonary lesions induced by CNT were mainly localized to the airways, again reflecting the formation of CNT agglomerates and their limited dispersion in the lungs. Ground CNT reached the alveolar spaces and induced the formation of parenchymal granulomas. Biochemical analyses indicated an accumulation of collagen in the lung of animals treated with CNT and ground CNT. Interestingly, the amplitude of the fibrotic response induced by CNT and ground CNT seemed to parallel the fraction of material retained in the lung after 2 months (Table 3).

Given all these effects of carbon nanotubes, we also measured a typical pro-inflammatory cytokine, TNF-α, which is well known for its implication in the pathogenesis of particle-induced lung diseases and pneumoconioses (Piguet et al., 1990). Again, the lung response to CNT and ground CNT was accompanied by increased levels of TNF-α in BALF. The fact that, after administration of intact CNT, TNF-α levels returned to that of controls after 2 months is possibly reflecting the entrapment of inflammatory cells that produce this mediator in intraluminal granulomas that are barely accessible to BAL.

In all the assays, Rhodesian chrysotile "A" fibers and ultrafine carbon black particles were included as reference materials. As expected, Asb induced an inflammatory and fibrotic lung reaction and stimulated the production of TNF-α. CB induced an early (day 3) inflammatory reaction which went paired with elevated TNF-α in BALF. Contrary to ground CNT, this reaction to CB was transient and after 60 days we could not detect any sign of fibrosis and TNF-α BALF levels were not significantly increased. These data point to a specific toxicity related to the unique properties of carbon nanotubes.

To further document the potential toxicity of multi-wall carbon nanotubes, we examined their effects on peritoneal macrophages. In vitro, we showed that ground CNT induced a TNF-α response similar to Asb and CB indicating again that, when they enter in contact with the target cells, carbon nanotubes have a toxic potential.

In conclusion, the present study shows that if multi-wall carbon nanotubes reach the lung they are biopersistent, have the capacity to stimulate lung cells to produce TNF-α and induce lung inflammation and fibrosis. Based on these initial observations, the precautionary principle should be applied and adequate industrial hygiene measures implemented to minimize human exposure during the manipulation of carbon nanotubes. Meanwhile, additional studies will be necessary to better characterize the toxicity of these materials in other animal models and by other modes of administration (e.g., inhalation study to take into account respirability). Further studies are also needed to examine the direct or indirect genotoxic potential of carbon nanotubes and whether they can be translocated from the respiratory

system to other organs. Comparative studies of different types of carbon nanotubes (single and multi-wall, functionalized, produced by metal catalysis or plasma, etc.) will also contribute to decipher the mechanisms that may govern the toxicity of these materials.

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References

Biondi, P.A., Chiesa, L.M., Storelli, M.R., Renon, P., 1997. A new procedure for the specific high-performance liquid chromatographic determination of hydroxyproline. J. Chromatogr. Sci. 35, 509–512.

Carbon Nanotubes—Worldwide Status and Outlook: Applications, Applied Industries, Production, R&D and Commercial Implications, 2002. http://www.researchandmarkets.com/reports/7730/.

Drew, R.T., Kuschner, M., Bernstein, D.M., 1987. The chronic effects of exposure of rats to sized glass fibres. Ann. Occup. Hyg. 31, 711–729.

Driscoll, K.E., Costa, D.L., Hatch, G., Henderson, R., Oberdorster, G., Salem, H., Schlesinger, R.B., 2000. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. Toxicol. Sci. 55, 24–35.

Huczko, A., Lange, H., Clko, E., Grubek-Jaworska, H., Droszcz, P., 2001. Physiological testing of carbon nanotubes: are they asbestos-like? Fuller. Sci. Technol. 9, 251–254.

Knaapen, A.M., Albrecht, C., Becker, A., Hohr, D., Winzer, A., Haenen, G.R., Borm, P.J., Schins, R.P., 2002. DNA damage in lung epithelial cells isolated from rats exposed to quartz: role of surface reactivity and neutrophilic inflammation. Carcinogenesis 23, 1111–1120.

Kohyama, N., Shinohara, Y., Suzuki, Y., 1996. Mineral phases and some reexamined characteristics of the international union against cancer standard asbestos samples. Am. J. Ind. Med. 30 (5), 515–528 (Nov.).

Lam, C., James, J.T., McCluskey, R., Hunter, R.L., 2004. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. Toxicol. Sci. 77, 126–134.

Lasfargues, G., Lison, D., Maldague, P., Lauwerys, R., 1992. Comparative study of the acute lung toxicity of pure cobalt powder and cobalt-tungsten carbide mixture in rat. Toxicol. Appl. Pharmacol. 112, 41–50.

Lison, D., Lauwerys, R., 1990. In vitro cytotoxic effects of cobalt-containing dusts on mouse peritoneal and rat alveolar macrophages. Environ. Res. 52 (2), 187–198 (Aug.).

Liu, C.H., Huang, H., Wu, Y., Fan, S.S., 2004. Thermal conductivity improvement of silicone elastomer with carbon nanotube loading. Appl. Phys. Lett. 84 (21), 4248–4250.

Martin, C.R., Kohli, P., 2003. The emerging field of nanotube biotechnology. Nat. Rev., Drug Discov. 2, 29–37.

Maynard, A.D., Baron, P.A., Foley, M., Shvedova, A.A., Kisin, E.R., Castranova, V., 2004. Exposure to carbon nanotube material: aerosol release during the handling of unrefined single-walled carbon nanotube material. J. Toxicol. Environ. Health, Part A 67, 87–107.

Miller, K., 1978. The effects of asbestos on macrophages. CRC Crit. Rev. Toxicol. 5 (4), 319–354 (Sep.).

Mossman, B.T., Bignon, J., Corn, M., Seaton, A., Gee, J.B., 1990. Asbestos: scientific developments and implications for public policy. Science 247, 294–301.

Muhle, H., Belmann, B., 1995. Biopersistence of man-made vitreous fibres. Ann. Occup. Hyg. 39 (5), 655–660 (Oct.).

Oberdörster, G., 2001. Pulmonary effects of inhaled ultrafine particles. Int. Arch. Occup. Environ. Health 74, 1–8.

Oberdörster, G., 2002. Toxicokinetics and effects of fibrous and nonfibrous particles. Inhalation Toxicol. 14, 29–56.

Pierard, N., Fonseca, A., Konya, Z., Willems, I., Van Tendeloo, G., Nagy, J.B., 2001. Production of short carbon nanotubes with open tips by ball milling. Chem. Phys. Lett. 335, 1–8.

Piguet, P.F., Collart, M.A., Grau, G.E., Sappino, A.P., Vassalli, P., 1990. Requirement of tumour necrosis factor for development of silica-induced pulmonary fibrosis. Nature 344, 245–247.

Warheit, D., Laurence, B., Reed, K., Roach, D., Reynold, G., Webb, T., 2004. Comparative pulmonary toxicity assessment of single wall carbon nanotubes in rats. Toxicol. Sci. 77, 117–125.

Watanabe, N., Kamei, S., Ohkubo, A., Yamanaka, M., Ohsawa, S., Makino, K., Tokuda, K., 1986. Urinary protein as measured with a pyrogallol red-molybdate complex, manually and in a Hitachi 726 automated analyzer. Clin. Chem. 32 (8), 1551–1554.

Willems, I., Konya, Z., Colomer, J.-F., Van Tendeloo, G., Nagajaru, N., Fonseca, A., Nagy, J.-B., 2000. Control of the outer diameter of thin carbon nanotubes synthesised by catalytic decomposition of hydrocarbons. Chem. Phys. Lett. 317, 71–76.