

# Tungsten Carbide-Cobalt as a Nanoparticulate Reference Positive Control in *In Vitro* Genotoxicity Assays

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**With the increasing human exposure to nanoparticles (NP), the evaluation of their genotoxic potential is of significant importance. However, relevance for NP of the routinely used *in vitro* genotoxicity assays is often questioned, and a nanoparticulate reference positive control would therefore constitute an important step to a better testing of NP, ensuring that test systems are really appropriate. In this study, we investigated the possibility of using tungsten carbide-cobalt (WC-Co) NP as reference positive control in *in vitro* genotoxicity assays, including 2 regulatory assays, the mouse lymphoma assay and the micronucleus assay, and in the Comet assay, recommended for the toxicological evaluation of nanomedicines by the French Agency of Human Health Products (Afssaps). Through these assays, we were able to study different genetic endpoints in 2 cell types commonly used in regulatory genotoxicity assays: the L5178Y mouse lymphoma cell line and primary cultures of human lymphocytes. Our results showed that the use of WC-Co NP as positive control in *in vitro* genotoxicity assays was conceivable, but that different parameters have to be considered, such as cell type and treatment schedule. L5178Y mouse lymphoma cells did not provide satisfactory results in the 3 performed tests. However, human lymphocytes were more sensitive to genotoxic effects induced by WC-Co NP, particularly after a 24-h treatment in the *in vitro* micronucleus assay and after a 4-h treatment in the *in vitro* Comet assay. Under such conditions, WC-Co could be used as a nanoparticulate reference positive control in these assays.**

**Key Words:** nanoparticle; comet assay; micronucleus assay; mouse lymphoma assay; regulatory test.

Nanoparticles (NP) have interesting physico-chemical properties, which explain their numerous applications. A NP is defined in the ISO/TS 27687 technical specification (ISO, 2008) as a material having its 3 dimensions in the nanoscale, (ie, between 1 and 100 nm). NP are used in many industries and are nowadays present in many consumer goods, resulting in an increasing human exposure. The question of their

toxicity, particularly their genotoxicity, is thus worth considering. Recent reviews indeed described different potential mechanisms for NP genotoxicity (Donaldson *et al.*, 2010; Magdolenova *et al.*, 2013). Genotoxicity may be mediated in the absence of inflammation by primary mechanisms, which can be classified as DNA reactive and non-DNA reactive. DNA-reactive mechanisms can be caused by NP themselves, as well as by reactive oxygen species (ROS) at the NP surface, which can induce oxidative DNA damage. Non-DNA-reactive mechanisms can be due to the interaction of NP with the cellular constituents involved in chromosome segregation during mitosis (eg, mitotic spindle), leading to aneuploidy. Non-DNA-reactive mechanisms can also result from an increase in the ROS production by cellular constituents (eg, mitochondria) induced by their interaction with NP and/or consecutively to the depletion in cellular antioxidants. Non-DNA-reactive mechanisms may also result from an inhibition of DNA repair. Finally, genotoxicity may also occur in the presence of inflammation through secondary mechanisms because of the generation of ROS by inflammatory cells.

However, genotoxicity assays routinely used to test bulk materials may not be perfectly adapted to nanomaterials (Doak *et al.*, 2009; Landsiedel *et al.*, 2009; Magdolenova *et al.*, 2012). One example is the Ames test, a commonly used bacterial reversion mutation test, which is probably not relevant for the testing of NP. Indeed, endocytosis does not occur in bacteria, and nanomaterials may not be able to diffuse through the bacterial wall, possibly leading to false-negative results (Doak *et al.*, 2012; Singh *et al.*, 2009). Some other tests require specific adjustments. For instance, cytochalasin B, used in the cytokinesis-block micronucleus assay, has been shown to inhibit the uptake of NP into the cells when added concomitantly with the NP (Doak *et al.*, 2009; Landsiedel *et al.*, 2009; Magdolenova *et al.*, 2012). Therefore, one of the recommended treatment schedules (OECD, 2010) for a long-term treatment, consisting of simultaneous exposure of cells with tested product and cytochalasin

B, may not be relevant for NP. The surrogate option, consisting in the addition of cytochalasin B after the exposure to NP, may therefore be preferable.

Another weakness, frequently pointed out, is the absence of any adequate nanoscaled reference positive control (Gonzalez *et al.*, 2008; Landsiedel *et al.*, 2009; Warheit and Donner, 2010). The finding that tungsten carbide-cobalt (WC-Co) microparticles were used as positive control in *in vitro* and *ex vivo* micronucleus assay in a rat lung epithelial (RLE) cell line and in rat type II pneumocytes (Muller *et al.*, 2008) lead us to study more specifically the nanoparticulate form of this alloy. WC-Co is used notably in the hard metal industry for the production of cutting tools and wear-resistant surfaces for its hardness. Occupational exposure to WC-Co has been associated with an increased risk of lung cancer (Moulin *et al.*, 1998; Wild *et al.*, 2000), and WC-Co was classified as probably carcinogenic to humans (group 2A) by the International Agency for Research on Cancer (IARC, 2006).

Most genotoxicity studies on WC-Co were conducted on microsized particles. *In vivo*, WC-Co microparticles induced positive responses in the comet and the micronucleus assays in rat type II pneumocytes (De Boeck *et al.*, 2003a). *In vitro*, they were found positive in the comet assay and the micronucleus test in human lymphocytes (Anard *et al.*, 1997; De Boeck *et al.*, 1998; Van Goethem *et al.*, 1997). More recently, WC-Co NP were studied in *in vitro* cytotoxicity assay in CaCo-2, HaCaT, A549, and OLN-93 cell lines and in primary rat brain cells (Bastian *et al.*, 2009), and in a micronucleus assay in HepG2 cell line (Kühnel *et al.*, 2012). Moreover, a comparison between WC-Co microparticles (average size: 4  $\mu$ m) and NP (average size: 80 nm) showed that NP were more reactive, inducing a greater oxidative stress (Ding *et al.*, 2009).

The aim of this study was to investigate the possibility of using WC-Co NP as reference positive control in *in vitro* genotoxicity assays. To accomplish this, the effects of WC-Co NP were studied in 2 regulatory tests, the mouse lymphoma assay (MLA-TK) and the micronucleus assay, and in the Comet assay, recommended for the toxicological evaluation of nanomedicines by the French Agency of Human Health Products (Afssaps, 2011). Different endpoints were covered by these tests: mutagenic effects with the mouse lymphoma assay, chromosomal damage with the micronucleus assay, and primary DNA damage with the comet assay.

