In vivo genotoxicity of hard metal dust: induction of micronuclei in rat type II epithelial lung cells

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Inhalation of hard metal dust (WC-Co particles) has been associated with an increased risk for lung cancer in occupational settings. In vitro, WC-Co was genotoxic in human lymphocytes producing DNA strand breaks and micronuclei. The aim of the present study was to evaluate the in vivo genotoxic effects of WC-Co dust in rat type II pneumocytes. DNA breaks/alkali-labile sites (alkaline comet assay) and chromosome/genome mutations (micronucleus test) were assessed after a single intra-tracheal (i.t.) instillation of WC-Co, including dose-effect and time trend relationships. In addition, the alkaline comet assay was performed on cells obtained after broncho-alveolar lavage (BAL) and on peripheral blood mononucleated cells (PBMC). As pulmonary toxicity parameters, protein content, lactate dehydrogenase activity, total and differential cell count in BAL fluid were evaluated in parallel. In type II pneumocytes, WC-Co induced a statistically significant increase in tail DNA (12 h time point) and in micronuclei (72 h) after a single treatment with 16.6 mg WC-Co/kg body wt, a dose that produced mild pulmonary toxicity. This observation provides the first evidence of the in vivo mutagenic potential of hard metal dust. In PBMC, no increase in DNA damage or micronuclei was observed. This study indicates the potential to detect chromosome/genome mutations (micronuclei) in relevant target cells (type II pneumocytes) after i.t. instillation of a particle mixture.

Introduction

Hard metal or cemented carbide (WC-Co) consists of tungsten carbide (WC) and metallic cobalt (Co), a unique combination of hardness and toughness with wide industrial applications. Occupational exposure to hard metal dust is associated with an increased risk of lung cancer (1).

Lasfargues et al. (2,3) demonstrated that in vivo, in the rat, a WC-Co mixture containing 6% of Co (5.0–16.67 mg/kg body wt) caused acute and delayed pulmonary toxicity, leading to a pulmonary fibrosis reaction. In vitro, in human lymphocytes, the WC-Co mixture was genotoxic as evaluated with the alkaline comet and elution assays (DNA breaks and alkali-labile sites) and with the in vitro cytokinesis-block micronucleus test (chromosome/genome mutations) (4–6). One of the possible mechanisms underlying the genotoxicity of hard metal dust is a specific physicochemical interaction between Co metal and WC particles, which leads to an excessive formation of active oxygen species (7), probably peroxides (8). No experimental data are available on the in vivo mutagenic and carcinogenic potential of hard metal dust in the target tissue, the lung (for review see ref. 9).

In a human biomonitoring study conducted in workers exposed to WC-Co particles, no significant genotoxic effects were detected in peripheral blood mononucleated cells (PBMC) (10). A limitation of the study was, however, that PBMC were used as reporter cells for possible genotoxic damage induced by dust inhalation.

Analogous to the in vivo gut micronucleus test for the evaluation of exposure to chemicals following the oral route (11), examining mutagenicity in lung cells in vivo appears very relevant for the evaluation of the carcinogenic potential of inhaled particles, especially in type II pneumocytes as they are the stem cells of the alveolar epithelium. Therefore, the main objective of the present study was to evaluate the in vivo genotoxicity of the WC-Co particle mixture in rat type II pneumocytes (AT-II). This was achieved by evaluation of DNA breaks and alkali-labile sites (alkaline comet assay) and of chromosome/genome mutations (micronucleus test) after a single intra-tracheal (i.t.) instillation. Animals treated with bleomycin were included as positive controls. In parallel, the alkaline comet assay was performed on cells obtained after broncho-alveolar lavage (BAL) and on PBMC.

This study provides the first proof of the in vivo mutagenic potential of hard metal dust since WC-Co particles induced micronuclei in type II pneumocytes of the rat after a single i.t. instillation. In PBMC of WC-Co-treated animals, no increase in DNA damage and micronuclei was observed.

