Additional Histopathologic Examination of the Lungs from a 3-Month Inhalation Toxicity Study with Multiwall Carbon Nanotubes in Rats

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For hazard assessment of multiwalled carbon nanotubes (MWCNTs), a 90-day inhalation toxicity study has been performed with Nanocyl NC 7000 in accordance with OECD 413 test guideline. MWCNTs produced no systemic toxicity. However, increased lung weights, multifocal granulomatous inflammation, diffuse histiocytic and neutrophilic infiltrates, and intra-alveolar lipoproteinosis were observed in lung and lung-associated lymph nodes at 0.5 and 2.5 mg/m³. Additional investigations of the lungs were performed, including special stains for examination of connective tissue, and electron microscopy was performed to determine the location of the MWCNTs. The alveolar walls revealed no increase of collagen fibers, whereas within the microgranulomas a slight increase of collagen fibers was observed. The pleura did not reveal any increase in collagen fibers. Only a slight increase in reticulin fibers in the alveolar walls in animals of the 0.5 and 2.5 mg/m³ concentration group was noted. In the 0.1 mg/m³ group, the only animal revealing minimal granulomas exhibited a minimal increase in collagen within the granuloma. No increase in reticulin was observed. Electron microscopy demonstrated entangled MWCNTs within alveolar macrophages. Occasionally electron dense particles/detritus were observed within membrane-bound vesicles (interpreted as phagosomes), which could represent degraded MWCNTs. If so, MWCNTs were degradable by alveolar macrophages and not persistent within the lung. Inhalation of MWCNTs caused granulomatous inflammation within the lung parenchyma but not the pleura in any of the concentration groups. Thus, there are some similarities to effects caused by inhaled asbestos, but the hallmark effects, namely pleural inflammation and/or fibrosis leading to mesotheliomas, are absent.

Key Words: MWCNT; 90-day inhalation study; fibrosis; lung; pleura; asbestos.

Many of the properties that make carbon nanotubes (CNTs) remarkable for engineering applications have also raised concern about their biocompatibility, especially in the lung. CNTs have fiber-like characteristics in terms of their elongated shape, dimensions, and aspect ratio. As particles with at least one dimension of less than 100 nm, they correspond to the high-aspect-ratio nanoparticles (Tran et al., 2008).

Muller et al. (2005) investigated the effects of intact or milled multiwalled carbon nanotubes (MWCNTs) by intratracheal instillation to female Sprague Dawley rats (0.5, 2, and 5 mg/animal). On examination of bronchoalveolar lavage, they found dose-dependent, persistent (60 days) inflammation and granuloma formation. Moreover, dose-related pulmonary fibrosis was diagnosed by measuring hydroxyproline in the lung tissue. When compared with asbestos and carbon black, the severity of the inflammation induced by MWCNTs was intermediate. However, these effects were not observed by Mitchell et al. (2007) after whole-body inhalation exposure of mice to 0.3–5 mg/m³ MWCNTs (6 h/day for 7 or 14 days).

Shvedova et al. (2005) reported diffuse interstitial fibrosis in C57Bl/6 mice lungs after pharyngeal aspiration of 10, 20, or 40 µg single-walled carbon nanotubes (SWCNT) per mouse. The animals were sacrificed 1, 3, 7, 28, and 60 days following exposure. In the animals exposed to 20 or 40 µg SWCNTs a significant increase in thickness of the alveolar walls and an increase in connective tissue were determined; such changes were not found in animals treated with ultrafine carbon black or SiO₂. Porter et al. (2010) exposed mice by pharyngeal aspiration to 10, 20, 40, or 80 µg MWCNT per mouse. When examined 7, 28, and 56 days postexposure, the animals revealed granulomatous to pyogranulomatous inflammation in the lung. Fibrosis accompanied the organizing pulmonary inflammation and persisted until postexposure day 56.

Mutlu et al. (2010) compared the effects of intratracheal instilled asbestos fibers with SWCNTs in mice. After instillation of asbestos fibers, dose-dependent fibrosis around medium- and small-sized airways was observed. Mice treated with SWCNTs revealed chronic inflammation in mid- to large-sized bronchi, but neither fibrosis nor an increase in collagen content within the lungs was detected.