## MATERIALS AND METHODS

### Chemicals

RPMI 1640, horse serum, fetal calf serum (FCS), nonessential amino acids, and PBS were purchased from GIBCO Invitrogen (Cergy-Pontoise, France). Giemsa reagent, penicillin, streptomycin, amphotericin B, mitomycin C (MMC, CAS No. 50-07-7), methyl methanesulfonate (MMS, CAS No. 66-27-3), Triton X-100, EDTA, trizma base, propidium iodide, KCl, NaCl, sodium bicarbonate, sodium pyruvate, trifluorothymidine (TFT), pluronic F68 solution, cytochalasin B, and bovine serum albumin (BSA) were obtained from

Sigma-Aldrich (Saint-Quentin Fallavier, France). Normal melting point agarose (NMPA) and low melting point agarose (LMPA) were purchased from Bio-rad (Marnes-la-Coquette, France); acetic acid was from VWR (Fontenay-sous-bois, France); dimethyl sulfoxide (DMSO) was from Acros Organics (Noisy le Grand, France); NaOH, L-glutamine, and absolute ethanol were from Merck (Darmstadt, Germany); heparin was from Choay (France); phytohemagglutinin A (PHA) was from Remel via Oxoid (Dardilly, France); and sterile water was from Fresenius Kabi (Sevres, France).

### Characteristics of WC-Co NP

WC-Co NP (8% wt/wt Co) were purchased from Nanostructured and Amorphous Materials, Inc. (Houston, Texas). Certificate of analysis indicated 8.26% wt/wt Co with 0.73% wt/wt additive Cr and 0.48% wt/wt additive V. Purity was 99.5%, with major impurities being O (0.2% wt/wt) and Fe (0.056% wt/wt). Average particle size given by the supplier was 60–250 nm; specific surface area was 1.5 m<sup>2</sup>/g. Crystallographic systems were hexagonal for WC and cubic for Co.

### Preparation of WC-Co NP Suspensions

Initial suspensions were prepared following a protocol described by Jensen *et al.* (2011). A prewetting step was performed with 0.5% vol/vol ethanol and the appropriate volume of a solution of BSA, hereafter mentioned as BSA-water, was added. BSA-water was prepared by dissolving 0.05% wt/vol BSA in distilled water and sterile-filtered. Dispersion was obtained by probe sonication for 16 min at 400 W and 10% amplitude using a 400 Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, Connecticut). NP suspensions were cooled on ice to minimize heat development during sonication.

Different concentrations of initial suspensions were prepared by diluting the dispersed suspension in BSA-water. These initial suspensions were then diluted 1:9 vol/vol in cell culture medium at the time of cell treatment to obtain final suspensions. Because of the low stability of WC-Co NP suspensions, initial and final suspensions were carefully vortexed before each pipetting.

### Characterization of WC-Co NP Suspensions

Dynamic light scattering (DLS) measurements were performed to measure the hydrodynamic diameter of WC-Co NP and agglomerates/aggregates using a Zetasizer Nano-ZS instrument (Malvern Instruments, Orsay, France). The polydispersity index, reflecting the range of NP and agglomerates/aggregates size, was also measured, with a value > 0.2 indicating a polydispersed size distribution. Measurements were carried out on WC-Co NP suspensions in BSA-water and in the 2 culture media used in this study, containing 10% and 20% vol/vol serum. Before measurements, suspensions were diluted in ultrapure water.

Zeta potential measurements were also performed using the Zetasizer Nano-ZS instrument on WC-Co NP suspensions in BSA-water and in culture media. pH was approximately 8 in all analyzed suspensions.

A transmission electron microscopy (TEM) analysis was performed on WC-Co NP suspensions in BSA-water and in culture media. One drop of NP suspension was deposited on a formvar-coated copper grid and dried. Observations were carried out at  $\times 10\,000$  and  $\times 40\,000$  magnifications using a Hitachi H7500 transmission electron microscope equipped with a 1 MP digital camera from AMT (Elexience, France). Analysis of size distribution of isolated NP was performed from TEM images of WC-Co NP suspension in complete RPMI medium using ImageJ software (Supplementary Figure 1).

### Cell Culture

**L5178Y cells.** L5178Y tk<sup>+/−</sup> mouse lymphoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia). Cells were thawed and seeded at a concentration of  $1.33 \times 10^5$  cells/ml in RPMI 1640 supplemented with 10% vol/vol heat-inactivated horse serum, 2 mg/ml sodium bicarbonate, 10 ml/l nonessential amino acids, 200 UI/ml penicillin, 5  $\mu$ g/ml streptomycin, 2.5  $\mu$ g/ml amphotericin B, 0.02% wt/vol L-glutamine, 0.02 mg/ml sodium pyruvate, 0.05% vol/vol pluronic F68 solution (hereafter referred to

as RPMI 10) and incubated in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. RPMI 20 corresponds to RPMI 10 but with 20% vol/vol heat-inactivated horse serum.

**Human lymphocytes.** Whole blood was obtained by venipuncture from nonsmoking male and female healthy donors younger than 45 years, not recently exposed to any known mutagen or radiation, into lithium heparin vacutainer tubes. For each condition, 0.5 ml whole blood was diluted 1:9 with complete RPMI medium, composed of RPMI 1640 medium supplemented with 20% vol/vol heat-inactivated FCS, 80 UI/ml heparin, 0.024% wt/vol L-glutamine, 400 UI/ml penicillin, 0.2 mg/ml streptomycin, and PHA (2%) for 44 h in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>.

#### Cell Treatment

**L5178Y cells.** Duplicate cultures of L5178Y cells were treated for 4 or 24 h in RPMI 10 with different concentrations of WC-Co NP. For all treatments except for the 4-h treatment in the MLA-TK, 1 ml of treatment suspension was added to 9 ml of cell suspension in RPMI 10 medium (cell concentrations of  $1.25 \times 10^5$  cells/ml for the 24-h MLA-TK,  $2 \times 10^5$  cells/ml for the 4-h comet and micronucleus assays, and  $5 \times 10^4$  cells/ml for the 24-h comet and micronucleus assays). For the 4-h treatment in the MLA-TK, 1 ml of treatment suspension was added to 8.5 ml of cell suspension in RPMI 10 medium (cell concentration of  $5 \times 10^5$  cells/ml) and 0.5 ml of KCl 150mM. Positive control for the MLA-TK and the comet assay was MMS at a final concentration of, respectively, 10 and 2 µg/ml for the 4-h and the 24-h treatments in the MLA-TK and 15 and 4 µg/ml for the 4-h and the 24-h treatments in the comet assay. Positive control for the micronucleus assay was MMC at a final concentration of 0.2 or 0.025 µg/ml for the 4-h and 24-h treatments. Treatments were conducted in a humidified atmosphere at 37°C with 5% CO<sub>2</sub> and under agitation on a rotor to allow interaction between NP and cells.

**Human lymphocytes.** At the end of the preculture, PHA-stimulated human lymphocytes (whole blood) were incubated during 4 or 24 h with different concentrations of WC-Co NP. Treatment suspensions were diluted 1:9 in complete RPMI medium containing blood. Positive control for the comet assay was MMS at a final concentration of 10 µg/ml for both treatment times. Positive control for the micronucleus assay was MMC at a final concentration of, respectively, 0.15 and 0.075 µg/ml for the 4-h and 24-h treatments. Treatments were conducted in a shaking water bath at 37°C.

