

# Structural Defects Play a Major Role in the Acute Lung Toxicity of Multiwall Carbon Nanotubes: Toxicological Aspects

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Experimental studies indicate that carbon nanotubes (CNTs) have the potential to induce adverse pulmonary effects, including alveolitis, fibrosis, and genotoxicity in epithelial cells. Here, we explored the physicochemical determinants of these toxic responses with progressively and selectively modified CNTs: ground multiwall CNTs modified by heating at 600 °C (loss of oxygenated carbon functionalities and reduction of oxidized metals) or at 2400 °C (annealing of structural defects and elimination of metals) and by grinding the material that had been heated at 2400 °C before (introduction of structural defects in a metal-deprived framework). The CNTs were administered intratracheally (2 mg/rat) to Wistar rats to evaluate the short-term response (3 days) in bronchoalveolar lavage fluid (LDH, proteins, cellular infiltration, IL-1 $\beta$ , and TNF- $\alpha$ ). The long-term (60 days) lung response was assessed biochemically by measuring the lung hydroxyproline content and histologically. In vitro experiments were also performed on rat lung epithelial cells to assess the genotoxic potential of the modified CNTs with the cytokinesis block micronucleus assay. The results show that the acute pulmonary toxicity and the genotoxicity of CNT were reduced upon heating but restored upon grinding, indicating that the intrinsic toxicity of CNT is mainly mediated by the presence of defective sites in their carbon framework.

## Introduction

Serious concerns have been raised regarding possible effects of carbon nanotubes (CNTs)<sup>1</sup> on human health (1–3). Available toxicological data are, however, still fragmentary and, to some extent, confusing. In vivo studies indicate that when CNTs (single- or multiwall CNTs) reach the lung, they have the potential to induce a strong inflammatory and fibrotic reaction (1, 4–6). It has also been shown that multiwall CNTs induce genotoxic effects in lung epithelial cells (7). Other in vivo studies indicate no or limited toxicity of CNTs (8–10). Diverging data from in vitro studies have also been reported. While several studies demonstrated that both single- or multiwall CNTs were able to induce cytotoxicity and apoptosis in different cell types (11–14), other studies indicated that CNTs caused very low or no cytotoxicity (15–17). The reason

for these discrepancies is not immediately evident but may depend on experimental protocols and/or interferences with test systems used (17). Moreover, the available data are not easily comparable mainly because of the variety of materials tested. Small variations in CNT morphology and physicochemical features might indeed modulate their toxicity. Therefore, identifying the main physicochemical characteristics that determine CNT toxicity appears very relevant to better characterize, and possibly control, their toxic potential.

It is well-known that the pathogenicity of inhaled particles is governed by their chemical and physical features (18, 19). In the case of CNT, different types are produced with a variety of physical and chemical properties depending on the mode of production and postsynthesis modifications. The crude postsynthesis material may be contaminated by amorphous carbon and by catalyst remnants (usually Al, Fe, Co, Ni, or Mo), which appear as metal residues present at the surface of the CNTs or as metallic clusters entrapped within the CNTs. Postsynthesis treatment can modify CNT characteristics, including purity, adsorptive nature, aspect ratio, surface reactivity, hydrophilic properties, and surface functionalization. Several sources of variation can therefore contribute to explain the varying toxic potential of CNTs. Some studies suggest that metallic contaminants play a role in CNT toxicity. The increase of intracellular reactive oxygen species (ROS) and the decrease of the mitochondrial membrane potential induced by a commercial preparation of CNT in rat NR8383 macrophages and human A549

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<sup>1</sup> BAL, bronchoalveolar lavage; BALF, BAL fluid; CNT, carbon nanotubes; CPBI, cytokinesis-block proliferation index; FCS, fetal calf serum; IL-1 $\beta$ , interleukin-1  $\beta$ ; H&E, hemalum-eosin staining; LDH, lacticodehydrogenase; MN, micronucleus; MNCB, micronucleated binucleated cell; PAH, polycyclic aromatic hydrocarbons; PBS, phosphate-buffered saline; RLE, rat lung epithelial cells; ROS, reactive oxygen species; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

lung cells were abolished with acid-washed CNTs with low metal content (16). Similarly, it was shown in activated mouse macrophages (RAW 264.7) that Fe-containing CNTs (30% of total mass) caused a greater oxidative stress and antioxidant depletion as compared with iron-stripped CNTs (20). These results are also in line with those reported by Shvedova et al. (14), showing that nonpurified CNTs treated with an iron chelator had less pronounced toxic effects on cultured keratinocytes than iron-rich CNTs. It has also been reported that CNT length can influence tissue reactivity. After implantation of CNT in subcutaneous tissue of rats, Sato et al. (21) found that the inflammatory response around shorter CNTs (220 nm) was lower than around longer CNTs (825 nm). The influence of surface chemistry and aspect ratio has also been investigated by Bottini et al. (11). They reported that hydrophobic pristine CNTs appeared less toxic for T cells than oxidized CNTs, which induced cell apoptosis. It has also been shown that while CNTs, carbon black, and carbon nanofibers inhibited proliferation and induced cell death in different human lung-tumor cell lines, these effects were more pronounced as the aspect ratio of carbon-based nanomaterials decreased and with the presence of chemically functional groups (carbonyl) on the graphene surface (22).

All of these studies indicate that the presence of metallic contaminants, the size, the degree of oxidation, and the hydrophilic properties of CNTs can modulate their toxic potential. However, most of these studies have been carried out *in vitro*, and it is not known whether these differences affect the *in vivo* response to CNTs, especially in the lung. Systematic studies carried out with standardized CNTs are lacking, and the influence of structural defects on the toxic potential of CNTs has not yet been explored. The aim of the present study was to investigate whether structural defects and/or entrapped metallic clusters determine CNT toxicity both *in vivo* and *in vitro*.