Summarizing the available data, the majority of the work published has demonstrated that exposure to CNTs could present a potential health hazard. Most studies to date have used intratracheal instillation, this route of administration being...
commonly used to assess the hazard potential of dust in the lungs. Both intratracheal instillation and pharyngeal aspiration assume that the particles can reach the lung in certain quantities, but do not take into consideration dust deposition behavior and consequently its possible effects on the upper respiratory tract. Most importantly they do not reflect the atmospheric concentration of potential exposure to workers. Data obtained by these administration routes may be useful to identify potential hazards but are insufficient for proper risk assessment.

Therefore, we developed a test system, which is capable of reliably generating dust aerosols with nanomaterials (Ma-Hock et al., 2007), and performed inhalation toxicity studies with a test design according to the OECD 413 test guideline (OECD, 1981), with additional exposure assessments based on nanomaterial-specific analytical data. The results of this study have already been published (Ma-Hock et al., 2009). Summarizing the findings, inhalation exposure to MWCNT for 90 days did not lead to any observed systemic toxicity. However, increased lung weights, pronounced multifocal granulomatous inflammation, diffuse histiocytic and neutrophilic inflammation, and intra-alveolar lipoproteinosis were observed in the lung at 0.5 and 2.5 mg/m³. These effects were accompanied by slight blood neutrophilia at 2.5 mg/m³. The incidence and severity of the effects were concentration related. At 0.1 mg/m³, there was still a minimal multifocal granulomatous inflammation in the lung and in lung-associated lymph nodes. The focus of this additional investigation was to elucidate whether treatment with MWCNTs over a period of 3 months could cause lung and/or pleura fibrosis in rats.

In a 13-week repeated exposure inhalation study performed with Wistar rats, Pauluhn (2010) concluded that the observed MWCNT-induced pulmonary toxicity was more likely due to the “poorly soluble particle overload paradigm” rather than the “fiber” paradigm. Warheit (2009) regarded Ma-Hock et al. (2009) as an excellent starting barometer for studying the toxicological effects of aerosolized MWCNTs. But he also mentioned additional questions that should be addressed in future testing: (1) the sustainability of the measured responses in the respiratory tract to delineate between Quartz-type effects and transient effects; (2) a more intensively focused investigation into the potential pleural effects.

Thus, in this additional work, an attempt was made to shed light on the asbestos-like or Quartz-type effects of MWCNTs. Special stains for the investigation of connective tissue were prepared and electron microscopy performed to examine the location of the MWCNTs within the lungs and their condition (processed or unprocessed).

MATERIALS AND METHODS

Test material and characterization. The test substance, Nanocyl NC 7000 thin MWCNTs, was provided by Nanocyl S.A. (Sambreville, Belgium). The purity was 90% carbon and 10% metal oxide, of which 9.6% was aluminum oxide with traces of iron and cobalt. The tubes were 5–15 nm in diameter and 0.1–10 μm in length. The specific surface area (Brunauer Emmet Teller method) was 250–300 m²/g according to the manufacturer. For further details, please refer to Ma-Hock et al. (2009).

Study design. A 90-day inhalation toxicity study on rats with MWCNT was performed with aerosol concentrations of 0.1, 0.5, and 2.5 mg/m³. The design of the main study was based on the OECD testing guideline 413 (OECD, 1981).

