Lack of Genotoxicity of Carbon Nanotubes in a Pilot Study

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Abstract. *Background: Different types of carbon nanotubes may represent toxic hazards due to their size distribution and massive surface area. They may adsorb other toxic agents that can consequently be transported into the body. The aim of the present study was to determine the possible genotoxicity of carbon nanotubes. Materials and Methods: In vivo bacterial mutagenicity and in vitro cytogenetic studies were performed on single-walled and multi-walled carbon nanotubes. Results: Oral exposure to nanotubes did not increase urinary mutagenicity in rats as studied using Ames test. No genotoxic effect was found in the in vitro micronucleus and sister chromatid exchange assays, either. Mitotic inhibition, a possible cytotoxic effect, however, was observed in the human lymphocyte cultures upon treatment with single-walled tubes. Conclusion: Due to the limited toxicity data on carbon nanotubes, these results may be particularly important for risk assessment purposes.*

During the past decade much technological effort has focused on nanomaterials. A technologically important type of nanotechnological products is that of carbon nanotubes (CNTs). They represent new members of carbon allotropes similar to fullerenes and graphite, forming single- (SWCNT) or multi-walled carbon nanotubes (MWCNT) with a diameter of <1 nm but a length of several micrometres. Their unique mechanical, electric and thermal properties predestine them for use in the electronic, aerospace and computer industries (1). Hundreds of metric tons are produced annually nowadays (2), however, only limited information has been published on their environmental and human health effects (3-9). It is especially true of both general toxicity (characterized *e.g.* by lethal dose (LD_{50}), no observed adverse effect level (NOAEL)) and specific toxicological features (*e.g.* genotoxicity and carcinogenicity). Oxidative stress, apoptosis, other cytotoxic properties and pulmonary toxicity have been described for SWCNTs (3, 5, 8, 9). The vast majority of the literature published in this field rather deals with fullerenes, than nanotubes.

The aim of the present study was to collect data on the further specific endpoints of possible genotoxicity. Two factors may determine possible environmental and health risks of CNTs: their size distribution and large surface area capable of adsorbing environmental toxicants. These characteristics of nanotubes are very similar to those of asbestos fibrils, the well-known carcinogens (10). Possible genotoxicity of chemically pure SW- and MWCNTs were studied in specific *in vitro* and *in vivo* genotoxicity tests involving a mutagenicity assay (Ames test) on urine samples of orally treated rats and cytogenetic tests on human cells (micronucleus and sister chromatid exchange assays). An additional task was to overcome technical problems of exact CNT dosage during the experiments because the 'official' solvent toluene did not produce homogenous suspensions, thus prohibiting proper measurement. Furthermore, toluene proved to be a confounding factor in the mutagenicity test (11).

Materials and Methods

The SWCNT (<2 nm x 4-15 μm, purity: 90% by vol) and the MWCNT (10-30 nm x 1-2 μm, purity: 95-98% by vol) samples were produced by Shenzhen Nanotech. Port Co. Ltd. China. Nanotubes were successfully dispersed in a carbopol-based semiliquid gel (12) by using magnetic stirrer.

All animal studies were performed in an ethical and humane way under the control of the authorized Ethical Board.

Urinary mutagenicity (Experimental design, treatment, sample collection and preparation). For testing urinary mutagenicity, the conventional Salmonella plate incorporation test was applied (13). Three groups of six inbred male Fischer-344 rats (each weighing about 100 g) were housed individually in metabolic cages to collect urine. Two groups were treated with a single dose of 50 mg/kg SWCNT or MWCNT, respectively, by oral gavage. The third group served as a vehicle (carbopol gel) control. The urine samples were collected during the first 24 h and were pooled separately. The creatinine concentrations in the urine samples were determined immediately. The chilled and filtered urine was passed over columns packed with Soxhlet-extracted (acetone diethylether methanol) macroreticular resins of different polarity (Sigma Amberlite XAD-4 and XAD-7, 1:1 v/v, Sigma-Aldrich Kft., Budapest, Hungary) (14). The dimethyl sulfoxide eluates

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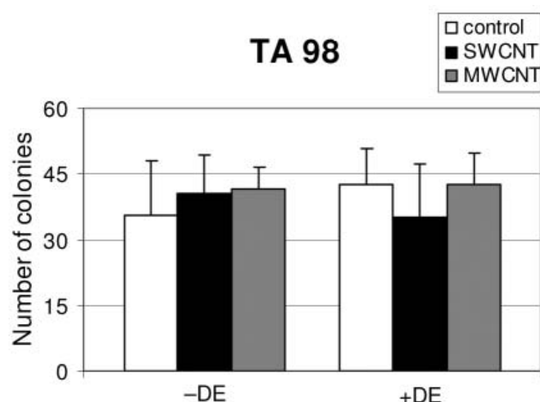