Materials and methods

Materials

DMEM (Dulbecco’s Modified Eagle Medium), fungizone (amphotericin 250 μg/ml), penicillin-streptomycin (10 000 U, 1000 μg/ml), l-glutamine (200 mM) and fetal calf serum (FCS) were purchased from Gibco (Merelbeke, Belgium). Percoll and trypsin type I (EC 3.4.214, Cat. No. T-8003) were purchased from Sigma-Aldrich (Antwerp, Belgium). Cell culture plastics were purchased from Iwaki and Nunc (International Medical, Belgium). PBS buffer was prepared as 130 mM NaCl, 5.4 mM KCl, 11 mM glucose, 10.6 mM HEPES, 2.6 mM Na2HPO4, 1.9 mM CaCl2, 1.29 mM MgSO4, adjusted to pH 7.4.

The WC-Co consisted of 6.3% cobalt, 84% tungsten and 5.4% carbon (median particle size 2 μm). The particles were sterilized during 4 h, at 200°C. The applied doses are always expressed as WC-Co per kg body weight (e.g. 16.6 mg WC-Co/kg body wt corresponding to 1.0 mg Co/kg body wt).
Animals

Wistar male rats of ~200 g were obtained from Elevage Janvier (Bagnues, France) and housed in plastic cages with wood dust bedding under controlled lighting conditions (12 h light/12 h dark). The rats were allowed free access to standard laboratory diet (Trouw Nutrition, Gent, Belgium) and tap water.

Preliminary study

A preliminary experiment was conducted to select the appropriate dose of WC-Co, which would cause minimum pulmonary damage. Based on this study, 16.6 mg WC-Co/kg body wt was chosen, because it induced up to 72 h post-instillation significant but relatively limited pulmonary toxicity as assessed by lung weight changes and cellular and biochemical (LDH, proteins) alterations in BAL fluid (Table I).

Treatment

The rats were treated, under mild anaesthesia (pentobarbitarial 20 mg/kg i.p.; Sanofi), by a single i.t. instillation (trans-oral) in a volume of 2 ml/kg body wt, followed by a bolus (3 ml) of air using a Becton Dickinson 14 Gauge catheter (2.1 × 45 mm). WC-Co particle suspensions were prepared freshly in sterile de-ionized water and stirred during 1 min just before use. The pulmonary toxicity and genotoxicity of 16.6 mg WC-Co/kg body wt in different cell types were evaluated at different sampling times: 12, 24, 48, 72, 96 and 120 h post-instillation. At 72 h post-instillation, the pulmonary toxicity and genotoxicity in different cell types of 1.8, 5.5, 16.6 and 49.8 mg WC-Co/kg body wt were assessed.

Blomycin (1.0 U/kg body wt) was dissolved in sterile de-ionized water and administered to the rats in the same way as WC-Co. Groups of animals receiving vehicle (sterile de-ionized water) were also included.

Determination of cobalt content

Cobalt was measured in blood, lung homogenates and BAL fluid by atomic absorption spectrometry (Varian Zeeman SpectraAA-30 equipped with a graphite furnace atomizer). Blood and homogenates were mineralized in concentrated nitric acid before analysis (12).

Pulmonary toxicity

A first set of animals (n = 30 in total) was killed with an overdose of nembutal (90 mg/kg, i.p.) at 12, 24, 48 or 72 h after i.t. dosing. The abdomen was opened and the abdominal aorta was cut to exsanguinate the animal. After opening the thorax, the left bronchi and pulmonary vessels were clamped and the left lung was removed, weighed (wet lung weight) and placed in an oven at 60°C for 48 h. Dry lung weight was obtained after the tissue had equilibrated to room temperature. Wet and dry lung weights are expressed relatively to body weight (mg/kg body wt).

