

Genotoxic and Inflammatory Effects of Depleted Uranium Particles Inhaled by Rats

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Depleted uranium (DU) is a radioactive heavy metal coming from the nuclear industry and used in numerous military applications. Uranium inhalation can lead to the development of fibrosis and neoplasia in the lungs. As little is known concerning the molecular processes leading to these pathological effects, some of the events in terms of genotoxicity and inflammation were investigated in rats exposed to DU by inhalation. Our results show that exposure to DU by inhalation resulted in DNA strand breaks in broncho-alveolar lavage (BAL) cells and in increase of inflammatory cytokine expression and production of hydroperoxides in lung tissue suggesting that the DNA damage was in part a consequence of the inflammatory processes and oxidative stress. The effects seemed to be linked to the doses, were independent of the solubility of uranium compounds and correlating with the type of inhalation. Repeated inhalations seemed to induce an effect of potentiation in BAL cells and also in kidney cells. Comet assay in neutral conditions revealed that DNA damage in BAL cells was composed partly by double strands breaks suggesting that radiation could contribute to DU genotoxic effects *in vivo*. All these *in vivo* results contribute to a better understanding of the pathological effect of DU inhalation.

Key Words: uranium; inhalation; acute-exposure; repeated-exposure; genotoxicity; inflammation.

Uranium (U) is a natural radioactive heavy metal. U is used as fuel in nuclear power plants and is present, in the different steps of the nuclear industry, in different forms with different isotopic compositions (natural, depleted, and enriched) and solubilities (Chazel *et al.*, 2000). Depleted uranium (DU) is a by-product of the nuclear industry (The Royal Society, 2001). Its specific activity is approximately 40% lower than that of naturally occurring U. Because of its high density and metallurgical properties, DU is used in heavy tank armor, armor-piercing bullets, and missiles by the military. There is

a risk of workers and military personnel being exposed to DU. Inhalation is the major route of exposure leading to internal contamination in workers and soldiers (ATSDR, 1999; The Royal Society, 2001). Moreover, U contamination by repeated or single inhalations can occur and the health effects of these different exposures are poorly known.

Different epidemiological studies have been performed on U miners and workers in the nuclear industry. These studies have shown an excess relative risk of lung cancer and fibrosis associated with the exposure to radon decay products (ATSDR, 1999) but it was difficult to ascertain the effects really attributable to U. Moreover, an increase in chromosomal aberrations or genetic damage in blood samples was observed among Gulf War veterans exposed by embedded DU shrapnel fragments (McDiarmid *et al.*, 2004), humans exposed to uranyl compounds (Prabhavathi *et al.*, 1995, 2000), and in U-exposed miners (Meszaros *et al.*, 2004; Zaire *et al.*, 1996). Assessment of the carcinogenic risks from DU is complicated by the dual toxicity of U, radiological as well as chemical. However, *in vitro*, DU has been shown to induce the transformation of human osteoblast cells to a tumorigenic phenotype (Miller *et al.*, 1998) and result in genomic instability manifested as delayed reproductive death and micronuclei formation (Miller, 2002; Miller *et al.*, 2003).

The majority of literature on U inhalation with animal models concerns the studies on U biokinetics and macroscopic effects (ATSDR, 1999). It has been demonstrated that exposure by inhalation to U dust particles can lead to a U accumulation predominantly in the lungs and tracheobronchial lymph nodes, in function of U solubility, as well as the development of neoplasia and fibrosis at the pulmonary level (ATSDR, 1999). Little is known, particularly *in vivo*, concerning the molecular processes leading to the pathological effects associated with exposure of DU. After inhalation and deposition of particulate matter, particles principally reach two main target cells: macrophages and epithelial cells (Schins and Borm, 1999). Macrophages are involved in particle clearance and retention in the alveolar compartment (Tasat and De Rey, 1987). Activated

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macrophages are known to secrete different mediators: pro- and anti-inflammatory cytokines (Driscoll, 2000; Driscoll *et al.*, 1997). *In vitro* studies with U exposure on macrophages have shown effects on cell viability (Kalinich *et al.*, 2002; Tasat and De Rey, 1987) and an induction of TNF- α secretion and MAPK activation (Gazin *et al.*, 2004). The inflammatory response is a key component of host defense but excessive or persistent inflammation contributes to the pathogenesis of disease (Oberdorster *et al.*, 1994). Genotoxicity can be caused by direct actions of particles or indirect mechanisms, often mediated by reactive oxygen species (ROS) produced by inflammatory cells (Kirsch-Volders *et al.*, 2003; Martin *et al.*, 1997).