Figure 1. *Standardized Salmonella (TA 98 tester strain) mutagenicity results of urine samples of treated and control groups. Significant differences were not detected at p<0.05.*

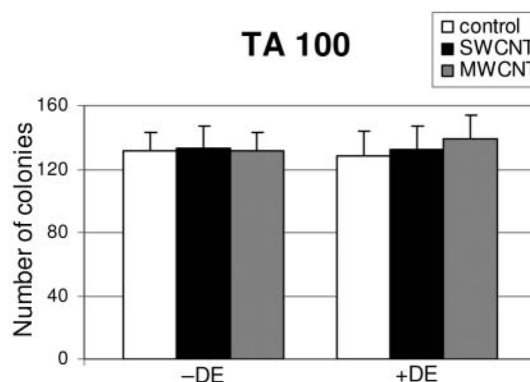

Figure 2. *Standardized Salmonella (TA 100 tester strain) mutagenicity results of urine samples of treated and control groups. Significant differences were not detected at p<0.05.*

represented 10x concentrated urine samples equalized to 1 mmol/L urinary creatinine concentration. Aliquots of the urine concentrates were incorporated into three paralel plates of TA 98 and 100 strains. The assays were performed both in the presence (+DE) and absence (-DE) of the deconjugation enzyme mix containing β-glucuronidase (250 U/plate) and sulfatase (100 U/plate). Revertant colony numbers were averaged for the statistical analysis.

Cytogenetic assays on human cells. Blood samples were taken from 3 healthy donors under sterile conditions, using lithium-heparinized vials. Negative control and exposed cultures prepared from each blood sample were analyzed in both cytogenetic assays applied. Ethyl methane sulfonate for the sister chromatid exchange (SCE) analysis and bleomycin for the micronucleus analysis (MN) were used as positive control. In this study, as usual in pilot studies, the applied doses were limited by practical considerations, since we were not aware of the LD_{50} or other basic toxicological data. *In vitro* micronucleus analyses were performed on binucleated cells prepared with cytochalasin-B in human lymphocyte cultures (15). With single measurement, 10 mg MWCNT or SWCNT were added to the culture medium of cells, respectively, to reach a final concentration of 1 mg/ml. Frequencies of micronuclei were calculated from 1000 scored Giemsa-stained binucleated cells in both groups. SCE analyses were also performed in 72 h lymphocyte culture using the 5-bromo-2'-deoxyuridine incorporation method and fluorescence-plus-Giemsa staining (14). Cultures contained 1 mg/ml final nanotube concentrations in this study. Twenty-five M2 cells were analyzed per slide. Cell kinetics (distribution of first, second, third+ mitoses) were also determined by scoring M1 and ≥M3 frequencies.

Statistical analyses. Student's paired *t*-tests were performed using SPSS for Windows 11.0 software. $P<0.05$ was considered as the criterion of significance.

Results

Statistically significant mutagenicity was not detected in the Ames test, neither in the TA98 nor the TA100 strains. These negative results were independent of the type of nanotube used and not affected by the enzymatic deconjugation of urine samples, either (Figures 1 and 2). Results of the MN test upon MWCNT-treatment are shown in Figure 3. The exposure did not affect frequency of binucleated cells, reflecting the lack of cytotoxic effects. In the culture cytotoxic effect can also be measured as the influence of a toxic agent preventing cells in G_0 from entering G_1, even in the presence of a mitogen (16). No statistically significant differences among paired control and exposed samples were detected ($p<0.05$). Since micronuclei are consequences of acentric fragment formation or extra chromosomes, these results cannot indicate clastogenic or aneugenic effects by using a high MWCNT dose. The SWCNT treatment at the same dose, however, indicated practical mitotic inhibition which was manifested in a considerable reduction of the binucleated cell number. Due to the insufficient cell quantity, we could not practically perform the MN analyses. Prior to the SCE analysis, cell kinetics of the control and exposed cultures were compared, based on the BrdU-incorporation pattern (Figure 4). Upon the MWCNT exposure, no significant differences were detected in the cell kinetics. This was also true for the SCE frequency analysis: a *t*-test of paired samples showed no significant differences at $p<0.05$ (Figure 5). In the case of SWCNT treatment, however, a complete shift in cell kinetics (towards M1) was observed, so the analysis of SCEs could not be performed.