#### L5178Y tk<sup>+/−</sup> MLA-TK

MLA-TK (microwell method) was performed in compliance with the OECD Test Guideline 476 (OECD, 1997) but only without metabolic activation. At the end of the treatment period, cells were washed with RPMI 10, counted using a hemocytometer, replated in RPMI 20 medium (mean of 1.6 cells/well, 2 microtiter plates/concentration), and placed in an incubator at 37°C and 5% CO<sub>2</sub>.

The remaining cells were incubated at a density of  $2 \times 10^5$  cells/ml in RPMI 10 medium for a 48-h expression time. Cell counts were performed 24 and 48 h after incubation in order to maintain exponential growth during all the expression time. Then the cells were plated: firstly in RPMI 20 medium at 1.6 cells/well (2 plates/concentration) to determine both the relative total growth and the plating efficiency at day 2, and secondly in selective medium containing 3 µg/ml TFT at  $2 \times 10^3$  cells/well (4 plates/dose) to determine mutation frequency (MF). After an incubation time of 10–14 days at 37°C and 5% CO<sub>2</sub>, plates containing cells in nonselective medium were scored for negative wells for assessment of survival rate and relative total growth (RTG), whereas plates containing TFT-selective medium were scored independently for “large” and “small” TFT-resistant colonies. The criteria for acceptance were the ones recommended (Moore *et al.*, 2006): Positive responses were defined as those where the induced MF in treated cultures exceeded the global evaluation factor of + 126 mutants per 10<sup>6</sup> cells, associated with a dose-related increase in MF.

#### Alkaline Comet Assay

The comet assay was performed under alkaline conditions (pH > 13) in compliance with a previously described protocol (Singh *et al.*, 1988; Tice *et al.*, 2000).

At the end of the treatment period, L5178Y cells were washed and resuspended in RPMI 10. Viable cells were counted using Trypan blue exclusion. For the comet assay,  $4 \times 10^4$  viable cells were mixed with 0.5% wt/vol LMPA kept at 37°C, whereas the remaining cell suspension was incubated in a humidified atmosphere at 37°C with 5% CO<sub>2</sub> for a 20-h recovery period before harvest for the micronucleus test and the cytotoxicity assessment (see hereafter Micronucleus assay in L5178Y cells section).

At the end of the treatment period, human lymphocytes were washed and centrifuged, supernatant was discarded, and 30 µl of the cell pellet was mixed with 0.5% wt/vol LMPA kept at 37°C for the comet assay, whereas the remaining cells were resuspended in complete RPMI and incubated in presence of 6 µg/ml cytochalasin B in a humidified atmosphere at 37°C with 5% CO<sub>2</sub> for a 20-h recovery period before harvest for the cytokinesis-block micronucleus assay and the cytotoxicity assessment (see hereafter Cytokinesis-block micronucleus assay in human lymphocytes section).

Cells embedded in LMPA were spread onto regular precoated microscopic slides (1.5% and 0.8% wt/vol NMPA). For each concentration and culture, 2 duplicate slides were prepared. All the following steps were sheltered from daylight to prevent the occurrence of additional DNA damage. Slides were immersed for at least 1 h at 4°C in a cold lysing solution (2.5M NaCl, 100mM EDTA, 10mM Trizma Base, pH 10, supplemented with 1% vol/vol Triton X-100 and 10% vol/vol DMSO) and washed in distilled water. The slides were then placed in a horizontal tank filled with fresh electrophoresis solution (1mM EDTA and 300mM NaOH, pH > 13) for 20 min to allow DNA unwinding and expression of single-strand breaks and alkali-labile sites. Next, electrophoresis was performed for 20 min using an electric current of 0.7V/cm. Slides were then placed for 10 min in a neutralization solution (0.4M Trizma base, pH 7.5), and gels were dehydrated by immersion in absolute ethanol for 5 min. Finally, slides were air-dried and stored at room temperature.

Slides were independently coded and analyzed blind after addition of propidium iodide (20 µg/ml in distilled water) and a coverslip on each slide. Slides were then examined at  $\times 250$  magnification using a fluorescence microscope (Leica Microscopy and Scientific Instruments Group, Heerbrugg, Switzerland) equipped with an excitation filter of 515–560nm and a 590-nm barrier filter, connected through a gated CCD camera to Comet Image Analysis System software, version 4.0 (Perceptive Instruments Ltd., Haverhill, United Kingdom). Four hundred randomly selected cells per test condition (100 cells from each of the 2 replicate slides in the 2 cultures) were scored. Tail intensity, defined as the percentage of DNA that had migrated from the head of the comet into the tail, was used as the measure of damage. Ghost cells, corresponding to either genotoxic or cytotoxic damage, were enumerated for the 100 cells per slide. Results were expressed as the percentage of ghost cells amongst total cells.

#### Micronucleus Test

Micronucleus test was performed in compliance with the OECD Test Guideline 487, but only without metabolic activation, in the presence of cytochalasin B in human lymphocytes and in the absence of cytochalasin B in L5178Y mouse lymphoma cells.

**Micronucleus test in L5178Y cells.** At the end of the 20-h recovery period, cells were counted using a hemocytometer, and the relative population doubling (RPD) was calculated to assess cytotoxicity. After centrifugation at 1000rpm for 6 min, supernatant was discarded and cells were treated for 4 min with a hypotonic solution (RPMI 1640 medium diluted 1:1 in distilled water). After the hypotonic shock, a pre-fixation step was performed by adding cold Carnoy's fixative (absolute ethanol/glacial acetic acid, 3:1 vol:vol). Cells were then centrifuged and suspended in Carnoy's fixative for 24 h at 4°C. After another centrifugation, cells were resuspended in 0.5 ml of cold Carnoy's fixative and spread on duplicate glass slides. Slides were air-dried at least overnight and stained for 10 min with 4% vol/vol Giemsa in water.

Slides were independently coded. Micronuclei, identified according to recommended criteria (Fenech *et al.*, 2003), were scored at  $\times 500$  magnification in 1000 intact mononucleated cells per slide. For each concentration, micronuclei were scored in 2000 cells per duplicate culture.

**Cytokinesis-block micronucleus assay in human lymphocytes.** At the end of the 20-h recovery period, cells were washed and centrifuged at 1000 rpm for 6 min. Cells were then treated for 10 min with a hypotonic solution (RPMI 1640 diluted 1:4 in distilled water supplemented with FCS 2% vol/vol). At the end of the hypotonic shock, a prefixation step was performed by adding cold Carnoy's fixative (absolute ethanol/glacial acetic acid, 3:1 vol:vol). Cells were then centrifuged and fixed with cold Carnoy's fixative for at least 10 min at room temperature. After another centrifugation, cells were spread on duplicate glass slides. Slides were air-dried at least overnight and stained for 10 min with 5% vol/vol Giemsa in water.