## Materials and Methods

**General Experimental Design.** To explore the main features responsible for CNT toxicity, ground multiwall CNTs (CNT containing structural defects, CNT<sub>g</sub>) were modified by heating at 600 °C (loss of oxygen from carbon functionalities and metal oxides, CNT<sub>g600</sub>) or 2400 °C (elimination of metal clusters and ablation of defects, CNT<sub>g2400</sub>) and by grinding material that had been heated before (introduction of defects in a metal-deprived carbon framework, CNT<sub>2400g</sub>) (23). Two biological responses, previously examined with CNT<sub>g</sub> (1, 7), were investigated as follows: (i) the short- and the long-term pulmonary responses were assessed in rats, respectively, 3 and 60 days after intratracheal administration of the modified CNT and (ii) the *in vitro* genotoxicity was assessed in rat lung epithelial cells (RLE) with the cytokinesis-block micronucleus assay. To study the short-term lung response, several parameters were measured in bronchoalveolar lavage [lactate dehydrogenase activity, total protein content, cellular parameters, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) concentration]. The long-term response was evaluated biochemically (hydroxyproline lung content) and histopathologically.

**CNTs. Modified CNTs.** The detailed physicochemical characterization of the different preparations (CNT<sub>g</sub>, CNT<sub>g600</sub>, CNT<sub>g2400</sub>, and CNT<sub>2400g</sub>), including their capacity to produce or to quench ROS, is reported in the companion manuscript (23).

**Pulmonary Response. Animals.** Female Wistar rats weighing 200–250 g were obtained from the local breeding facility (UCL, Brussels, Belgium). The animals were kept in a conventional animal facility and housed in positive-pressure air-conditioned units (25 °C and 50% relative humidity) on a 12 h light/dark

cycle with free access to water and laboratory animal food. The experimental protocol was approved by the local ethical committee for the use of animals in biomedical research.

**CNT Administration.** After heat inactivation of any possible trace of endotoxin (200 °C, 2 h), the CNTs were dispersed in sterile 0.9% saline containing 1% Tween 80. The suspensions were injected directly into the lungs by intratracheal (i.t.) instillation (24). All administrations (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. Five rats per group were included in each experiment. Control animals were treated with an equivalent volume of sterile 0.9% saline containing 1% Tween.

**Bronchoalveolar Lavage (BAL) and Lung Homogenates.** At 3 and 60 days after CNT administration, the animals were sacrificed with sodium pentobarbital (60 mg/rat, i.p.), and a BAL was performed by cannulating the trachea and perfusing the lungs with a volume of 10 mL of saline. The recovered BAL fluid (BALF) was centrifuged (250g, 10 min, and 4 °C), and the cell-free supernatant was used for biochemical measurements [lactate dehydrogenase (LDH) activity, total proteins, TNF-α, and IL-1β]. The cell pellets were resuspended in saline and used to determine total and differential cell numbers. Leukocytes were differentiated on cyt centrifuge preparations fixed in methanol and stained with Diff Quick (Dave, Brussels, Belgium).

The lavaged lungs were then perfused via the right ventricle, excised, and placed into a Falcon tube (Becton-Dickinson) chilled on ice to which 18 mL of phosphate-buffered saline (PBS) was added. The content of each tube was then homogenized using an Ultra-Turrax T25 homogenizer (Janke and Kunkel, Brussels, Belgium) for 1 min to measure the hydroxyproline lung content.

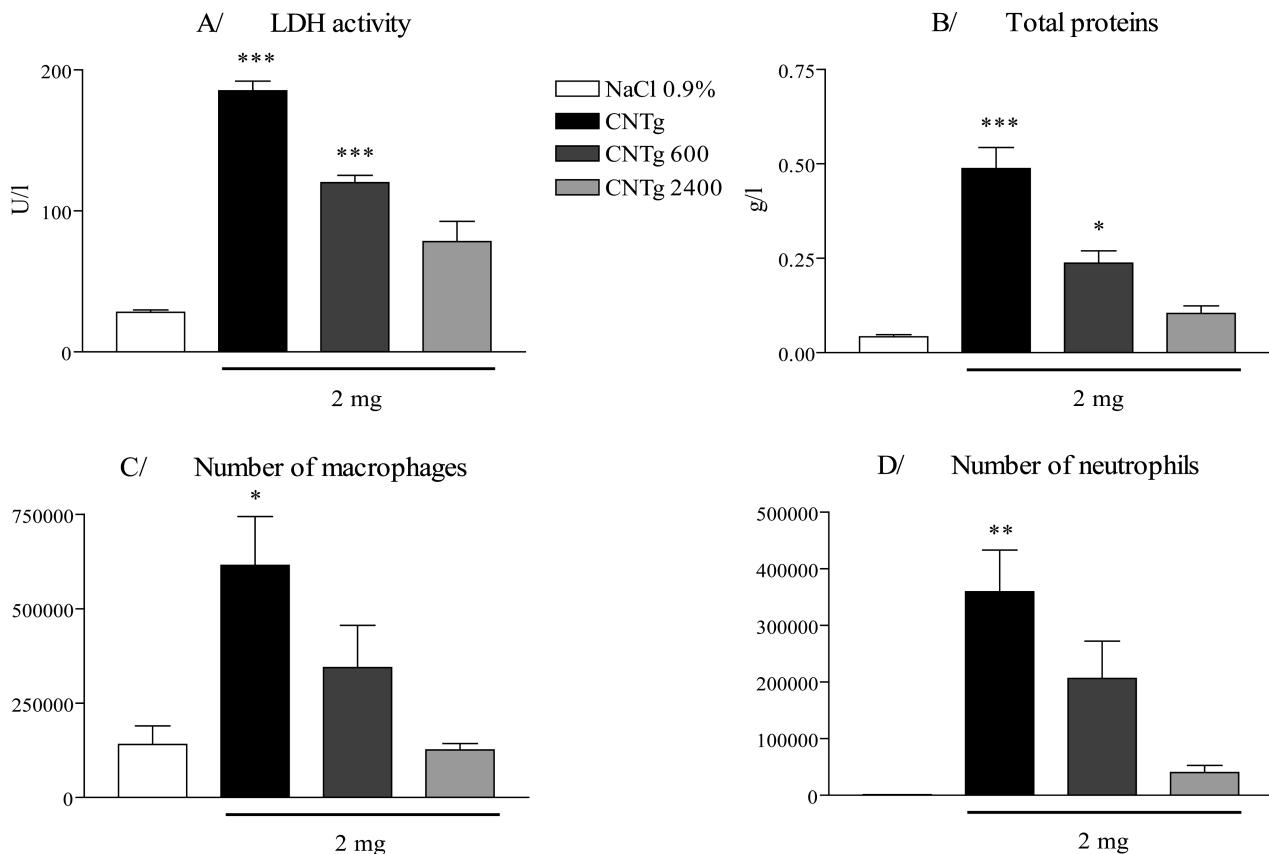
**Biochemical Assays.** The LDH activity was measured by following the reduction of NAD<sup>+</sup> at 340 nm (Technicon RA systems, Bayer Diagnostics, Domont, France). The total protein concentration was estimated spectrophotometrically at 600 nm after complexation with molybdate pyrogallol red (15). IL-1β and TNF-α concentrations in BALF were measured by ELISA (detection limit 5 and 7 pg/mL, respectively) according to the manufacturer's instructions (Pharmingen, BD Biosciences, San Diego, CA). BALFs were concentrated up to eight times (Amicon Ultra-15, Millipore, United States) before cytokine measurement.