The right lung was lavaged in situ four times with 4 ml 0.9% NaCl at room temperature via a 14 gauge Insyte-W catheter inserted into the trachea through a cut in the cricoid membrane. After recovery, the pooled BAL fluid was kept on ice until centrifugation at 250 g for 10 min at 4°C. The supernatant was assayed for total protein concentration and the activity of LDH with the Bio-Radmicro-assay procedure (Bio-Rad Laboratories N.V., Nazareth, Belgium). The pellet was resuspended in 200 μl PBS. One aliquot of the BAL cell suspension was used to calculate total cell count. Aliquots containing approximately 30,000 cells were diluted in 0.5 ml PBS and placed in the sample chamber of a Cytospin-3 cell centrifuge (Shandon Scientific, Cheshire, UK). Slides were stained using the Dade Diff-Quik (Baxter Diagnostics AG, Duedingen, Switzerland) staining set for differential cell counting (200 cells per animal were differentially counted).

Lung histology

After lavage, the right lung was instilled, using a syringe, with 6% formaldehyde in PBS until full expansion of the different lobes. Slices from all lung lobes were embedded in paraffin, 5 μm sections were prepared, stained with haematoxylin-eosin and examined by light microscopy by an experienced pathologist, who evaluated lung injury blindly. Observations were done in a semi-quantitative way, using a score from ‘−’, normal lung tissue; ‘+’, slight changes; ‘+++’, moderate and ‘++++’, severe change or damage.

Sampling and type II pneumocyte isolation

Another group of animals (n = 56 in total) was anaesthesized (pentobarbitarial 90 mg/kg i.p.), blood was taken by venipuncture of the vena cava with a pre-heparinized syringe to be used in the comet assay and for isolation of PBMC for the micronuclear test and then the rats were killed by exsanguination. After opening of the chest, the trachea was cannulated and the lungs were perfused in situ with 0.9% NaCl via the pulmonary artery.

BAL was performed by filling and emptying the lungs four times with 10 ml 0.9% NaCl. The BAL fluid was centrifuged, the total cell count was determined and the cells were processed in the alkaline comet assay.

AT-II cells were isolated following the procedure of Richards et al. (13) and as described previously in ref. 14. The lungs were trypsinised (250 mg/lung) at 37°C during 30 min and the enzyme activity was arrested by addition of 5 ml FCS. The digested tissue was then minced with scissors in the presence of DNase, shaken and filtered to obtain individual and/or clumps of a few cells. Further purification was achieved by centrifugation onto a discontinuous Percoll gradient (20 min, 300 g). The cells of the layer above the heavy gradient were plated in a Petri dish and incubated for 1 h at 37°C allowing fibroblasts and remaining macrophages to attach. The viability of the finally purified AT-II was assessed using the Trypan blue exclusion technique.

The cells were resuspended in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin, 0.5% fungizone and 1% glutamine and used immediately in the alkaline comet assay or plated onto chamber slides (16 well glass slides, Nalgen Nunc, IL) (~300,000 cells/cm²). This procedure yielded an average purity of 90% of AT-II cells (14).

Peripheral blood mononucleated cells isolation

After 1:1 dilution with PBS, the blood was layered onto Ficoll Paque. After 40 min centrifugation at 400 g, PBMCwere isolated and washed with PBS (10 min centrifugation at 400 g). For the micronuclear test, the cells were cultured in RPMI 1640 medium supplemented with 10% fetal clone II, 2% PHA, 1% l-glutamine and 1% penicillin/streptomycin (triplicate 1 ml aliquots kept at 37°C). Cytochalasin B (6 μg/ml) was added 4 h after the start of the culture in order to block cytokinesis. After a total of 72 h culturing the PBMC were used in the cytokinesis-block micronucleus test (see below).