A satellite group foreseen for transmission electron microscopy (TEM) (consisting of two male animals per concentration group) was run in parallel to the main group described in the preceding sentence. The satellite animals were exposed in the same way as the main group animals. At the termination of the exposure time after 90 days the animals were subjected to deep anesthesia with isoflurane (Isoflo; Essex GmbH, Munich, Germany). After opening the thorax the animals were sacrificed and fixed by whole-body perfusion using cacodylate buffer as a rinsing solution, followed by perfusion of 5% buffered glutaraldehyde as fixation solution. From each of the five lung lobes, several samples of non-subpleural tissue were taken for TEM. To this end, the tissue samples of the lungs were refixed with 2% buffered osmium tetroxide. They were then embedded in Epon mixture (Polysciences Europe GmbH, Eppelheim, Germany) and further processed to semithin and ultrathin sections. The semithin sections (500 nm thick) were stained with azure-methylene blue-basic-fuchsin (Ambf). Based on the evaluation of the semithin sections, five ultrathin sections of 40–70 nm thickness per cube were cut. When preparing the ultrathin sections in the areas where the MWCNTs were located, notches appeared due to the extreme hardness of the MWCNTs that damaged the diamond knife. This led to drag marks in every section where MWCNTs were present.

Further to the published results, additional slides of the lung sections were stained with modified Masson’s trichrome stain according to Ladewig to determine any increase in collagen fibers and Gomori stain to detect any reticulin fibers. Trichrome stain, which stains collagen blue, serves to detect an increased amount of collagen (fibrosis) (Titford, 2009). With the Gomori methenamine silver stain, reticulin fibers appear as delicate, dark staining fibrils and distortances in the reticulin pattern can be detected that occur, for example, in lung fibrosis (Titford, 2009). For assessment of increase of collagen/reticulin fibers, a semiquantitative grading system from minimal (grade 1) to excessive (grade 5) was used. The first evaluation was followed by an internal peer review, both evaluations performed by well-experienced, board-certified toxicopathologists.

RESULTS

The results of clinical observations, clinical chemistry, hematology, gross pathology and histopathology (H&E stained sections) have already been published (Ma-Hock et al., 2009). This work focuses on the additional morphological evaluation performed on the special stained sections (main group animals) and ultrathin sections (satellite animals) that were obtained from the lung tissue.

Observation of Collagen Elements with Masson’s Trichrome Stain (Ladewig)

To detect connective tissue, especially mature collagen fibers, the modified Masson’s trichrome stain according to Ladewig was performed on all lung sections of all treated and control animals. The stained slides (one section of each lung lobe) were examined by light microscopy. When compared to control animals (Fig. 1), there was no increase of connective tissue in the alveolar wall or the pleura (Fig. 2). Only in areas of granulomatous inflammation a minimal focal increase of collagen was observed (Fig. 3). Minimal (grade 1) was scored when the collagen was approximately double the size of an
unaltered alveolar wall. As an increase of collagen was noted
in granulomas only, the scoring of collagen increase was linked
to the granulomatous inflammation reported in Ma-Hock et al.
(2009). Here the incidence was 0/1/10/10 (control group/low
concentration group/mid concentration group/high concentra-
tion group) with an increasing severity from minimal (grade
1) to moderate (grade 3). However, the amount of collagen
within the granulomas did not increase with increasing num-
bers and severity of granulomas.

**Observation of Reticulin Fibers with Gomori Stain**

Reticulin fibers support tissues and are one of the main
structural elements of the alveolar wall. The Gomori stain was
performed on all lung sections of all treated and control animals.
The stained slides (one section of each lung lobe) were exam-
ined by light microscopy. Control animals (Fig. 4) revealed very
delicate, single black filaments within the alveolar wall that were not continuous. In animals exposed to 0.5 and 2.5 mg/
ml MWCNTs, a minimal to moderate increase in reticulin fibers was observed in a dose-response-related manner (Table 1).
Grade 1 was selected when there was a continuous pattern of
reticulin fibers, occasionally showing two fibers in one loca-
tion. The severity rose to grade 3 when the thickness of the
single fibers increased, and up to five strands of reticulin fibers
were present in one location. Grade 2 was used for findings

![FIG. 1. Control group, lung, normal collagen content of pleura and septae. Masson trichrome stain according to Ladewig.](image)

![FIG. 2. High concentration group (2.5 mg/ml), lung, black particles (MWCNTs) lying free within the alveolar space (arrow) adjacent to blue-staining clusters (lipoproteinosis) or within subpleural located alveolar macrophages. No increase in collagen of the pleura (arrow head). Masson trichrome stain according to Ladewig. This figure can be viewed in color online.](image)