To better understand the processes leading to the pathological effects associated with DU inhalation, we examined some of the events in terms of genotoxicity and inflammation after DU exposures to inhalation in rats. The effects were studied for different situations: dose, solubility of the compounds, and type of inhalation.

MATERIALS AND METHODS

Animals. Pathogen-free adult male OFA Sprague Dawley rats weighing around 500 g were obtained from Charles River Laboratories, France. Rats were housed by pairs in traditional cages. General health parameters of the rats (animal weight, food and water consumption) were monitored weekly. The study was conducted in accordance with French legislation concerning the protection of animals used for experimental purposes. The non-parametrical statistical comparison between the different groups was made predominantly by a Mann-Whitney test ($p < 0.05$).

Aerosol generation. The industrial U dioxide (UO₂) and U peroxide (UO₄) powders found at workplaces in U fuel cycle facilities were supplied by COGEMA (France). The UO₂ characteristics were: insoluble DU; specific alpha activity = 13.10^3 Bq.g⁻¹; isotopic composition by mass: ²³⁸U = 99.75%, ²³⁵U = 0.24%, ²³⁴U = 0.001%, ²³⁶U < 0.0003%, ²³²U < 0.00001%. The UO₄ characteristics were: soluble reprocessed DU; specific alpha activity = 25.10^3 Bq.g⁻¹; isotopic composition by mass: ²³⁸U = 99.54%, ²³⁵U = 0.39%, ²³⁴U = 0.005%, ²³⁶U = 0.061%, ²³²U < 0.00001%. The device used for aerosol generation was a Small-Scale Powder Dispenser (SSPD, model 3433, TSI, U.S.). The particle size distribution of the aerosols administered to the rats was determined using a cascade Andersen impactor and an Aerodynamic Particle Sizer (APS, model 3310A, combined with a diluter, model 3302, TSI, U.S.). The aerodynamic median activity diameter (AMAD) of UO₂ aerosols was 2.53 μ m (geometric standard deviation, gsd = 1.93), the mass median

aerodynamic diameter (MMAD) was 1.8 μ m (gsd = 1.66) and the number median aerodynamic diameter = 0.91 μ m (gsd = 1.48). The AMAD of UO₄ aerosols was 2.34 μ m (gsd = 2.01), the MMAD was 1.31 μ m (gsd = 1.70), and the number median aerodynamic diameter = 0.74 μ m (gsd = 1.38). These DU compounds and these aerosol types are found in the nuclear industry and after impacts involving DU munitions (Chazel *et al.*, 2000, 2003; Salbu *et al.*, 2005). The DU particles could reach the alveoli of the lungs because of their size.

Animal exposure and euthanasia. The aerosols were administered using a nose-only inhalation system previously described (Monleau *et al.*, in press). The concentration of particles in the inhalation chamber was calibrated by sampling onto membrane filters (pore size 0.8 μ m, 25 mm diameter, in cellulose acetate, Millipore). Rats were acclimatized to housing facilities and contention tubes during a period of at least two weeks. Different inhalation exposures were carried out with several rat groups presented in Table 1.