As a measure for cell cycle delay and/or cytotoxicity, the replication index (RI) was calculated. Slides were finally independently coded. Micronuclei, identified according to recommended criteria (Fenech *et al.*, 2003), were scored at  $\times 500$  magnification in 500 intact binucleated cells and 500 intact mononucleated cells per slide. For each concentration, the frequency of micronuclei was obtained from 1000 binucleated cells and 1000 mononucleated cells per duplicate culture.

#### Statistical Analysis

In MLA-TK, each treatment was compared with the control using the one-sided Dunnett's test for multiple comparisons with the same control.

For the comet assays, the nonparametric Mann-Whitney *U* test was used to evaluate the statistical difference in tail intensity between each dose and the negative control. Statistical analyses were performed with StatView Software (version 5.0, SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27513). Differences were considered statistically significant ( $p < .05$ ). Percentage of ghost cells in treated groups was compared to control with the chi-square test. Differences were considered statistically significant ( $p < .05$ ).

For the micronucleus assays, the statistical significance of difference between groups was determined using the chi-square test. Differences were considered statistically significant ( $p < .05$ ).

## RESULTS

### Characterization of WC-Co NP Suspensions

Initial WC-Co NP suspension in BSA-water and final suspensions in RPMI 10 medium and complete RPMI medium were characterized in terms of size and zeta potential (Table 1). In each analyzed suspension, size measurements by DLS were not relevant because of the broad-ranged size distribution, indicated by a high polydispersity index. This was particularly noticeable in initial suspension, and confirmed by the TEM

**TABLE 1**  
**Size Dispersion and Zeta Potential of WC-Co NP Suspensions in Different Media**

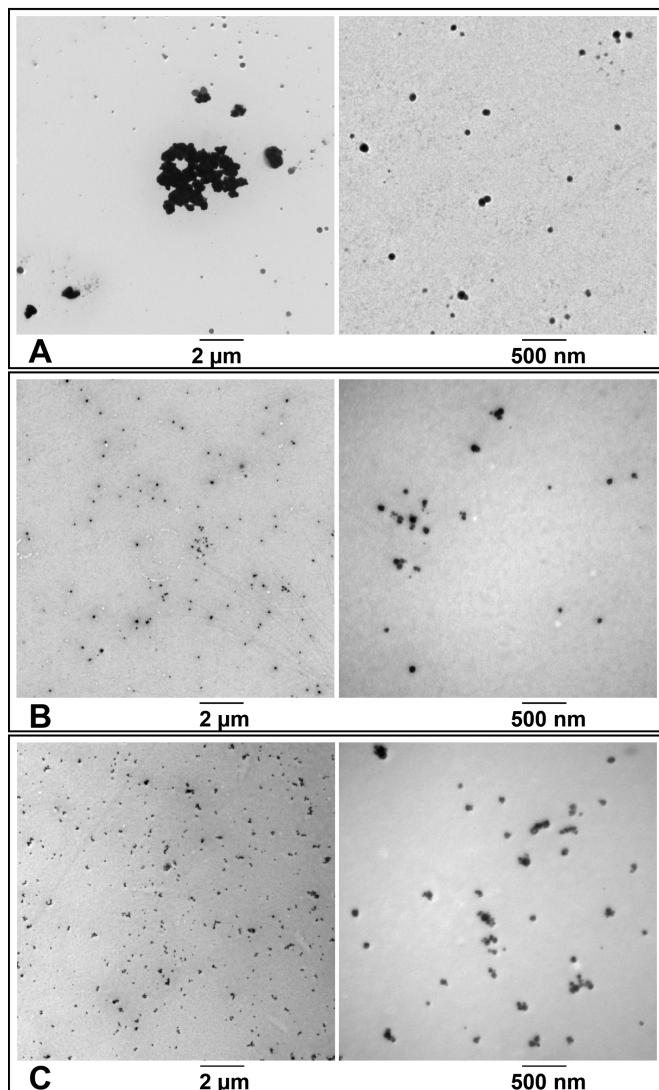
Suspension Medium	Polydispersity Index	Zeta Potential (mV)
BSA-water <sup>a</sup>	0.643	-26.7
RPMI 10 <sup>b</sup>	0.428	-8.96
Complete RPMI <sup>c</sup>	0.386	-8.81

*Note.* <sup>a</sup>0.05% wt/vol BSA in water.

<sup>b</sup>RPMI medium with 10% vol/vol horse serum.

<sup>c</sup>RPMI medium with 20% vol/vol fetal calf serum.

analysis, showing both isolated NP and agglomerates or aggregates of different sizes, up to a few micrometers. (Fig. 1A). In return, in final suspensions in both culture media, size distribution seemed to be narrower, with the presence of isolated NP and agglomerates or aggregates of limited size, according to the slightly lower polydispersity indexes and the TEM analysis (Figs. 1B and C). These final suspensions were characterized by a higher protein content than initial suspension. A similar observation was made on WC and WC-Co NP, which aggregated in medium without FCS, but not in presence of 5% or 10% vol/vol FCS (Bastian *et al.*, 2009; Kühnel *et al.*, 2009, 2012). Size distribution of isolated NP measured from TEM images of WC-Co NP suspension in complete RPMI medium ranged from 20 to 160 nm, with 67.8% NP between 50 and



**FIG. 1.** TEM images of tungsten carbide-cobalt nanoparticles initial (A) and final suspensions obtained from 1:9 dilution of initial suspensions in RPMI 10 medium (10% vol/vol horse serum) (B) or complete RPMI medium (20% vol/vol fetal calf serum) (C).

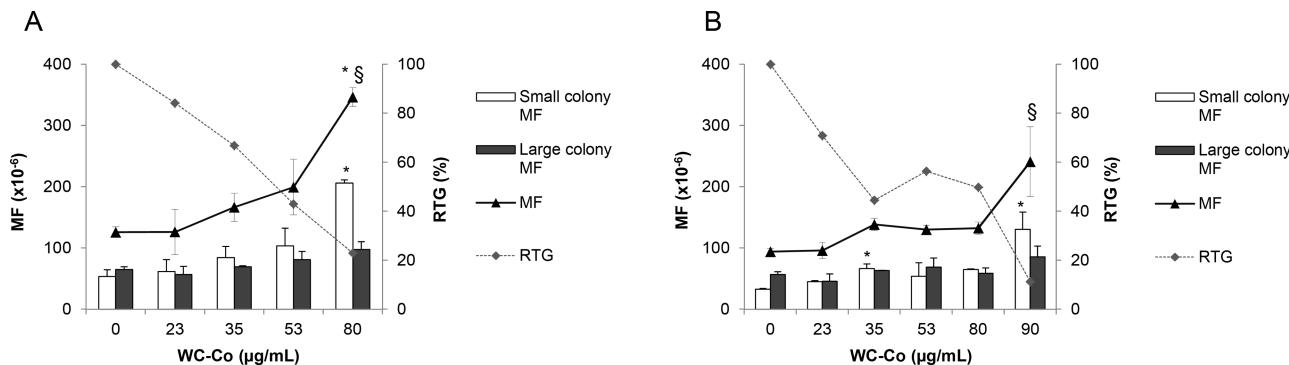
90 nm (Supplementary Figure 1). Zeta potential was negative for all suspensions, and absolute value decreased between initial and final suspensions.