Table I. Pulmonary toxicity endpoints (change in body weight, wet and dry left lung weight, protein and LDH content of BAL fluid) at different time points after i.t. treatment with WC-Co; mean (SD) values of three animals

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Treatment (mg/kg body wt)</th>
<th>Body weight change (g)</th>
<th>Wet left lung weight (mg/kg body wt)</th>
<th>Dry left lung weight (mg/kg body wt)</th>
<th>BAL protein (μg/ml)</th>
<th>BAL LDH (IU)</th>
<th>Total BAL cell count × 10⁶</th>
<th>Tissue damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>WC-Co 16.6</td>
<td>8 (5)</td>
<td>1860 (120)</td>
<td>380 (30)</td>
<td>4094 (1592)</td>
<td>6.52 (0.80)</td>
<td>922 (771)</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>WC-Co 16.6</td>
<td>7 (3)</td>
<td>2000 (90)</td>
<td>400 (10)</td>
<td>1355 (169)</td>
<td>6.88 (1.46)</td>
<td>351 (258)</td>
<td>+</td>
</tr>
<tr>
<td>48</td>
<td>WC-Co 16.6</td>
<td>0 (8)</td>
<td>1950 (150)</td>
<td>390 (40)</td>
<td>2079 (277)</td>
<td>7.30 (0.64)</td>
<td>201 (107)</td>
<td>+</td>
</tr>
<tr>
<td>72</td>
<td>Untreated</td>
<td>27 (8)</td>
<td>1780 (80)</td>
<td>480 (110)</td>
<td>429 (74)</td>
<td>3.31 (0.75)</td>
<td>107 (91)</td>
<td>–</td>
</tr>
<tr>
<td>72</td>
<td>H₂O</td>
<td>12 (4)</td>
<td>1720 (170)</td>
<td>350 (20)</td>
<td>350 (57)</td>
<td>2.81 (0.77)</td>
<td>227 (44)</td>
<td>–</td>
</tr>
<tr>
<td>72</td>
<td>WC-Co 1.8</td>
<td>15 (5)</td>
<td>1910 (290)</td>
<td>360 (20)</td>
<td>846 (61)</td>
<td>3.91 (0.30)</td>
<td>217 (33)</td>
<td>+/+</td>
</tr>
<tr>
<td>72</td>
<td>WC-Co 5.5</td>
<td>15 (3)</td>
<td>2050 (200)</td>
<td>410 (60)</td>
<td>937 (380)</td>
<td>4.92 (1.69)</td>
<td>413 (240)</td>
<td>+/+</td>
</tr>
<tr>
<td>72</td>
<td>WC-Co 16.6</td>
<td>15 (3)</td>
<td>2000 (410)</td>
<td>350 (10)</td>
<td>2501 (291)</td>
<td>4.87 (0.54)</td>
<td>413 (240)</td>
<td>+/+</td>
</tr>
<tr>
<td>72</td>
<td>WC-Co 49.8</td>
<td>9 (3)</td>
<td>3150 (420)</td>
<td>880 (310)</td>
<td>8683 (4424)</td>
<td>7.30 (2.80)</td>
<td>740 (79)</td>
<td>+/+</td>
</tr>
</tbody>
</table>

LDH, lactate dehydrogenase; ‘−’, normal tissue; ‘+’, slight changes; ‘+++’, moderate changes; ‘++++’, severe changes.

*P < 0.05 as compared with untreated rats (for the earlier sampling times no concurrent vehicle-treated rats were available) (one-way ANOVA).