![FIG. 3. High concentration group (2.5 mg/ml), lung, multifocal granulomas within the septal walls composed of alveolar macrophages containing black particles (MWCNTs) within their cytoplasm. A single neutrophil is located in the alveolar lumen (arrow). There is an increase of blue-staining fibers within the alveolar septae (collagen). Masson trichrome stain according to Ladewig. This figure can be viewed in color online.](image)

![FIG. 4. Control, lung, normal content of delicate, undulated, dark staining fibers (reticulin fibers) within the alveolar septae. Gomori stain.](image)
that were in between. This increase was diffusely distributed all over the lungs but was slightly more pronounced in the deep parenchymal region, especially at the bronchiolar-alveolar transition (Fig. 5). Furthermore, female animals seemed to be slightly more affected, as they showed a higher severity compared to males (Table 1). The alveolar wall itself did not appear to be thicker when compared to control animals (based on H&E section, data not shown).

Identification of MWCNTs with TEM

To locate the MWCNTs within the lung, TEM was performed on ultrathin sections of the 2.5 mg/m³ concentration group. Within alveolar macrophages large (> 2 µm), electron-dense clews of entangled fibers (MWCNTs, arrow) within membrane-bound structures (phagosomes) located in the cytoplasm. TEM.

Table 1

<table>
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<tr>
<th>Increased Reticulin Fiber Index in the Alveolar Wall</th>
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<tr>
<td><strong>Male</strong></td>
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<tr>
<td>Number of animals</td>
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<td>Increased reticulin fibers</td>
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<tr>
<td><strong>Female</strong></td>
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<td>Number of animals</td>
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<td>Increased reticulin fibers</td>
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*Note. Severity grades: 1, minimal; 2, slight; 3, moderate.

aMean severity: grade × number of animals/total number of animals with findings.

DISCUSSION

This study was conducted to obtain sound data on inhalation hazard caused by MWCNTs. During the routine (H&E stained sections) investigation according to OECD 413, a multifocal granulomatous inflammation in animals of all concentration groups in a concentration-response-related manner was observed. In addition, lipoproteinosis was present in animals of the 0.5 and 2.5 mg/m³ concentration groups (Ma-Hock et al., 2009).
This study investigated whether the inhalation of the MWCNTs led to an increase of connective tissue (collagen, reticulin), which could lead to lung fibrosis. Pulmonary fibrosis is characterized by abnormal lung physiology and by the excessive production of extracellular matrix molecules (e.g., collagen).

Evaluation of the special stained slides for detection of either collagen (Masson’s trichrome according to Ladewig) or reticulin (Gomori) fibers revealed a minimal focal increase of collagen fibers within the (multi)focal granulomas only. There was no increase in collagen fibers, neither in the alveolar walls or surrounding small to middle airways, nor was plaque formation observed on the pleura. In the case of the reticulin fibers, there was a minimal to moderate diffuse increase inside the alveolar wall, which was concentration-response related in the animals of the 0.5 and 2.5 mg/m³ concentration groups. Females seem to be slightly more susceptible compared with male animals based on the slightly higher severity of findings. But also in females, the pleura was not affected. In animals of the 0.1 mg/m³ concentration group, no increase in intra-alveolar wall reticulin fibers was found.

Pauluhn (2010) observed in rats exposed to 0.4 mg/m³ or higher concentrations of Baytubes for 90 days a concentration-dependent increase of neutrophilic granulocytes and of soluble collagen in the bronchoalveolar lavage. After a recovery period of 6 months, there were no clearly significant differences between treated and control animals. This points in the direction that the effects caused by MWCNTs are reversible, although for our study this cannot be proven as no recovery group was run in parallel. With regard to the questioned sustainability of findings by Warheit (2009), only a minimal increase in severity of the findings (granulomas) was observed after 90 days of treatment.
when compared with 5 days of treatment observed in a range-finding study for the present study. In this range-finding study, animals were treated for 5 days with concentrations of 8 and 32 mg/m\(^3\) Nanocyl MWCNTs and revealed minimal to mild granulomatous inflammation, respectively. Animals treated with 2 mg/m\(^3\) MWCNTs showed no treatment-related findings (data not shown). Therefore, sustainability of the granulomas is present as they are still observable in the present study after 90 days of treatment. It would be important to investigate the further progress of these findings and whether they would persist after a recovery period.