#### MLA-TK

MLA-TK was performed in L5178Y cells after 4 and 24 h of treatment. WC-Co NP concentrations were chosen following preliminary cytotoxicity studies (data not shown), with the highest analyzed concentration resulting in a RTG approximately between 10% and 20%, as requested in the OECD Test Guideline 476 (OECD, 1997). The RTG was 22.9% at the highest concentration of 80  $\mu\text{g}/\text{ml}$  in the 4-h assay and 11.2% at the highest concentration of 90  $\mu\text{g}/\text{ml}$  in the 24-h assay. According to the acceptance criteria for negative and positive controls (Moore *et al.*, 2006), the suspension growth value of the negative control (mean of the 2 cultures) ranged from 8 to 32 in the 4-h assay (suspension growth of 16), and above 32 in the 24-h assay (suspension growth of 61). The cloning efficiency of the negative control (mean of the 2 cultures) ranged from 65% to 120% (94% for the 4-h assay and 108% for the 24-h assay) at the time of mutant selection. The MF of the negative control ranged from  $50 \times 10^{-6}$  to  $170 \times 10^{-6}$  mutants ( $126 \times 10^{-6}$  for the 4-h assay and  $94 \times 10^{-6}$  for the 24-h assay). MMS, used as positive control, yielded a small colony induced mutation frequency (IMF) of  $577 \times 10^{-6}$  for the 4-h treatment and  $182 \times 10^{-6}$  for the 24-h treatment, ie, higher than  $150 \times 10^{-6}$ .

After the 4-h treatment, WC-Co NP induced a concentration-related increase in MF, statistically and biologically significant, ie, with an IMF greater than the global evaluation factor of  $126 \times 10^{-6}$ , at the highest analyzed concentration of 80  $\mu\text{g}/\text{ml}$  (Fig. 2A). After 24 h of treatment, WC-Co NP also induced a biologically significant increase in the MF of total mutants at the highest concentration of 90  $\mu\text{g}/\text{ml}$  (Fig. 2B).

Interestingly, small colony MF was particularly increased, with a statistically significant increase at the highest concentration in both treatment schedules.



**FIG. 2.** MF ( $\times 10^{-6}$ ), small colony MF ( $\times 10^{-6}$ ) and large colony MF ( $\times 10^{-6}$ ) induced by WC-Co NP in L5178Y cells after a 4-h (A) or 24-h (B) treatment. Cytotoxicity was assessed by the RTG (% of negative control). Mean  $\pm$  SD data for 2 duplicate cultures. The IMF for methyl methanesulfonate, used as positive control, was  $880 \times 10^{-6}$  in the 4-h treatment (10  $\mu\text{g}/\text{ml}$ ) and  $258 \times 10^{-6}$  in the 24-h treatment (2  $\mu\text{g}/\text{ml}$ ). \* $p < .05$  (Dunnett's test). §Biologically significant ( $>$  global evaluation factor, + 126). Abbreviations: IMF, induced mutation frequency; MF, mutation frequency; RTG, relative total growth.

#### Micronucleus Assay in L5178Y Cells

Micronuclei formation was assessed in L5178Y cells, after 4 or 24 h of treatment with WC-Co NP, followed by a 20-h recovery period. Studied concentrations were chosen after preliminary cytotoxicity assays (data not shown) and induced a RPD greater than  $45 \pm 5\%$ , as recommended by the OECD Test Guideline 487 (OECD, 2010). The RPD was 43.2% for the highest analyzed concentration after the short-term treatment and 51.7% after the long-term treatment. The population doubling value of the negative control was 1.7 in the short-term treatment and 3.5 in the long-term treatment.

WC-Co NP induced a statistically significant increase in the number of micronucleated cells (MN cells) at the concentrations of 80 and 100  $\mu\text{g}/\text{ml}$  in the 4-h treatment (Fig. 3A) and at the concentration of 80  $\mu\text{g}/\text{ml}$  in the 24-h treatment (Fig. 3B). These increases, associated with decreased RPD, were relatively weak, with only twice the number of micronuclei of the negative control.

#### Cytokinesis-Block Micronucleus Assay in Human Lymphocytes

Micronuclei formation was also assessed in human lymphocytes from 2 different donors, after 4 or 24 h of treatment with WC-Co NP, followed by a 20-h recovery period with cytochalasin B. Studied concentrations were chosen after preliminary cytotoxicity assays and induced a RI greater than  $45 \pm 5\%$ , as recommended by the OECD Test Guideline 487 (OECD, 2010). The RI was 54.4% for the highest analyzed concentration after the short-term treatment and 59.8% after the long-term treatment. The cytokinesis-block proliferation index (CBPI) value of the negative control was 1.89 in the short-term assay and 1.75 in the long-term assay. Binucleated and mononucleated MN cells were scored, as aneugenic compounds may induce an increase in the number of mononucleated MN cells (Elhajouji *et al.*, 1998).

WC-Co NP induced a statistically significant increase in the number of binucleated MN cells at the intermediate

concentration of 80  $\mu\text{g}/\text{ml}$  after the 4-h treatment (Fig. 4A) and at each analyzed concentration after the 24-h treatment (Fig. 4B). This was associated with a decrease in RI. After the long-term treatment, the increase in the number of binucleated MN cells was thus more important, and a statistically significant increase in the number of mononucleated MN cells was also observed.

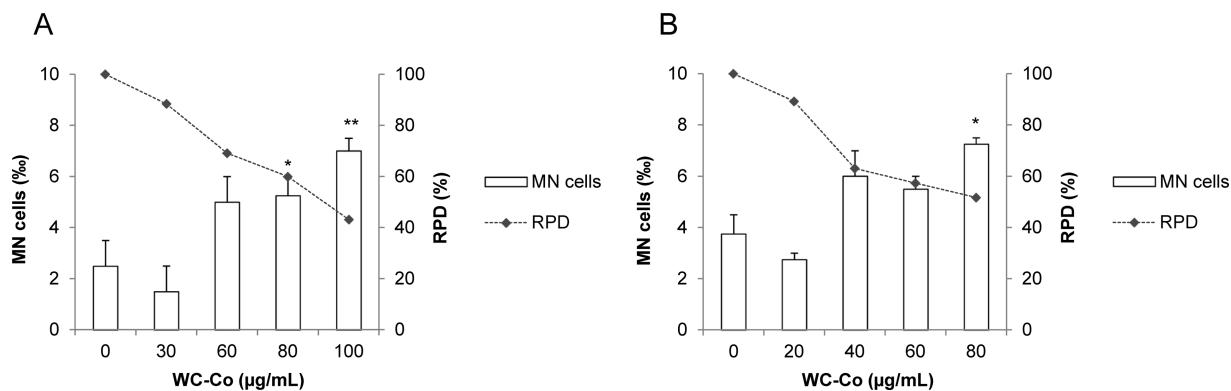
#### Comet Assay in Human Lymphocytes and in L5178Y Cells