The aerosol concentrations inhaled by rats were high comparing with the derived air concentrations for workers. The concentrations for intakes of DU by workers are, for inhalation of 5 μ m particles, around 0.075 mg.m⁻³ for an insoluble compound and 0.72 mg.m⁻³ for a soluble compound (ICRP, 1994). On the other hand, our aerosol concentrations were in the range of concentrations estimated for inhalation of DU during the different battlefield scenarios (0.05 to 5000 mg.m⁻³) (The Royal Society, 2001). The U contents in organs (especially lungs and kidneys) were determined in rats, exposed similarly for another study, by Kinetic Phosphorescence Analysis (KPA, Chemcheck, U.S.) (Monleau *et al.*, in press). The lung intake in the AcUO2-3 group was comparable to the cumulated intake in the RepUO2 group. The lung intake in the AcUO2-1 group was comparable to the intake in the AcUO4 group, one day after exposure. Therefore, with these different exposure groups, we can study the DU effects as a function of dose with the different single inhalations of UO₂ (AcUO2-1, -2, -3), as a function of compound solubility with the single inhalations of insoluble UO₂ (AcUO2-1) and soluble UO₄ (AcUO4) and in relation to the type of inhalation with the single or repeated inhalations of UO₂ (AcUO2-3 and RepUO2). Uranium contents have showed that the nephrotoxic dose was not exceeded. The effects, which could be observed in this study, were not a consequence of a renal dysfunction. Three rats per group were anesthetized at each time post-exposure by ip injection of pentobarbital (1 μ l.g⁻¹) and exsanguinated via the abdominal artery. The protocols "Isolation of epithelial nasal cells" to "Comet assay" were used for each rat from each group at each time post-exposure and performed on the day of euthanasia.

Biochemical analyses. The serum of blood from the groups AcUO4, RepUO2, and Control was isolated and maintained at -20°C until analysis of the plasma biochemical parameters. The concentrations in alanine aminotransferase (ALT, U.L⁻¹), aspartate amino-transferase (AST, U.L⁻¹), creatinine (CREAT, μ mol.L⁻¹) and urea (mmol.L⁻¹) were measured by routine methods (using a Konelab 20/20i, Thermo Electron, France).

Isolation of epithelial nasal cells. The *in situ* enzyme incubation technique described by Steele and Arnold was used to isolate the nasal turbinate epithelial cells (Steele and Arnold, 1985). Briefly, the nasal cavity was filled with an

TABLE 1
Experimental Protocol for Inhalation Study

Inhalation type	Group name	Inhalation duration	Aerosols concentration	Euthanasia post-exposure
Acute UO ₂	AcUO2-1	30 min	190 mg.m ⁻³ \pm 41 mg.m ⁻³	4 h, 1, 3, and 8 days
Acute UO ₂	AcUO2-2	2 h	375 mg.m ⁻³ \pm 70 mg.m ⁻³	1, 3, and 8 days
Acute UO ₂	AcUO2-3	3 h	375 mg.m ⁻³ \pm 70 mg.m ⁻³	1, 3, 8, and 14 days
Repeated UO ₂	RepUO2	30 min, 4 days/week, for 3 weeks	190 mg.m ⁻³ \pm 41 mg.m ⁻³	1, 3, 8, and 14 days
Acute UO ₄	AcUO4	30 min	116 mg/m ³ \pm 60 mg.m ⁻³	4 h, 1, 3, and 8 days
Air	Control	30 min, 4 days/week, for 3 weeks	Air	1, 3, 8, and 14 days

Note. h, hours; min, minutes.

enzyme mix (protease type XIV 0.5%, collagenase type IV 0.1%, and hyaluronidase type IV-S 0.1%) via a tube inserted in trachea. After incubation, the cell suspension was retrieved in a centrifuge tube by washing and centrifuged at 1500 rpm for 15 min at 4°C. The pellet was resuspended in 250 µl of PBS. One-hundred seventy µl was used for the comet assay and 80 µl for the cell viability.

Isolation of BAL cells. After removing the lungs with trachea (and heart), broncho-alveolar lavage fluid (BALF) was collected by cannulating the trachea and washing with 50 ml of sterile saline solution at 37°C. BALF was centrifuged at $900 \times g$ at 4°C for 10 min and the BAL cells were resuspended in 2 ml of PBS. BAL cells are mainly composed of alveolar macrophages (around 95%; Derelanko and Hollinger, 2002). After BAL, the lungs were minced and kept at -80°C until protein or RNA extractions.