**P < 0.05 as compared with vehicle-treated rats.
The alkaline comet assay was performed on freshly isolated AT-II, BAL cells and whole blood. An appropriate amount of cells was mixed with 300 μl of low melting point agarose (0.8% w/v in PBS) and loaded onto a regular pre-coated microscope slide (normal melting point agarose, 1% w/v in water). The comet assay was performed as described previously (6) including internal standards of untreated and ethylmethane sulfonate exposed K562 cells according to De Boeck et al. (15). Briefly, after lysis at 4°C (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, supplemented with 1% Triton X-100 and 10% DMSO just before use), unwinding of the DNA was performed in a buffer containing 300 mM NaOH, 1 mM Na2EDTA (pH >13, 17°C) during 40 min. Alkaline electrophoresis was performed in the same buffer for 20 min at 0.7 V/cm (25 V, 300 mA). The slides were then washed three times for 5 min each with neutralization buffer (0.4 M Tris, pH 7.5) and dehydrated in 100% ice-cold ethanol (10 min). To visualize and analyze the DNA damage, the slides were rehydrated with deionized water (200 μl, 10 min) and stained with ethidium bromide (20 μg/ml, 10 min). Fifty randomly chosen, non-overlapping, comets per coded duplicate slide (100 cells/animal) were captured using a Leitz fluorescence microscope (25× objective) coupled to a CCD camera and an image analysis system (Komet 3.0, Kinetic Imaging, Liverpool, UK). All slides were scored by a single person. Percentage of DNA in the tail (TD) was recorded as DNA migration parameter. Data are presented as corrected TD values, i.e. recalculated by applying the formula presented in (15), in which the median TD for a given animal/cell type (100 comets) is divided by the median TD of the concurrent negative internal standard.

**Micronucleus test**

The frequency of micronuclei was assessed in isolated type II pneumocytes (ex vivo) and in PBMC (ex vivo, cytokinesis-block method).

Type II pneumocytes were fixed with 100% methanol (20 min) after 2 days of culture and stained with acridine orange (0.012% in phosphate buffer) prior to analysis with a Zeiss Axioscope fluorescence microscope (magnification 400×). Multiple culture wells on different coded chamber slides were analysed for each animal. In total, 2000 AT-II cells per animal were evaluated for the presence of micronuclei.

PBMC were cytopspun onto microscope slides and fixed with 100% methanol (20 min). The cells were stained with 5% Giemsa in S buffer (pH 6.8). Slides from triplicate cultures were analysed for each treatment point. Cytokinesis-blocked (binucleated) lymphocytes were examined for the presence of micronuclei (1000–3000 cells/animal). All coded slides were analysed with a Zeiss light microscope (1250× magnification).

The microscopical analysis of the micronucleus test was performed by a single person.

**Statistical analysis**

For pulmonary toxicity, differences between vehicle-treated animals and WC-Co or bleomycin-treated animals were evaluated using one-way ANOVA followed when appropriate by a Dunnett multiple comparison test. For genotoxicity, pairwise differences between pooled vehicle-treated animals and WC-Co or bleomycin-treated animals were assessed using the Student’s t-test and the Mann–Whitney U test for the micronucleus test and comet assay, respectively.

**Results**

**Dose- and time-finding studies**

**Time-dependent toxicity.** Considering the parameters of pulmonary toxicity over time (Table I), 16.6 mg WC-Co/kg body wt led to an early (12 h) significant increase in protein content, LDH activity and total cell content in the BAL indicating cytotoxicity and the development of an inflammatory reaction. These indices lowered progressively with time but were still (not significantly) elevated at 72 h. Differential cell count showed that, at all time points, the percentage of lymphocytes, neutrophils and eosinophils remained relatively high until 72 h, and almost returned to normal after 120 h (Figure 1a and b). In the case of bleomycin, an even higher percentage of inflammatory cells was observed in the BAL, which remained elevated at later sampling times (Figure 1a and c).

**Dose-dependent toxicity.** At 72 h post-instillation, the degree of toxicity was found to be dose-dependent (Table I and Figure 2). The two lowest doses, 1.8 and 5.5 mg WC-Co/kg body wt, did not induce any significant pulmonary toxicity. The pulmonary toxicity in rats exposed to 16.6 mg WC-Co/kg body wt was moderate; the total BAL cell count was increased (not statistically significant) and showed a significant relative increase in neutrophils accompanied by a relative decrease in macrophages. The highest dose, 49.8 mg WC-Co/kg body wt, induced severe pulmonary damage as indicated by all measured parameters: reduced body weight, higher wet and dry lung weight, increased cell number in BAL, a higher protein content and LDH activity. The differential cell count of the BAL cells showed an accumulation of neutrophils and eosinophils.