Primary DNA damage was assessed using the comet assay. Cytotoxicity was measured with the same methods as those used for the micronucleus assays, ie, after the 1.5–2 cell cycle recovery periods following treatments, ensuring evaluation of viable cells at the selected concentrations. For technical convenience, comet assays may have been performed separately or concurrently to micronucleus assays. In the case of separate assays, analyzed concentrations in the comet assay and in the micronucleus assay might be different, due to a slight variability of cytotoxicity between the 2 assays. However, this had no impact on the interpretation. In human lymphocytes, the RI was

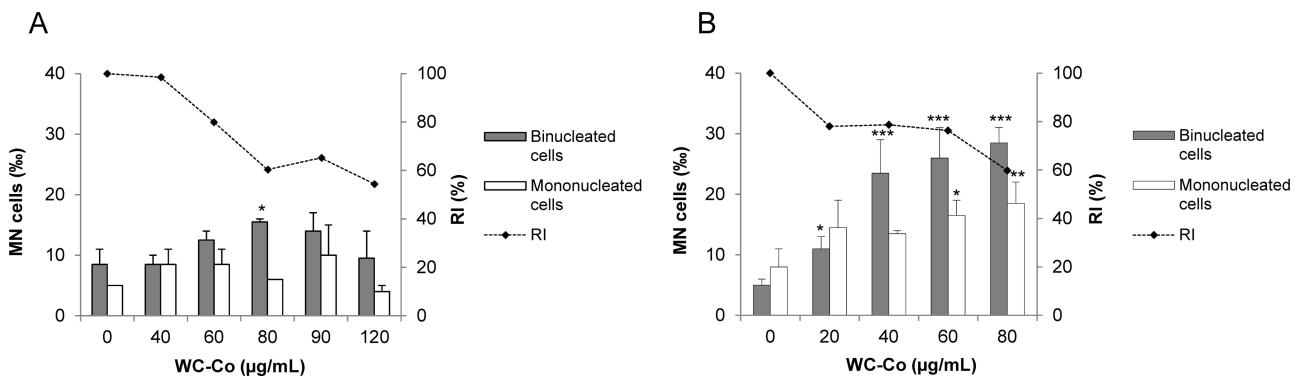
46.2% for the highest concentration of 120  $\mu\text{g}/\text{ml}$  in the short-term assay and 52.5% for the highest concentration of 60  $\mu\text{g}/\text{ml}$  in the long-term assay. In L5178Y cells, the RPD was 43.2% for the highest concentration of 100  $\mu\text{g}/\text{ml}$  in the short-term assay and 55.1% for the highest concentration of 80  $\mu\text{g}/\text{ml}$  in the long-term assay.

In human lymphocytes, a concentration-dependent increase in DNA migration was observed after 4 h of treatment. The response was statistically significant at the concentrations of 60, 80, and 120  $\mu\text{g}/\text{ml}$  (Fig. 5A). The percentage of ghost cells increased alongside DNA migration, showing the same dose-response relationship. Based on the simultaneous increase in DNA fragmentation and cytotoxicity data in the associated micronucleus assay (Fig. 4A), ghost cells could represent both genotoxic and cytotoxic damage.

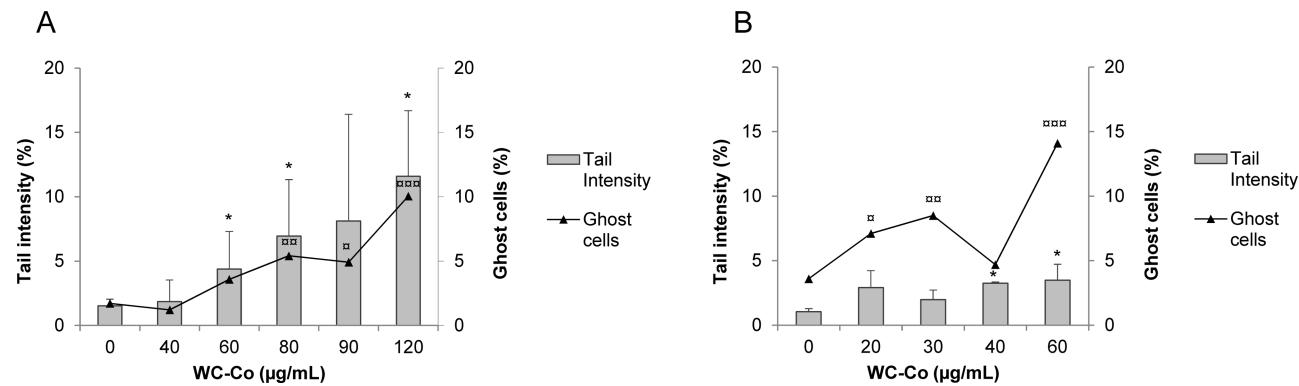
Interestingly, an important variability of response was observed between the two donors. To confirm this observation, a second assay was performed using the same donors, and similar results were obtained. Figure 6 represents DNA migration induced by WC-Co NP for each donor and experiment.



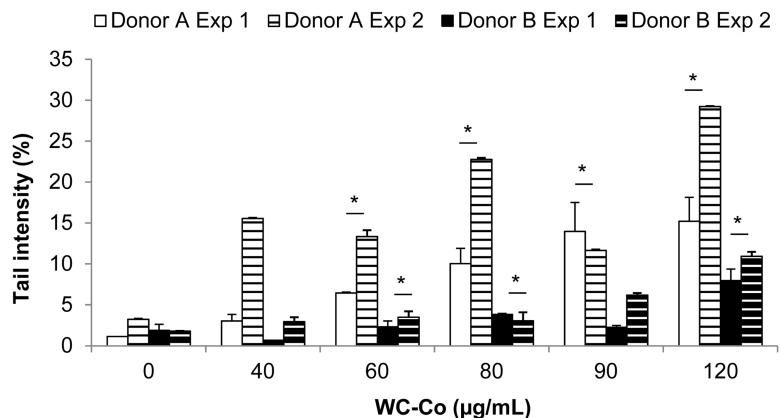
**FIG. 3.** Frequency of MN cells (%) in L5178Y cells after a 4-h (A) or 24-h (B) treatment with WC-Co NP followed by a 20-h recovery period. Cytotoxicity was assessed by the RPD (% of negative control). Micronuclei were scored in 1000 intact cells per duplicate slide per culture. Mean  $\pm$  SD data for 2 duplicate cultures. Mitomycin C was used as positive control and induced 82% and 85.5% MN cells in the short-term and long-term treatment (0.2 and 0.025  $\mu\text{g}/\text{ml}$ ). \* $p < .05$ ; \*\* $p < .01$  (chi-square test). Abbreviations: MN, micronucleated; RPD, relative population doubling.