**Collagen Assays.** Collagen deposition was estimated by measuring the hydroxyproline content by high-performance liquid chromatography analysis in lung homogenates hydrolyzed in 6 N HCl overnight at 110 °C (25).

**Histopathology.** A separate set of animals was euthanized 2 months after administration of CNT for histopathological analysis. The lungs were excised and inflated with 6 mL of 3.7% formaldehyde solution. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome for light microscopic examination.

**Genotoxicity. Cell Cultures.** Immortalized RLEs were kindly provided by Dr C. Albrecht (Heinrich-Heine-University Düsseldorf, Germany) and cultured in Ham's F 12 medium containing 5% fetal calf serum (FCS), 1% penicillin, 1% streptomycin, and 1% glutamine. The cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. For cell exposure, CNTs were dispersed in culture medium without Tween 80.

**Cytokinesis-Block Micronucleus Assay.** The frequency of micronuclei was assessed with the *in vitro* cytokinesis-block assay. RLE cells (cell cycle length, 12 h) were seeded into 25 cm<sup>2</sup> flasks and allowed to attach for 12 h. After attachment, the medium was replaced with fresh medium containing CNT. After 3 h, 2.5 µg cytochalasin B/mL culture was added. The



**Figure 1.** Short-term lung response 3 days after i.t. administration of CNT<sub>g</sub>, CNT<sub>g600</sub>, CNT<sub>g2400</sub> (all 2 mg/animal), or saline (0.9% NaCl containing 1% Tween) in Wistar rats. Levels of lactate dehydrogenase (LDH, A), total proteins (B), number of macrophages (C), and neutrophils (D) were measured in bronchoalveolar lavage fluid. Each bar represents the mean  $\pm$  SEM of five observations. \* $P$  < 0.05, \*\* $P$  < 0.01, and \*\*\* $P$  < 0.001 denote significant differences between mean values measured in the indicated group as compared to saline, as analyzed by the Student–Newman–Keuls multiple comparison test.

RLE cells were harvested by trypsinisation after 24 h of exposure. Then, they were spotted directly onto slides using a cytocentrifuge (Shandon, Pittsburgh, PA) at 700 rpm for 5 min and immediately fixed with 100% methanol (20 min) and stained with 5% Giemsa (Merck, Darmstadt, Germany) in Sorønsen buffer, pH 6.8 (Prosan, Gent, Belgium), for 20 min.

**Micronucleus Analysis.** The slides were coded and analyzed on a Zeiss transmission light microscope at a magnification of 1000 $\times$ . The micronucleus analysis was described previously (26). In brief, two cultures per concentration of the test substance were analyzed; 1000 binucleated cells (CB) were examined per culture for the presence of one, two, or more micronuclei (MN) and expressed per thousand CB (MNCB). The scoring criteria included round or oval-shaped micronuclei with no connection to the main nucleus, a size between 1/16 and 1/3, and similar staining characteristics of the main nucleus. From the data of the micronucleus analysis, the cytokinesis-block proliferation index (CPBI) (27) was calculated as follows: CPBI = number mononucleated cells + 2  $\times$  number binucleated cells + 3  $\times$  number multinucleated cells/total number of cells.