Isolation of kidney cells. Five-hundred mg of the kidney was minced and washed with Ca^{2+} - and Mg^{2+} - free Hanks' balanced salt solution (HBSS free) supplemented with 20 mM HEPES and 0.8 mM EDTA. The suspension was incubated in a solution of 0.25% trypsin and 0.05% EDTA in HBSS free at 37°C with gentle stirring for 10 min (Robbiano *et al.*, 1996; Sasaki *et al.*, 1997). The suspension was centrifuged at $700 \times g$ at 4°C for 10 min and the pellet was resuspended in 1 ml of PBS.

Comet assay. Comet assay was used to determine DNA stand breaks *in vivo*. The comet assay under alkaline conditions simultaneously detects DNA single and double strand breaks and alkali-labile sites, while the neutral conditions allow the detection of DNA double-strand breaks considered to be the "biological relevant" lesions for radiation damage (Olive, 1999). For all samples, cell viability was >85% (determined with a Malassez haemocytometer using the trypan blue exclusion technique). The comet assay was performed, essentially following the procedure of Singh *et al.*, under alkaline conditions (De Meo *et al.*, 1991; Singh *et al.*, 1988) with epithelial nasal cells (85 µl per slide), BAL cells (3.10^4 cells per slide) and kidneys cells (3.10^5 cells per slide). The comet assay was performed also under neutral conditions for BAL cells (Singh, 2000; Singh *et al.*, 2003). Two slides were prepared for each sample. During the entire test, samples and slides were maintained at 4°C in the dark. Briefly, the isolated cells were mixed with a solution of low melting point agarose in PBS (0.5% final) and placed onto a slide pre-coated with 0.5% agarose in distilled water. The slide was dipped in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA , 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10, 4°C) for 1 h. The slide was placed on an electrophoretic unit containing an electrophoretic buffer for 30 min in an alkaline condition or for 20 min under neutral conditions for DNA unwinding. The alkaline electrophoretic buffer was composed of 300 mM NaOH and 1 mM EDTA, pH > 13 whereas the neutral electrophoretic buffer was composed of 100 mM Tris and 300 mM Sodium acetate, pH 9. The slide was subjected to an electric field at 4°C of 0.84 V/cm (25 V, 350 mA) for 20 min under alkaline conditions and of 0.4 V/cm (12 V, 100 mA) for 1 h under neutral conditions. Following electrophoresis, the slide was neutralized in 0.4 M Tris-HCl (pH 7.5) for 10 min, rinsed with water, dehydrated in 100% ethanol, and air-dried at room temperature overnight. The dried slide was stained with 2.5 µg/ml propidium iodide and examined at 250× magnification using an Olympus BX61 fluorescent microscope equipped with a UPlanFI 20XUV objective. Image analysis was performed by using Cohu camera and Fenestra Komet software (version 5.5, Kinetic Imaging, BFI Optilas, France). For each sample (one tissue/one rat/one exposure condition), 100 cells (50 cells/duplicate) were scored. The increase in the Olive tail moment (OTM) was used to quantify DNA damage. A major advantage of using the OTM as an index of DNA damage is that both the amount of damaged DNA and the distance of migration of the genetic material in the tail are represented by a single number. Normalized distribution frequencies of OTM were calculated using 40 OTM classes between the minimal and maximal values for each set of data. Non linear regression analysis was performed using a chi-square function model. The degree of freedom (n), called OTMchi2, of the model has been previously shown to be a quantitative parameter to describe the level of DNA damage (Bauer *et al.*, 1998; Jean *et al.*, 2001). The degree of freedom was calculated using the curve-fitting software Table Curve 2D (Jandel Scientific

Software). Each sample (100 cells/cell type/time post-exposure/animal) corresponds to an OTMchi2, expressed in arbitrary units. For a time post-exposure and cell type, the OTMchi2 from three exposed rats were calculated and compared to the OTMchi2 from the control group performed the same day.