**Co measurements**

The cobalt content was measured in the lung, BAL fluid and blood in order to assess the persistence of WC-Co particles in the lung tissue. Figure 3 shows the mean cobalt measurements in three animals at different time points after i.t. instillation of different doses of WC-Co. It is clear that the applied dose of cobalt was relatively rapidly eliminated from the lung. The first statistically significant decrease in cobalt content was observed after 48 h in the lung and already after 24 h in BAL and blood. As could be expected, the cobalt levels in the three investigated tissues decreased in parallel. Background levels of Co in lungs of vehicle-treated animals were below the detection limit. In BAL fluid and blood of vehicle-treated animals, Co content was 0.3 ± 0.3 and 1.8 ± 0.3 μg/l, respectively.

**Lung histology**

Black pigmentation indicating the presence of metal particles was found in pulmonary macrophages at all time points after treatment of animals with WC-Co. No pigmentation was found in control or vehicle (water)-treated rats. The lungs of both lowest dose groups (1.8 and 5.5 mg WC-Co/kg body wt) did not show any tissue damage after 72 h, the group treated with 16.6 mg WC-Co/kg body wt showed moderate damage, while severe damage was observed in the highest dose group (Table I). Also, a dose-dependent accumulation of eosinophils and hyperplasia of AT-II cells was observed 72 h post-installation. At earlier time points, no AT-II hyperplasia was found in the 16.6 mg WC-Co/kg body wt group.

**Induction of DNA migration in AT-II, BAL cells and PBMC:**

Alkaline comet assay

Figure 4a–c presents the results of the alkaline comet assay (relative tail DNA) performed on AT-II, BAL cells and PBMC of rats at different time points after i.t. instillation of WC-Co (16.6 mg WC-Co/kg body wt) or bleomycin (1.0 U/kg body wt). The dotted line indicates the average DNA migration in vehicle-treated animals (n = 8; AT-II: 1.22 ± 0.51; BAL cells: 13.23 ± 3.69; PBMC: 0.61 ± 0.28).

In AT-II, a statistically significant increase in DNA migration was observed at 12 h after i.t. treatment with WC-Co in comparison with vehicle controls. An increase was also seen at this time point in bleomycin-treated rats but this was, however, not statistically significant. Increased DNA migration was no longer present at later time points.

In BAL cells of vehicle-treated rats, the first observation was a higher level of DNA migration as compared with AT-II and PBMC (dotted line); the DNA migration levels were similar to those of untreated animals (data not shown). BAL cells of
WC-Co and bleomycin-treated animals showed statistically significantly lower levels of DNA migration as compared with the vehicle controls.

In PBMC, no statistically significant difference in the level of DNA migration between WC-Co or bleomycin-treated and vehicle-treated animals was observed.
Fig. 2. Composition of BAL cell population of rats 72 h after i.t. treatment with vehicle (water), different doses of WC-Co or 1.0 U bleomycin/kg body wt: percentage of macrophages, lymphocytes, neutrophils and eosinophils. Mean ± SD of three animals. *P < 0.05 in comparison with vehicle-treated animals (one-way ANOVA and Dunnett multiple comparison test).

Fig. 3. Cobalt content of lungs (A), BAL fluid (B) and peripheral blood (C) of rats at different time points after i.t. treatment with different doses of WC-Co. Mean ± SD of three animals. *P < 0.05 as compared with 12 h sampling time (Student’s t-test).

Fig. 4. Relative tail DNA of AT-II (A), BAL (B) and PBMC (C) cells at different time points after i.t. treatment of rats with bleomycin (1.0 U/kg body wt) or WC-Co (16.6 mg/kg body wt). *P < 0.05 in comparison with vehicle-treated rats (Mann–Whitney U test). The dotted line represents the relative DNA migration level in vehicle-treated rats. Error bars (SD) indicate variation across animals (number of animals in parentheses).
Discussion

The European legislation imposes an in vivo rodent micronucleus assay on bone marrow for the evaluation of new chemicals. Performing mutagenicity assessment on one tissue might, however, give misleading results since problems of organ-specificity and influence of the administration route on bioavailability at the target tissue have been reported (16–19). In the case of exposure to particles by inhalation, obviously the lung is the first (and often the main) target tissue.