**FIG. 4.** Frequency of MN cells (%) in human lymphocytes after a 4-h (A) or 24-h (B) treatment with WC-Co NP followed by a 20-h recovery period in presence of cytochalasin B. Cytotoxicity was assessed by the RI (% of negative control). Micronuclei are scored in 500 binucleated and 500 mononucleated cells per duplicate slide per donor. Mean  $\pm$  SD data for 2 donors. Mitomycin C, used as positive control, induced 77% and 9% binucleated and mononucleated MN cells in the short-term treatment (0.15  $\mu\text{g}/\text{ml}$ ) and 184% and 68% binucleated and mononucleated MN cells in the long-term treatment (0.075  $\mu\text{g}/\text{ml}$ ). \* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$  (chi-square test). Abbreviations: MN, micronucleated; RI, replication index.



**FIG. 5.** DNA migration (tail intensity, %) and frequency of ghost cells (%) in human lymphocytes after a 4-h (A) or 24-h (B) treatment with WC-Co NP. A total of hundred cells were scored per duplicate slide per donor. Mean  $\pm$  SD data for 2 donors. Methyl methanesulfonate (10 µg/ml) was used as positive control and induced 20.5% DNA migration and 34.1% ghost cells after the 4-h treatment and 8.9% DNA migration and 20.7% ghost cells after the 24-h treatment.  $^*p < .05$  (Mann-Whitney test);  $^{*p} < .05$ ;  $^{**p} < .01$ ;  $^{***p} < .001$  (chi-square test).



**FIG. 6.** DNA migration (tail intensity, %) in human lymphocytes after a 4-h treatment with WC-Co NP, determined in 2 experiments with the same 2 donors. A total of hundred cells were scored per slide. Mean  $\pm$  SD data for 2 duplicate slides. \*Statistically significant difference ( $p < .05$ , Mann-Whitney test) between treated cells and negative control for the combined results of the 2 experiments for each donor.

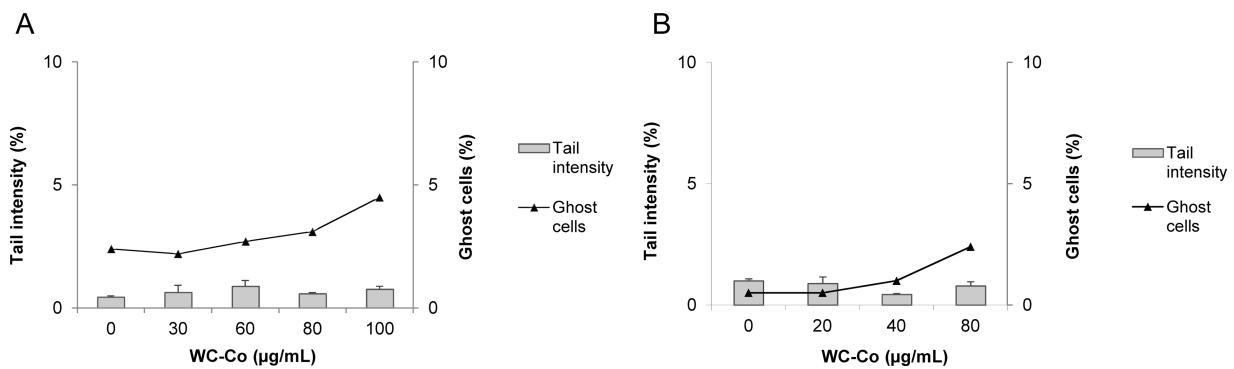
DNA migration was greater for donor A than for donor B in both experiments, and an interexperiment heterogeneity was also noticed. However, when combining results of the 2 experiments for each donor, a statistically significant increase was still observed for both donors at the WC-Co NP concentrations of 60, 80, and 120 µg/ml compared with the negative control values.

After 24 h of treatment, a statistically significant increase in DNA migration, yet weaker than after the 4-h treatment, was observed at the highest concentrations of 40 and 60 µg/ml (Fig. 5B). The number of ghost cells was increased in a statistically significant manner at the concentrations of 20, 30, and 60 µg/ml.

In L5178Y cells, no increase in DNA migration was observed after treatment for either 4 or 24 h (Figs. 7A and B), but the number of ghost cells slightly increased in a statistically nonsignificant manner at the highest concentrations of 100 µg/ml in the short-term treatment and 80 µg/ml in the long-term treatment.

## DISCUSSION

A nanoparticulate reference positive control for *in vitro* genotoxicity assays would constitute an important step to a better testing of nanomaterials, ensuring that test systems are actually appropriate to such test items. Some NP have been proposed as positive control for nanotoxicology. The most used of them, ZnO NP, induced positive but also equivocal responses in several cell lines (Di Bucchianico *et al.*, 2013). A recent study proposed differently shaped CuO NP (spheres, spindles, and rods), inducing positive responses in the micronucleus assay and the comet assay in the RAW 264.7 murine macrophage cell line and, with a lower amplitude, in human lymphocytes (Di Bucchianico *et al.*, 2013). The heterogeneity of nanomaterials should also be taken into account for the choice of positive control. Indeed, every nano-objects, with 1, 2, or 3 external dimensions in the nanoscale (NP, nanofiber and nanoplate) and nanostructured materials, with internal or surface nanostructure (ISO, 2008), may not interact with test systems exactly in the same way.



**FIG. 7.** DNA migration (tail intensity, %) and frequency of ghost cells (%) in L5178Y cells after a 4-h (A) or 24-h (B) treatment with WC-Co NP. A total of hundred cells were scored per duplicate slide per culture. Mean  $\pm$  SD data for 2 cultures. Methyl methanesulfonate was used as positive control and induced 17.6% DNA migration and 52% ghost cells after the 4-h treatment and 4.1% DNA migration and 0.2% ghost cells after the 24-h treatment. No statistically significant differences with negative controls were observed.

The aim of this study was to evaluate WC-Co NP as a candidate reference positive control. For this purpose, different endpoints were analyzed using 3 *in vitro* genotoxicity assays, among them 2 regulatory assays: OECD Test Guideline 476 (OECD, 1997) for the MLA-TK and OECD Test Guideline 487 (OECD, 2010) for the micronucleus assay.

To assess the mutagenic activity, we chose to use the MLA-TK, performed in mammalian cells. Our results showed that WC-Co NP have a weak mutagenic potential *in vitro*. For each treatment time, significant increases in MF were observed at the highest concentrations of 80 µg/ml in the 4-h treatment and 90 µg/ml in the 24-h treatment, associated with a high level of cytotoxicity (RTG between approximately 10% and 20%). Small colony MF was particularly increased, which suggested a clastogenic activity. No other results were available in literature concerning the *in vitro* mutagenic effects induced by WC-Co, even for its microparticulate form. Due to the weak response and the narrow range of concentrations leading to a biologically significant effect due to a steep curve, the routine use of WC-Co NP as positive reference control in the MLA-TK seems to be difficult.