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

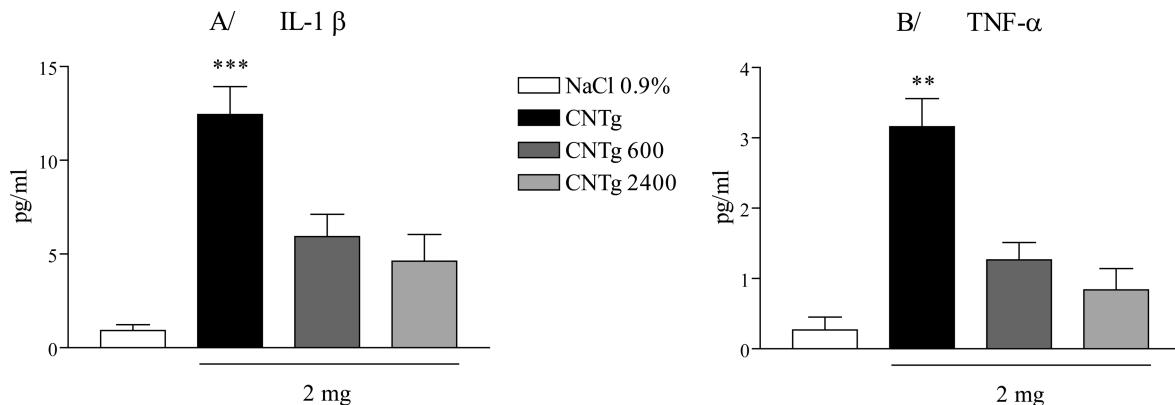
## Results

**Short-Term Lung Response.** We first tested whether heating the particles modified their lung toxicity. We examined the short-term lung response by measuring different markers (LDH activity, total protein, and cellular parameters) in BALF, 3 days

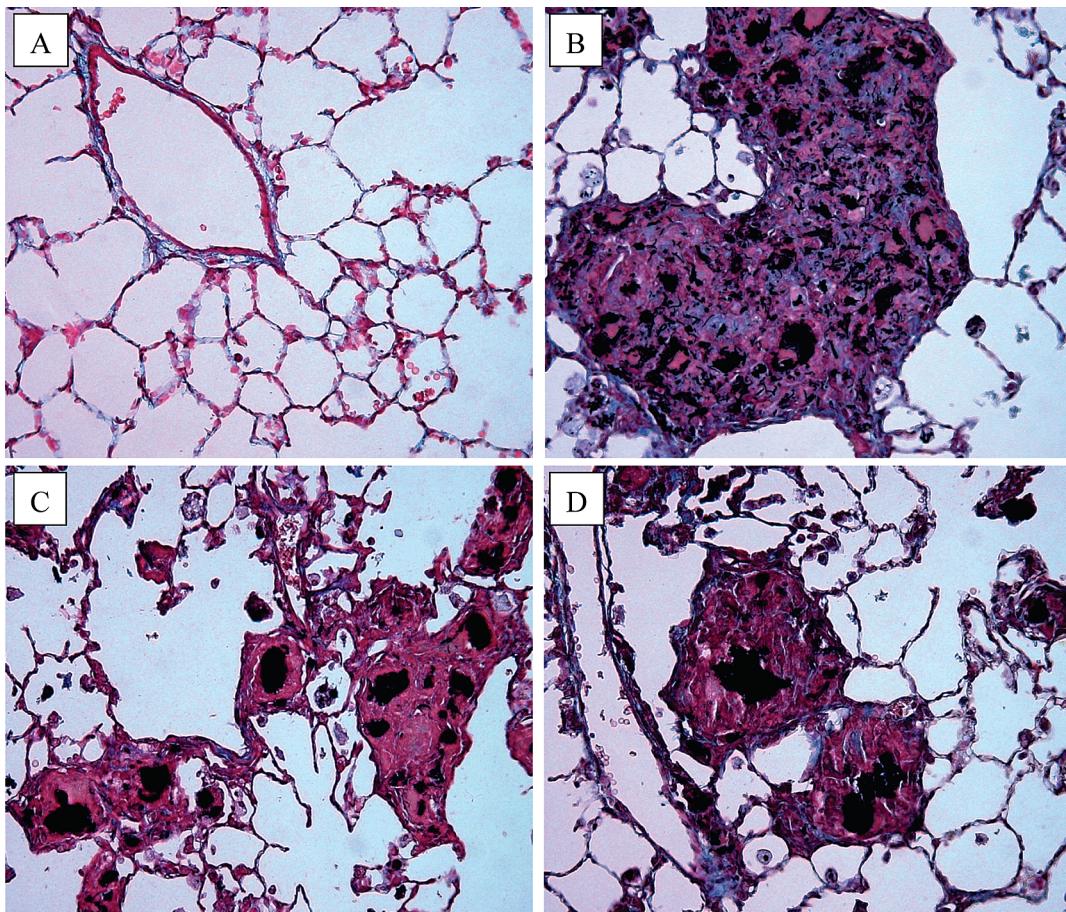
after administration. The dose used was selected from previous studies, which indicated clear lung inflammatory responses in rats after instillation of 2 mg CNT<sub>g</sub>/rat (1). LDH activity was significantly increased as compared to controls after exposure to CNT<sub>g</sub> and CNT<sub>g600</sub> but not CNT<sub>g2400</sub> (Figure 1A). LDH activity was, however, less marked after administration of CNT<sub>g600</sub> than CNT<sub>g</sub>. A similar trend was observed with the protein concentration in BAL fluid, which is considered to reflect alveolocapillary permeability, denoting again that CNT<sub>g2400</sub> was much less toxic to the lung (Figure 1B). Accumulation of both macrophages and neutrophils in the lung was also evident after treatment with CNT<sub>g</sub> (Figure 1C,D). Again, we noted a reduced cellular accumulation as the temperature used to heat CNT<sub>g</sub> increased; the animals treated with CNT<sub>g2400</sub> showed almost no cellular accumulation. The production of IL-1 $\beta$  and TNF- $\alpha$ , two pro-inflammatory cytokines, was also assessed (Figure 2). We observed that the BAL levels of IL-1 $\beta$  and TNF- $\alpha$  were significantly increased after instillation of CNT<sub>g</sub> but not after CNT<sub>g600</sub> or CNT<sub>g2400</sub> (Figure 2A,B).

We concluded at this stage that the acute inflammatory response to CNT decreased upon heating, possibly resulting from modifications of the CNT physicochemical features. When considering the possible association with modifications induced by thermal treatments, the decrease in acute lung toxicity paralleled better the reduction in structural defects than changes in metallic residues.