Discussion

Biological impacts of chemically pure fibrous materials are affected by three important parameters: their length, diameter and persistence. The inflammatory action of the same material can frequently be more significant in the nanosize range. To date, results published on the *in vitro* and *in vivo* effects of

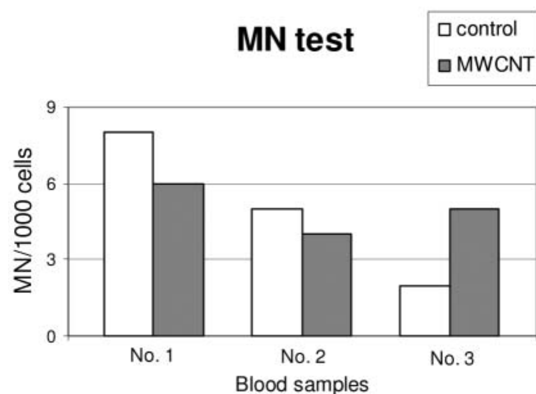

Figure 3. *MN test upon MWCNT-treatment. Significant differences among paired control and exposed samples were not detected at $p<0.05$.*

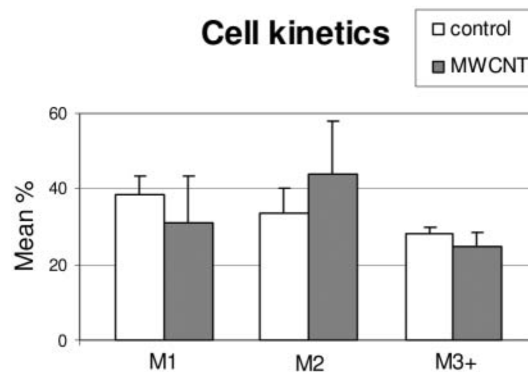

Figure 4. *Comparison of cell kinetics of the control and exposed cultures to MWCNT. Significant differences were not detected at $p<0.05$.*

CNTs have been mostly limited to SWCNTs. Upon the intratracheal exposure of rats to 5 mg/kg SWCNT, 15% lethality was detected as a consequence of mechanical blockage of the upper airways. Lower doses caused multifocal granulomas as proof of foreign body reaction (3, 9). In our previous chronic studies, CNTs were investigated in an *in vivo* mesothelioma model originally developed to study asbestos carcinogenesis. After direct peritoneal exposure (implantation into Kertai's fold, an omental envelope lined with mesothelial cells) granulomatous reaction of the foreign body type with epitheloid and multinucleated giant cells but no mesothelioma was observed (11, 17). In our present study the 50 mg/kg applied single oral dose of CNTs was exactly ten times higher than the dose used in the intratracheal instillation studies.

As we suggested earlier in the *in vitro* Ames test (11), clear negative results were also observed in the urinary mutagenicity studies upon exposure to both types of CNTs. These results are in accordance with other genotoxicity studies performed with SWCNT in human keratinocyte and bronchial epithelial cultures (8). Oxidative stress mechanisms may play an important role in the cytotoxicity of CNTs; this has been suggested for SWCNTs and fullerenes which are redox active, lipophilic, localized in the lipid-rich cell membranes, and can cause oxyradical induced damage *in vitro* (7, 8). Harboring of MWCNT by human epidermal keratinocytes and the consequent release of proinflammatory cytokine interleukin-8 were also reported (18). Although, our preliminary *in vitro* studies did not detect any cytogenetic alteration (clastogenic, aneugenic or SCE-inducing effect) caused by MWCNTs, a specific cytotoxic effect, mitotic inhibition, was clearly observed at the same dose of SWCNTs. Since we did not study dose dependence in this pilot experiment, we can only state that the cytotoxiciy of the dose applied inhibited the genotoxicity analysis. Oxidative or radical-inducing agents are frequently genotoxic but the tests applied might not be highly sensitive to this type of genotoxicity. Therefore, use of higher resolution cytogenetic and other assays (involving *in vivo* comet assay on target tissues or testing further strains of *Salmonella typhimurium*) can yield mechanistic information.

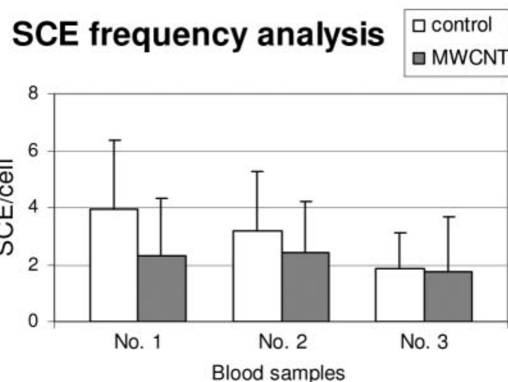

Figure 5. *T-test of paired samples did not show significant differences at $p<0.05$.*

The wide size-scale of the two main CNT types, their large surface-to-mass ratio and great adsorptive capacity may also be crucial in any assessment. This means that the investigation of chemically pure nanotubes may be insufficient in order to accumulate relevant data for risk assessment in a real life scenario. We should not neglect the problems which are faced in risk assessment of asbestos: fibre size distribution and compounds adsorbed by the fibres are in close association to the final health effect.

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