This study provides the first proof of the in vivo mutagenicity of hard metal dust in a target cell type, i.e. type II pneumocytes in the rat. Seventy-two hours after i.t. instillation of a subtoxic dose of WC-Co, a statistically significant increase in micronucleated AT-II was found. This observation was preceded by an increase in DNA damage detected with the alkaline comet assay in AT-II at 12 h after exposure. In BAL cells, the alkaline comet assay showed decreased DNA migration in exposed animals and, in PBMC, no significant

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**Table II. Induction of micronuclei: frequency of micronucleated type II pneumocytes (AT-II) per 1000 cells (%) of rats at 72 h after i.t. instillation of vehicle (water), WC-Co or bleomycin.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animal</th>
<th>MN AT-II (%)</th>
<th>Mean ± SD MN AT-II (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>1</td>
<td>0.0</td>
<td>3.5 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>WC-Co (1.8 mg WC-Co/kg body wt)</td>
<td>1</td>
<td>6.0</td>
<td>5.1 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>WC-Co (5.5 mg WC-Co/kg body wt)</td>
<td>1</td>
<td>12.5</td>
<td>8.1 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>14.0</td>
<td></td>
</tr>
<tr>
<td>WC-Co (16.6 mg WC-Co/kg body wt)</td>
<td>1</td>
<td>30.9</td>
<td>16.1 ± 7.3*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>16.4</td>
<td></td>
</tr>
<tr>
<td>WC-Co (49.8 mg WC-Co/kg body wt)</td>
<td>1</td>
<td>6.0</td>
<td>9.2 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12.4</td>
<td></td>
</tr>
<tr>
<td>Bleomycin</td>
<td>1</td>
<td>19.4</td>
<td>14.7 ± 5.3*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8.9</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05 in comparison with vehicle-treated rats (Student’s t-test).
differences could be detected in animals treated with WC-Co both with the alkaline comet assay or the cytokinesis-block micronucleus test. Particles, together with fibres, form a rather specific group of toxic agents. Genotoxicity by inhalation of particles is believed to follow two predominant pathways (20,21): (i) active oxygen species, produced because of specific surface properties or the presence of transition metals, together with other parameters such as particle size, shape and uptake, play a major role in the primary genotoxicity of particles; (ii) secondary genotoxicity, mediated by the excessive and persistent formation of active oxygen species by inflammatory cells, combined with increased proliferation of type II pneumocytes, may act to initiate and promote genotoxic effects in lung epithelial cells after chronic exposure to toxic particles. Either or both mechanisms are likely to be at play in the case of WC-Co particles as they were demonstrated to cause excessive formation of active oxygen species (7) and pulmonary inflammation (2,3). In addition, WC-Co was found to inhibit DNA repair, to interact with the mitotic spindle inducing chromosome loss, and to cause less apoptosis than its single components in vitro in human PBMC (22).

The observation in the present study of the highest increase in micronucleated AT-II at 72 h after i.t. instillation of WC-Co, is not unexpected because it parallels the time required for these cells to complete one division (23). The nature of the micronuclei induced in the AT-II is not clear at this point, i.e. whether they originate from a whole chromosome (aneugenic event) or from an acentric fragment (clastogenic event). Given the presence of intruding inflammatory cells, one cannot exclude that the mutagenic events detected in the AT-II are, besides being due to genotoxicity of the WC-Co particles, the result of inflammation (secondary genotoxicity as suggested in ref. 21). Indeed, already at 12 h post-instillation of WC-Co a substantial increase in inflammatory cells was observed. Their presence in the alveoli before and during the proliferation of the AT-II could contribute to the formation of the observed micronuclei. The sporadic presence of cells other than AT-II on the chamber slides did not hamper the microscopical analysis because AT-II could easily be distinguished due to their specific cytoplasmic and nuclear characteristics.