**Long-term lung response.** We previously showed that CNT<sub>g</sub> induced major long-term reactions in the rat lung, characterized by alveolitis and well-organized collagen-rich granulomas (1). We examined whether these long-term lung responses could also be modulated by modifying CNT characteristics. Sixty days after the



**Figure 2.** Wistar rats were instilled intratracheally with CNT<sub>g</sub>, CNT<sub>g</sub>600, CNT<sub>g</sub>2400 (all 2 mg/animal), or saline (0.9% NaCl containing 1% Tween). Levels of IL-1 $\beta$  (A) and TNF- $\alpha$  (B) were measured in bronchoalveolar lavage fluid 3 days after treatment. Each bar represents the mean  $\pm$  SEM of five observations. \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001 denote significant differences between mean values measured in the indicated group as compared to saline, as analyzed by the Student-Newman-Keuls multiple comparison test.

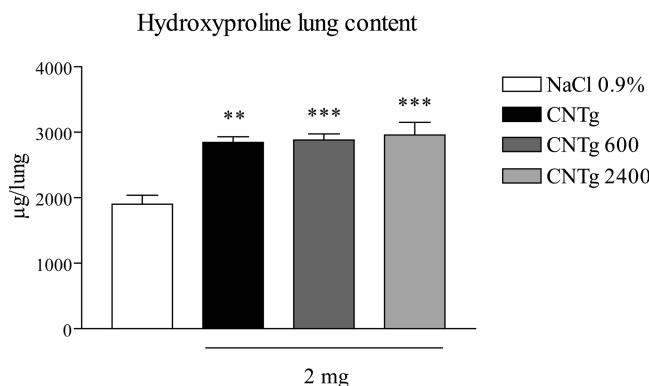


**Figure 3.** Lung sections in the lung of Wistar rats administered with a single dose of saline (0.9% NaCl containing 1% Tween; A), CNT<sub>g</sub> (B), CNT<sub>g</sub>600 (C), or CNT<sub>g</sub>2400 (D; all 2 mg/animal) 60 days prior. The panels show Masson trichrome stainings. Original magnification was 20 $\times$ .

intratracheal administration of CNT in rats, the long-term lung responses were assessed by histological analysis. Masson's trichrome and hemalum-eosin staining revealed, as previously observed (1), that the administration of ground CNT (2 mg CNT<sub>g</sub>/rat; Figure 3B) induced the formation of numerous granulomas containing collagen, which were well-dispersed in the parenchyma. These findings were reflected by the measurement of different markers of collagen deposition (hydroxyproline, Figure 4; fibronectin and soluble collagen, data not shown). In rats treated with heated CNT (CNT<sub>g</sub>600 and CNT<sub>g</sub>2400), the granulomas were smaller (Figure 3C,D). The increased hydroxyproline lung content observed following exposure to the heated CNT was of the same amplitude than after administration of CNT<sub>g</sub> (Figure 4). We concluded that

the long-term lung response induced by CNT was modulated by the modifications of CNT physicochemical features, although to a lesser extent than the acute response.

**Genotoxic Response.** To examine whether CNT modifications affected their genotoxic activity, we used the *in vitro* cytokinesis-block MN assay (27) with RLE exposed to 25  $\mu$ g of the different CNT preparations per mL. This dose was selected from a previous study, which indicated a clear genotoxic response (7). Figure 5A shows that the frequency of MNCB induced by CNT<sub>g</sub> or CNT<sub>g</sub>600 was significantly higher than in control cells. Exposure to CNT<sub>g</sub>2400 did not induce any significant increase in the MNCB frequency. We did not observe any effect of the different types of CNT on the proliferation



**Figure 4.** Long-term lung response 60 days after i.t. treatment of Wistar rats with CNT<sub>g</sub>, CNT<sub>g600</sub>, CNT<sub>g2400</sub> (all 2 mg/animal), or saline (0.9% NaCl containing 1% Tween). Hydroxyproline content was measured in lung homogenates. Each bar represents the mean  $\pm$  SEM of five observations. \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001 denote a significant difference between the mean values measured in the indicated groups as compared to saline, as analyzed by the Student–Newman–Keuls multiple comparison test.

indices (Figure 5B). Here, the reduction in genotoxicity went better paired with the decrease of structural defects induced by the thermal treatments than with the metal content.

**Structural Defects Appear as the Main Determinants of CNT Toxicity.** From the data obtained above, we suspected that structural defects rather than metallic residues played a major role in CNT toxicity. To test this hypothesis, an additional CNT preparation was tested (CNT<sub>2400g</sub>). In a second experiment, ground CNT (CNT<sub>g</sub>/containing defects), ground CNT heated at 2400 °C (CNT<sub>g2400</sub>/elimination of metals and ablation of defects), and heated CNT that were subsequently ground (CNT<sub>2400g</sub>/introduction of defects in a metal-deprived carbon framework) were administered intratracheally in rats to examine the same end points at the same time points.

We first studied the short-term lung response and found that the different BAL parameters (LDH activity, total protein, and cellular parameters) increased upon grinding (CNT<sub>g</sub>, CNT<sub>2400g</sub>) (Figure 6), supporting the hypothesis that surface defects are involved for a major part in CNT lung toxicity. The histological study revealed that after 60 days the particles accumulated in alveolar lumens where they were phagocytosed by alveolar macrophages or multinuclear giant cells. In animals treated with CNT<sub>2400g</sub>, these cells merged to form granulomas similar to those induced by CNT<sub>g2400</sub> (data not shown). No difference in the hydroxyproline lung content was observed between the animals exposed to CNT<sub>g</sub> or CNT<sub>g2400</sub> (data not shown). Finally, the results of the in vitro cytokinesis-block micronucleus assay on RLE indicated that while the frequency of MNBC induced by CNT<sub>g</sub> or CNT<sub>2400g</sub> was significantly higher than in control cells, exposure to CNT<sub>g2400</sub> did not induce any significant increase in the MNBC frequency (Figure 7A). We did not observe any effect of the different types of CNT on the proliferation index (Figure 7B).