A micronucleus assay using the mouse lymphoma L5178Y cell line, used in MLA-TK, was performed. We also carried out the cytokinesis-block micronucleus assay using primary cultures of human peripheral blood lymphocytes, where cytochalasin B was added at the end of the treatment periods in order to avoid any potential interaction with NP uptake by cells (Doak *et al.*, 2009). The use of both cell types was validated in international studies for regulatory micronucleus assay (Clare *et al.*, 2006; Oliver *et al.*, 2006). In L5178Y cells, WC-Co NP induced a statistically significant increase in the number of MN cells after both short- and long-term treatments. In human lymphocytes, a weak response was observed in the short-term treatment (4 h followed by a 20-h recovery period). The maximum and statistically significant response was observed at the intermediary concentration of 80 µg/ml. However, after a long-term treatment (24 h followed by a 20-h recovery period), this assay was clearly positive, with a dose-response relationship

and a statistical significance for each WC-Co NP concentration, from 20 to 80 µg/ml in binucleated cells and for the 2 highest concentrations of 60 and 80 µg/ml in mononucleated cells. One explanation for this treatment schedule-dependent difference in response could be that the long-term treatment allows exposing cells all along the cell cycle, and the NP access to DNA may be facilitated when the nuclear membrane disassembles during mitosis (Doak *et al.*, 2012). Cellular uptake of similar WC-Co NP was previously studied (Bastian *et al.*, 2009), who demonstrated the presence of NP or agglomerates inside the cytoplasm of HaCaT, A549, and OLN-93 cells incubated with WC-Co NP for 2 days but did not detect any NP inside the nucleus.

Our positive results in the micronucleus assay are in accordance with published data on WC-Co microparticles, in isolated human leukocytes (Van Goethem *et al.*, 1997) and RLE cells (Muller *et al.*, 2008). However, WC-Co NP did not induce micronuclei in HepG2 cells (Kühnel *et al.*, 2012). One explanation could be that they chose a 3-h treatment with low concentrations inducing only weak cytotoxicity, whereas our positive results were associated with a significant decrease in cell viability and were maximized in human lymphocytes after the 24-h treatment. Moreover, the cell type was different.

After the long-term treatment in human lymphocytes, WC-Co NP unexpectedly induced an increase in the number of micronuclei in both binucleated and mononucleated cells. This was not observed in another study (De Boeck *et al.*, 2003b), where only binucleated MN cells were increased after treatment of human lymphocytes with WC-Co microparticles. Besides the particle size, the short 15-min duration of treatment could explain the difference in results, as we observed a statistically significant increase in the number of mononucleated MN cells in the long-term treatment.

Based on this study, the use of WC-Co NP as a positive control in the micronucleus assay is applicable only for the long-term treatment of human lymphocytes. Indeed, even if a statistically significant effect was observed in L5178Y cells and after the short-term treatment in human lymphocytes, the

response amplitude was rather low. The use of WC-Co NP as positive reference control in the micronucleus assay is thus conceivable, depending on the cell type and the treatment schedule.

However, WC-Co NP does not constitute a perfect positive control for the *in vitro* comet assay. Although WC-Co NP induced a dose-dependent increase in DNA migration in human lymphocytes after the 4-h treatment, the response amplitude was lower after the long-term treatment. Concurrently, no DNA damage was detected in L5178Y cells after neither treatment schedules, meaning that the response depends on the origin of the cell type.

An oxidative mechanism of action was proposed for WC-Co microparticles (Lison *et al.*, 1995): Electrons released by cobalt oxidation could be transferred to the surface of WC where they could reduce oxygen and generate ROS. Therefore, the difference of response observed between the 2 cell types in the comet assay could be due to a variability in antioxidant capacities, leading to an overfragmentation of DNA in L5178Y cells, while in human lymphocytes WC-Co NP induced detectable damage. This hypothesis is supported by the significant heterogeneity observed for the highest WC-Co NP concentrations between the 2 donors in the comet assay on human lymphocytes. This was also observed for WC-Co microparticles in a comet assay on isolated human lymphocytes (De Boeck *et al.*, 1998) and could be partly due to interindividual differences in antioxidant status. In our study, this may be the particular case as we used whole blood, with erythrocytes that contain antioxidants. Such interindividual variability was not observed in the micronucleus assay, which could be related to the nature of the cells scored in each assay, ie, PHA-stimulated T lymphocytes in the micronucleus assay and peripheral blood mononuclear cells in the comet assay (De Boeck *et al.*, 2003b).

In general, our results show an apparent lack of sensitivity of L5178Y mouse lymphoma cells compared with human lymphocytes to detect the effects of WC-Co NP. Besides the hypothesis of different antioxidant capacities, this could be explained by the different origins of these cells (ie, human or murine), or by their p53 status, human lymphocytes being p53 proficient and L5178Y cells being p53 deficient. The importance of the choice of cell type was highlighted by a study showing different sensitivity of 10 cell lines toward cytotoxicity induced by 23 nanomaterials (Kroll *et al.*, 2011). This is, however, a concern even for studies on soluble chemicals, and a recommendation was made for *in vitro* genotoxicity tests to use human p53-competent cells (Pfuhler *et al.*, 2011).

## CONCLUSIONS

We showed in this study that several experimental parameters directly influence the nature of the response to WC-Co NP. First, cell type has to be chosen carefully, as WC-Co NP induced

different results in human lymphocytes and in L5178Y cells, particularly in the *in vitro* comet assay. Moreover, when using human lymphocytes, the recommendation is to use several blood cultures from multiple donors because of the interindividual variability. The choice of treatment schedule is also important: In human lymphocytes, the maximal response was observed after the short-term treatment in the comet assay, whereas a long-term treatment was required for the micronucleus assay. Furthermore, attention should be paid to the range of concentrations used, as statistically and biologically significant genotoxic effects were sometimes observed only at subtoxic concentrations. The use of several different concentrations may therefore be relevant to ensure a positive response associated with an acceptable cytotoxicity. Under the conditions of our study, the optimal WC-Co NP concentration for its use as a positive control in the *in vitro* micronucleus assay was 80 µg/ml after a long-term treatment in human lymphocytes. For the *in vitro* comet assay, the maximal response was obtained with a concentration of 120 µg/ml after the 4-h treatment in human lymphocytes. In the case of L5178Y mouse lymphoma cells, neither the comet assay nor the micronucleus assay was clearly positive. In the MLA-TK, WC-Co NP induced only a weak positive response, making their use as positive reference control difficult in this assay. This corroborates the Afssaps report on nanomedicines (Afssaps, 2011), which advises the use of human rather than murine cells. Taking into account these technical issues, WC-Co NP could be used as positive reference NP in *in vitro* standard genotoxicity assays.

## SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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