With regard to pulmonary fibrosis caused by asbestos, Bernstein et al. (2006) exposed Wistar rats for 13 weeks to an aerosol of Brazilian chrysotile asbestos (1.3 and 3.3 mg/m\(^3\) target concentration equivalent to 3413 and 8941 fibers/cm\(^3\)). Animals of the 1.3 mg/m\(^3\) concentration group revealed after the 90-day treatment period multifocal microgranulomas occasionally with minimal fibrosis in the granulomas. The 3.3 mg/m\(^3\) concentration group animals also showed slight fibrosis at the bronchiolar-alveolar junction. There was no clear difference in animals allowed to recover for 45 or 92 days. In a 5-day inhalation study with the amphibole tremolite in rats and after a 90-day recovery period, there was an increase in severity of collagen deposition in granulomas in the lung and a progression of the fibrosis into the interstitium (Bernstein et al., 2005a). The aerosol concentrations in the tremolite study (100 fibers with a length (l) > 20 \(\mu\)m/cm\(^3\)) were lower than the 1.3 mg/m\(^3\) in the chrysotile study (Bernstein et al., 2006) and exposure was only for 5 days. This clearly shows that there are differences in the lung toxicity of different types of asbestos.

Nevertheless, there are some similarities between histopathologic findings in rat lungs exposed to MWCNTs for 90 days and rats exposed also for 90 days to chrysotile asbestos (Bernstein et al., 2006). Similar exposure concentrations of chrysotile asbestos caused similar findings as MWCNTs in the present study with formation of microgranulomas, slight fibrosis at the bronchiolar-alveolar junction, and a minimal increase in alveolar macrophages. In addition, animals exposed via aerosol to chrysotile asbestos fibers for 5 days were allowed to live for up to 1 year after cessation of treatment (Bernstein et al., 2005b). The clearance of the chrysotile fibers was investigated, and 1 year postexposure no fibers > 20 \(\mu\)m in length were observed. Bernstein et al. (2005b) showed that biopersistence of fiber is one important factor in development of severe lung damage. In our study the rats were treated for 90 days, but only mild findings despite the granulomas were observed. It would be important to investigate the biopersistence or fate of the MWCNT to come to a definitive conclusion on the hazard potential of MWCNT, especially for longer exposure periods.

To evaluate the exact location and persistence of the inhaled MWCNTs within the lung on an ultrastructural basis, two animals per concentration group were treated as the main group and then the lungs were processed for TEM. In the investigations by Ma-Hock et al. (2009), TEM examination of the test atmosphere after dust generation was performed. Approximately 2 \(\mu\)m in diameter, wool-like clumps formed by Nanocyl were observed. Similar findings were found in the TEM evaluation of the lungs of the satellite animals. The MWCNTs were observed within alveolar macrophage located in membrane-bound vesicles (interpreted as phagosomes) and also free within the alveolar lumen. This is comparable to the dark staining particles within granulomas and free in the alveolar lumen detected in H&E-stained slides. The lipoproteinosis observed by light microscopy could be confirmed ultrastructurally, as within the alveoli many fingerprint-like structures were seen. These result from excessive secretion of surfactant by alveolar Type II cells. This phenomenon is readily induced in rats by repeated exposure to cytotoxic materials such as quartz (Dungworth et al., 1992). The intracytoplasmatic clumps of MWCNTs were often large (> 2 \(\mu\)m in diameter), thus making it difficult for the alveolar macrophages to process and clear them. Nevertheless, in some alveolar macrophages beside the unprocessed MWCNTs, round, small (up to 100 nm in diameter), electron-dense clumps located within membrane-bound vesicles (interpreted as phagolysosomes) were observed together with single, short (approximately 100 nm in length) fibrils interpreted as small parts of the MWCNTs. This could mean that phagocytized MWCNTs were processed within the phagolysosome and the degradation product is seen as electron-dense clumps inside the membrane-bound vesicles. This would mean that MWCNTs, although quite persistent in the lung and causing toxic effects, can be processed by alveolar macrophages. In this study the degradation products could not be proven to be processed MWCNTs. Such electron-dense clumps could also be digested cell detritus or inhaled substances other than MWCNTs. The coexistence of degradation products and single MWCNT fibrils could point in the direction that degradation of MWCNTs within the macrophage took place. Further investigations, for example, labeling MWCNTs, or comparing findings in animals allowed to recover after exposure, are necessary to underpin this assumption; Vlasova et al. (2012) recently demonstrated in vitro that SWCNTs attract neutrophils and are degraded by neutrophilic myeloperoxidase products. In a recent review, Manke et al. (2013) state that the factors influencing the toxicological potential of CNTs are size, surface area, functionalization, and metal impurities. This has to be kept in mind when performing or assessing a study.