All of these results indicate that the acute toxicity and genotoxicity of CNT are, for a major part, due to the presence of structural defects in CNT.

## Discussion

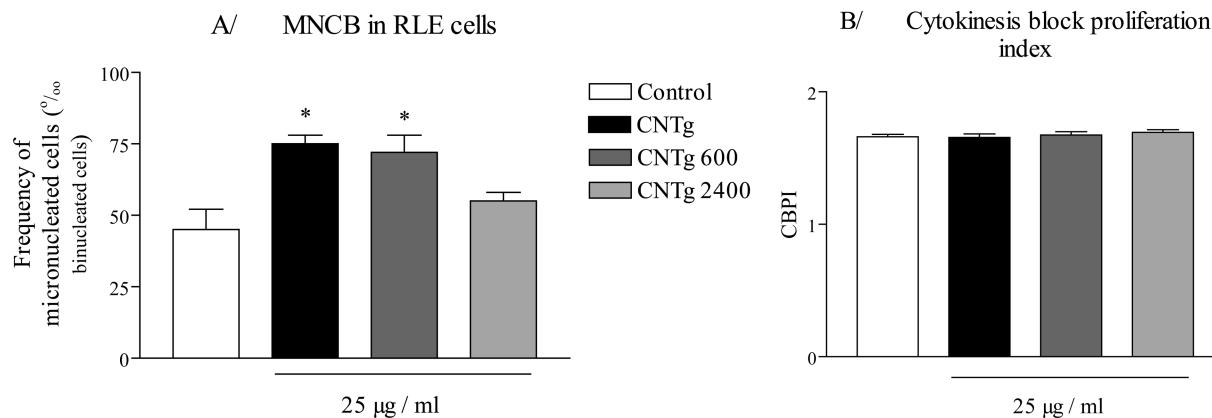
Several physicochemical features of CNT (metallic contaminants, size, degree of oxidation, or hydrophilic properties) have been found to modulate their toxicity (11, 14, 20–22), but the influence of structural defects and hydrophilic properties, as well as the presence of metal clusters within CNTs, had not yet been

explored systematically. We were able here, for the first time, to show that the acute lung response to CNT as well as their genotoxic potential can mainly be ascribed to the presence of structural defects in CNT.

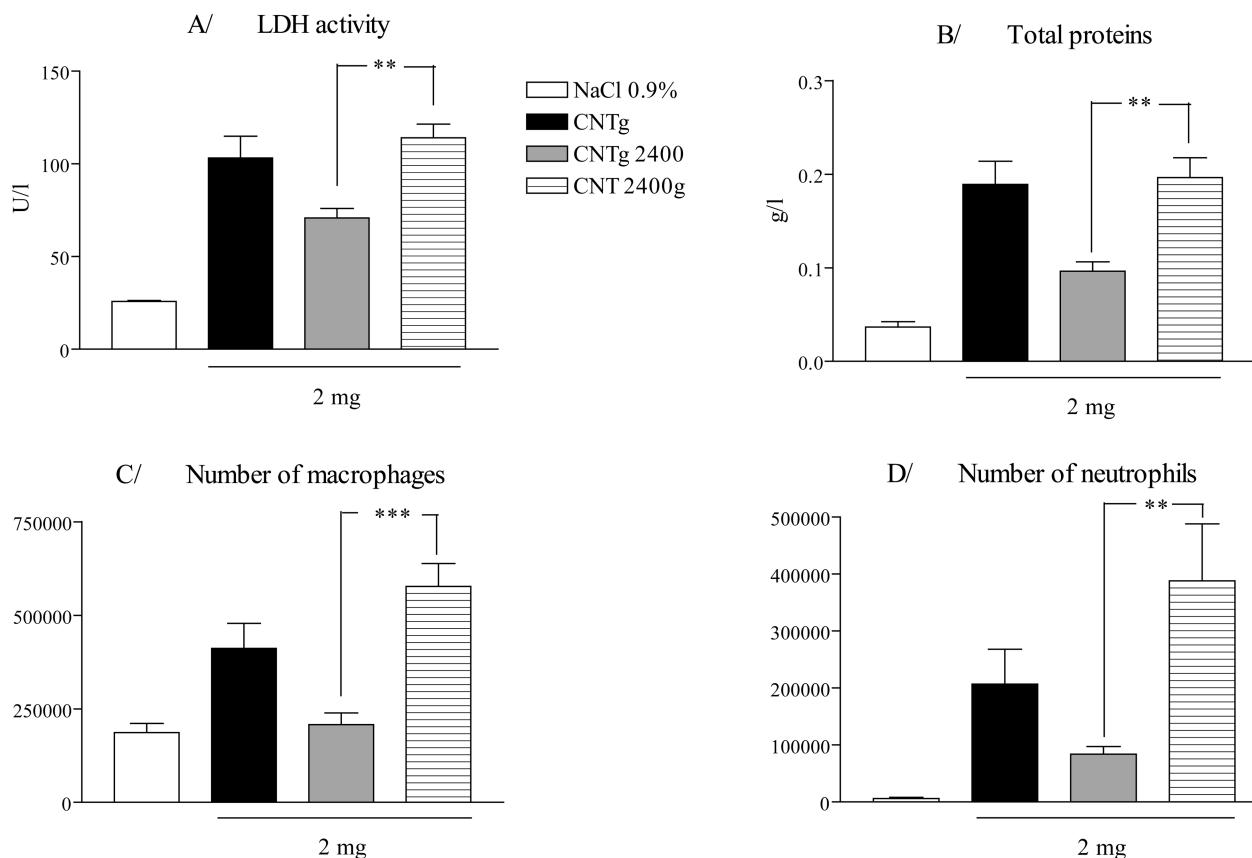
We modulated CNT toxicity by specifically modifying the metallic content and/or the structural defects in the same original material through thermal treatment and grinding (23). High temperature treatment has already been shown to eliminate surface defects and metallic contaminants of CNT without altering their intrinsic structure (28). The results obtained with the modified CNT indicate that the acute lung reactions as well as the in vitro genotoxicity albeit with some differences are all significantly reduced upon heating but restored upon grinding of the metal-deprived material. The fact that the long-term lung response was not or less modulated by the same treatments may reflect a progressive passivation of the CNT with the time of residence in the lungs (e.g., through the deposition of endogenous proteins), which may gradually reduce differences between the various CNT tested. Another explanation might be that the fibrotic process induced by CNT is determined by other physicochemical parameters than acute inflammation and therefore less or not influenced by the modified parameters. It might also be considered that the long-term response to CNT develops independently of the acute response and might reflect an aspecific foreign-body reaction or a chronic process such as observed in the rat with several low toxicity insoluble particles including TiO<sub>2</sub>, carbon black, or diesel exhaust particles (29). Whatever the exact explanation, this observation has important implications for the testing strategy of new nanomaterials because the results of acute toxicity tests may not be predictive of the long-term lung response (CNT<sub>g2400</sub> did not induce an acute reaction but elicited the formation of granulomas after 2 months).