The fact that, at 72 h, the highest dose of WC-Co (49.8 mg/kg) induced a lower frequency of micronucleated AT-II than the second highest tested dose (16.6 mg WC-Co/kg body wt) possibly indicated that this 3-fold higher dose caused either mitotic delay or cell death. It should, however, be kept in mind that for most time points other than 72 h, results from only a limited number of animals were available and they should therefore not be considered as conclusive. At later time points, the number of micronucleated type II pneumocytes after WC-Co treatment (16.6 mg WC-Co/kg body wt) decreased, while this frequency remained higher in the bleomycin exposed rats. This difference might be related to the fact that cobalt was rapidly removed from the lungs and that, at that time point, a lower frequency of inflammatory cells was present in the alveoli, both factors contributing to reduce the burst of active oxygen species released in the alveoli. Another possibility is the occurrence of cell death and/or mitotic delay at later time points after WC-Co treatment.

The observation of a statistically significant increase in DNA migration in AT-II at 12 h (and the return to baseline afterwards) after instillation of WC-Co, could be related to the presence of inflammatory cells exerting oxidative stress. The non-significant increase observed for bleomycin could, however, suggest that, for this endpoint, earlier sampling times (immediately after instillation) would be required to detect a significant effect. This would be in line with the idea that the alkaline comet assay detects transiently occurring DNA strand breaks and alkali labile sites. However, it should be kept in mind that the isolation of the AT-II itself requires at least 6 h, a period during which DNA strand break repair could take place.

The decrease in DNA migration observed in BAL cells of WC-Co- and bleomycin-treated rats is probably related to changes in the relative proportion of exposed (resident macrophages) and non-exposed cells (recruited inflammatory cells consisting of lymphocytes, neutrophils and eosinophils) as indicated by the differential cell counts of the BAL cell population. Unfortunately, and in contrast to the microscopical analysis of AT-II for the micronucleus test, no distinction could be made between the macrophages and the other cell types on the basis of comet appearance of the BAL cells.

In PBMC, no significant difference could be detected in animals treated with WC-Co both with the alkaline comet assay or the ex vivo/in vitro cytokinesis-block micronucleus test at any of the considered time points. The observation of a small but not statistically significant increase in micronucleated binucleated cells 72 h after bleomycin exposure suggests a potential of PBMC as reporter cells for genotoxic damage induced in the lung, when the agent is, at least partially, systemically distributed. This is not the case for a particulate agent such as the WC-Co mixture. Therefore, there is a need to re-evaluate cautiously the relevance of PBMC as reporter cells to assess lung effects induced by inhaled particulate agents. In order to draw firm conclusions, other relevant positive controls should be tested and further confirmation with regard to biomonitoring strategies is required.

In summary, this study introduced the use of isolated type II pneumocytes for the assessment of in vivo genotoxicity. WC-Co particles are able to induce micronuclei in type II pneumocytes of the rat after i.t. instillation. Further characterization of micronuclei with pan-centromeric probes to distinguish between the induction of chromosome and genome mutations would provide valuable mechanistic information. Parallel decreased DNA migration in BAL cells reflected change in BAL cell populations, accompanying the inflammatory response. No corresponding change in micronucleus frequencies could be detected in PBMC. Induction of apoptosis is another endpoint that should be taken into account, as it may specifically eliminate mutated cells. It could also be considered to look at more relevant target cell types for lung cancer (e.g. bronchial epithelial cells) and the specific involvement of inflammatory cells in genotoxic effects.

Acknowledgements

The authors wish to thank E. Verbeken (MD, PhD) of the Laboratory of Morphology and Molecular Pathology (Catholic University of Leuven) for examining the lung tissue samples. This study was supported by the Belgian Federal Offices for Scientific, Technical and Cultural Affairs (contract PS/04/35) and by FRSM grant no. 3.4505.03F.

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Received January 7, 2003; revised and accepted August 5, 2003