At the lowest tested concentration of 0.1 mg/m\(^3\), the incidence of granulomatous inflammation was low and the severity grade minimal; only single granulomas were observed (versus multifocal at higher concentrations). Although a no observed effect concentration (NOEC) could not be established and 0.1 mg/m\(^3\) was the low observed effect concentration regarding the granuloma formation, no increase in reticulin fibers was observed. Increase in collagen fibers was limited within the granulomatous lesions at a minimal severity. Therefore, when MWCNTs are administered in low concentrations (< 0.5 mg/m\(^3\)), they do not provoke a diffuse increase in collagen or reticulin...
fibers within the alveolar wall. With regard to diffuse fibrosis development in the lung after inhalation of MWCNTs, 0.1 mg/m³ can be regarded as NOEC.

In conclusion, the results in this study clearly show that inhaled MWCNTs produce lesions in the lung (inflammation, lipoproteinosis, and granuloma formation) similar to those after inhalation of chrysotile asbestos. However, the available long-term studies performed with chrysotile asbestos are difficult to interpret due to an overload effect (Bernstein and Hoskins, 2006). But there are indications from both animal and epidemiological studies that different types of asbestos have a different tumorigenic and fibrogenic potential. Yarborough (2007) compared epidemiological studies where exposures to chrysotiles and amphiboles were investigated. He came to the conclusion that the risk of pleural mesothelioma in human populations is probably negligible for exposures to chrysotile asbestos not contaminated by amphiboles. Also Pierce et al. (2008) came to the conclusion that chrysotile is of lower risk of causing mesotheliomas in humans. There are some limitations in evaluating epidemiological studies (low cohort size, limited information about fiber length, potential contamination by amphiboles, limited information about smoking) as the authors in this study only considered one group of workers being exposed exclusively to chrysotile. These were vehicle mechanics working with friction products in the 1970s that typically contained chrysotile. It was concluded that the vehicle mechanics were not at increased risk of developing asbestos-related diseases.

It would be important to investigate the biopersistence of MWCNTs in the lungs and also to perform a long-term study to investigate potential risk of lung tumor or mesothelioma development, especially as mesotheliomas are known to have a very long latent period in rats (Davis and Cowie, 1990).

Like asbestos, inhalation of MWCNTs causes inflammation in the lungs at low concentrations and the MWCNTs are relatively biopersistent. This effect is also known for other biopersistent particles in the lung but with varying potencies. The progression of the inflammation observed with MWCNTs is as yet unknown. In the present examination, no precursor lesions for mesotheliomas-like fibrosis, chronic/active inflammation, and mesothelial hypertrophy/hyperplasia of the pleura were observed. As different types of asbestos cause different effects in the lungs, there is no general “asbestos effect.” From the information gathered so far from studies where rats were treated with MWCNTs, the lung effects seem to be similar to those caused by chrysotile asbestos, resulting in granulomas and mild fibrosis but not developing into mesotheliomas or other lung tumors.

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REFERENCES