The modulation of the acute lung response observed with the modified CNT could theoretically be ascribed to the presence of three major factors: (i) adsorbed molecules such as polycyclic aromatic hydrocarbons (PAH), (ii) metals residues, and (iii) structural defects. It is well-known that carbon-based nanomaterials present an adsorptive nature characterized by some interaction or binding of a variety of organic or endogenous molecules (30–33). The adsorbed components may hide the toxic effects of CNT or may interact to generate a toxic response. On the basis of the grid of the sample characteristics reported in the companion paper (23), we can exclude a major contribution of PAH in the toxic response. If present on the original CNT<sub>g</sub>, these compounds would have been completely evaporated already at 600 °C and could, therefore, not explain the difference in response observed between CNT<sub>g600</sub>, CNT<sub>g2400</sub>, and CNT<sub>2400g</sub>.

Some published data indicate that the presence of bioavailable metallic contaminants plays a role in CNT toxicity (14, 20), but the contribution of the entrapped metallic residues had never been investigated. In this paper, we used an intermediate temperature (600 °C) that allowed us to partially reduce oxidized functionalities/metals at the surface of CNT but did not eliminate metal cluster residues (23). Interestingly, this material showed a reduced acute toxicity as compared to the original material but remained more toxic than the CNT heated at 2400 °C, which did not contain any defects or metal-entrapped clusters. We cannot totally exclude a role of metallic residues to explain the overall modulation of CNT toxicity, even if the data rather suggest a major role of defective sites reactivity. It is well-known that transition metals may contribute to particle-induced ROS generation through mechanisms such as a Fenton reaction, leading, together with cell-derived ROS, to oxidative stress, which is crucial for the development of particle-related responses (34, 35). However, we demonstrated that the same modified



**Figure 5.** Frequency of micronucleated binucleated cells (per 1000 binucleated cells) (A) and CPBI (B) in RLE cells incubated with CNT<sub>g</sub>, CNT<sub>g600</sub>, or CNT<sub>g2400</sub> (all 25 µg/mL) as assessed with the cytokinesis-block MN assay. The values represent the means of two experiments ± SEM. For each experiment, duplicates were performed. \*P < 0.05 denotes a significant difference between the mean values measured in the indicated group as compared to control (culture medium), as analyzed by the Student–Newman–Keuls multiple comparison test.

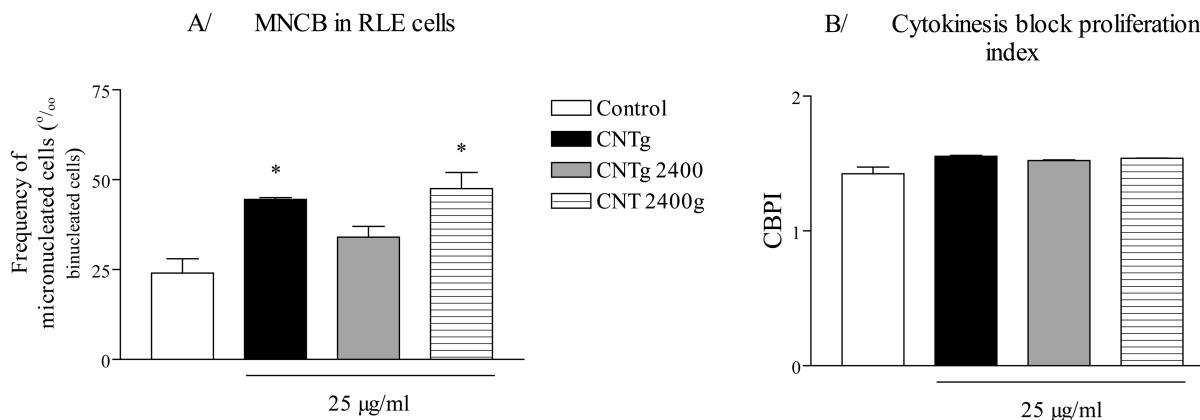


**Figure 6.** Short-term lung response 3 days after i.t. treatment of Wistar rat with CNT<sub>g</sub>, CNT<sub>g2400</sub>, CNT<sub>2400g</sub> (all 2 mg/animal), or saline (0.9% NaCl containing 1% Tween). Levels of lactate dehydrogenase (LDH, A), total proteins (B), number of macrophages (C), and neutrophils (D) were measured in bronchoalveolar lavage fluid. Each bar represents the mean ± SEM of five observations. \*\*P < 0.01 and \*\*\*P < 0.001 denote significant differences between mean values measured in the indicated group, as analyzed by the Student–Newman–Keuls multiple comparison test.

CNT failed to generate any form of oxygen radical in an acellular model but, in contrast, exhibited, except CNT<sub>g2400</sub> (23), a remarkable radical scavenging capacity (36). Whether and how this quenching activity is involved in acute toxicity remains unclear, and additional studies will be needed to further elucidate the toxic mechanism of CNTs. This observation lends, however, further support to the hypothesis that metallic clusters are not the main source of toxicity in the present study.

It is known that surface properties, including oxygenated functions, of a particle (e.g., silica and asbestos) are an important determinant to elicit the production of oxidants and toxicity (37, 38). It has been demonstrated in a human lung epithelial cell line (A549) that surface modification of quartz particles

either with aluminum lactate or with polyvinyl-pyridine-N-oxide inhibits its hydroxyl radical-generating capacity, cytotoxicity, particle endocytosis, and oxidative DNA damage as well as neutrophil burst activation (39). These authors also highlighted with the same modified particles that the surface properties of quartz appear crucial for the uptake by macrophages in vitro and that it can play a role in pulmonary inflammation and lung clearance in vivo (40). Some of us have previously reported that thermally modified cristobalite dusts were not only less effective in inhibiting cell proliferation but also did not damage the cell membrane integrity in comparison with the pristine material (41). As thermal treatment did not affect overall particle crystallinity and morphology, it was postulated that the varia-



**Figure 7.** Frequency of micronucleated binucleated cells (per 1000 binucleated cells) (A) and CPBI (B) in RLE cells incubated with CNT<sub>g</sub>, CNT<sub>g2400</sub>, or CNT<sub>g2400g</sub> (all 25 µg/mL) as evaluated with the cytokinesis-block MN assay. The values represent the means of two experiments ± SEM. For each experiment, duplicates were performed. \*P < 0.05 denotes a significant difference between the mean values measured in the indicated group in comparison with the control, as analyzed by the Student–Newman–Keuls multiple comparison test.

tions in cytotoxicity had to be ascribed to some modifications at the surface level, reflecting the conversion of silanol into siloxane functions (dehydroxylation) upon heating of the particles. Because, in the present study, the various biological responses elicited went paired with the modulation of surface oxygen and the defective sites, we confirm here that structural defects, probably exposed at the surface, may mainly contribute to the toxicity of CNTs. We confirmed by Raman spectroscopy the presence of imperfections in the carbon framework. Ground CNTs (CNT<sub>g</sub> as well as CNT<sub>g2400g</sub>) exhibited a higher  $I_D/I_G$  ratio than heated CNTs (CNT<sub>g2400</sub>), reflecting an increased structural disturbances (23). The role of the abundance of defects is also confirmed by microcalorimetry as the heat of adsorption of water vapor showed that heating CNTs at high temperature increased hydrophobicity and fully eliminated hydrophilic sites. Conversely, grinding the material lead to the generation of defects, thus creating sites interacting with water molecules, suggesting the formation of defects at the surface, which are subject to oxidation or the opening of the internal pores where water may condense. It is already known that there is a relationship between the extent of hydrophilic sites and the interaction with various biological macromolecules as the degree of hydrophobicity of the surface interferes notably with cell surface adhesion and adsorption (42). In general, hydrophilic mineral particles show the strongest interaction with biological molecules (18). Those findings support our hypothesis upon which surface defects are key parameters determining acute CNT toxicity.

Interestingly, while all data reported here show a minimum acute toxicity for the sample heated at 2400 °C, a comparison among the intensities of the responses to the four samples examined reveals at least three different behaviors: (i) Genotoxicity was not modified at 600 °C, whereas it was fully eliminated at 2400 °C; (ii) long-term effects did not show remarkable differences among the samples, except for smaller granulomas with heated samples; and (iii) all of the inflammatory response parameters examined showed a decreased effect at 600 °C but even more at 2400 °C. Interestingly, acute cell recruitment showed large differences between the two heated samples, whereas cytokine release showed already a substantial decrement in their production after treatment at 600 °C, only slightly further reduced with the 2400 °C heated sample.

On the basis of the grid of physicochemical features reported in the companion paper (23), we may hypothesize the following: Genotoxicity appears only related to the structural defects evidenced by Raman spectroscopy. These structural defects are closely related with the capacity of the CNTs to scavenge ROS, which may,

therefore, be critical for their genotoxic potential. This would imply a completely different mode of action than other genotoxic fibers such as asbestos for which a paradigm based on the generation of ROS is generally admitted to account for their genotoxic potential. Various factors contribute to the acute inflammatory response, including framework defects and active metals. Finally, the long-term effects could be governed by factors different from those causing the acute inflammatory response.

In conclusion, all of the data obtained in this study clearly indicate that defects in the structure of CNT constitute one of the main physicochemical features determining the acute toxic potential of CNT. This finding has important implications for hazard identification and also indicates that it is possible to abrogate, at least in part, the acute toxic potential of CNT by eliminating their surface defects.

While our paper was under review, another study was published indicating that long (>20 µm) MWCNTs have the potential to induce an acute inflammatory reaction when injected in the peritoneal cavity of mice. This acute inflammatory response was similar to that induced by long amosite fibers, suggesting that long MWCNTs may exert an asbestos-like activity (43). We show here that short (0.7 µm) MWCNTs are also inflammogenic and that, besides length, the presence of defects in the structure of MWCNT is another important determinant of their toxicity. Our results also indicate that the severity of the acute inflammatory reaction induced by CNT in the lung may not be predictive of the long-term response.

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