Quantification of cytokine mRNA levels: RT-PCR analysis. These experiments were performed on lung samples from RepUO2, AcUO2-3, and Control groups at 1, 3, and 14 days post-exposure. Total RNAs were extracted from lung tissue (60 mg) using the RNA isolation mini kit (Qiagen, France). The RNA concentration was determined by measuring the absorbance at 260 nm. The integrity of the RNA was evaluated by measuring the 260:280 nm ratios and confirmed by visualization of intact 18S and 28S RNA bands after agarose gel electrophoresis. Total RNAs (1 µg) were used to generate cDNA by reverse transcription using 200U SuperScript Reverse Transcriptase (GIBCO) in a 20-µl reaction buffer containing 1X Superscript buffer (GIBCO), 1 mM 2-deoxynucleotide 5'-triphosphate, 20 ng random hexamer, 10 mM DTT, and 20U Rnase inhibitor. After incubation for 60 min at 42°C, the reaction was terminated by a denaturing enzyme for 15 min at 70°C. The mRNA levels of the pro-inflammatory cytokines: tumour necrosis factor alpha (TNF- α), interleukin-8 (IL-8), macrophage inflammatory protein-2 (MIP-2) and interferon-gamma (IFN- γ); the anti-inflammatory cytokine interleukin-10 (IL-10) and the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) were measured in duplicate by real-time (RT) PCR. Sequences for the forward and reverse primers used in the present study are listed in Table 2. The ABI PRISM 7000 Sequence Detection System was used for detected RT-PCR products with the SYBR Green I assay (Applied Biosystems, France), according to the manufacturer's recommendations. The PCR cycling conditions were performed for all samples as follows: 50°C, 2 min; 95°C, 10 min and 40 cycles with 95°C, 15 s and 60°C, 1 min. PCR fluorescent signals were normalized to the fluorescent signal obtained from the housekeeping gene HPRT for each sample.

Hydroperoxide level determination. Measuring peroxides in samples was an important factor in determining the degree of free radicals present in specific tissues that reflect a potential oxidative stress. Lung samples (600 mg) from RepUO2, AcUO2-3, and control groups at 1, 3, and 14 days post-exposure were homogenized in 1 ml phosphate buffer (10 mM KH_2PO_4 , 40 mM Na_2HPO_4 , 0.01 mM EDTA, pH 7.5) on ice then centrifuged at $15,000 \times g$ for 15 min at 4°C. The supernatants were stored at -80°C for later assessment. Protein levels in supernatants were determined by Bradford dosage. The peroxide levels, aqueous and lipid hydroperoxides, were determined using a PeroxiDetect kit (Sigma, France). Briefly, the samples were incubated for 30 min at 25°C with an aqueous reagent (1 ml solution of 100 mM sorbitol and 125 µM xylenol orange in water and 10 µl of 25 mM ferrous ammonium sulfate in 2.5 M

TABLE 2
SYBR Green Primer Sequences Used for RT-PCR Reactions

Gene name	Primers	5'-3' Sequence
HPRT	Sense	GCTCGAGATGTCATGAAGGAGA
	Antisense	TCAGCGCTTTAATGTAATCCAGC
TNF- α	Sense	CATCTTCTCAA AATTCGAGTGACAA
	Antisense	TGGGAGTAGACAAGGTACAACCC
MIP-2	Sense	GCTTGAGTGTGACGCC
	Antisense	CTTTTGGACCGCCCTTGAGA
IL-8	Sense	GACTGTTGTGGCCCGTGAG
	Antisense	CCGTC AAGCTCTGGATGTTCT
IFN- γ	Sense	CACCCCGCTCTTGTTGGT
	Antisense	TCTAGGCTTTCAATGAGTGTGCC
IL-10	Sense	GTTGCCAAGCCTGTGACGAAA
	Antisense	TTTCTGGCCATGGTTCTCT

TABLE 3
General Health Parameters of Different Rat Groups

Days post-exposure	Control	AcUO4	AcUO2-1	AcUO2-2	AcUO2-3	RepUO2
Rat weight (g)						
0	563 ± 25	554 ± 35	577 ± 26	552 ± 50	513 ± 57	565 ± 32
10	578 ± 29	431# ± 36	590 ± 37	528# ± 36	469# ± 60	575 ± 36
14	592 ± 31	483# ± 27	600 ± 29	541# ± 38	520# ± 81	610 ± 29
Food (g)						
0	21 ± 2	20 ± 3	20 ± 3	21 ± 6	21 ± 6	18 ± 4
4	24 ± 3	1# ± 1	23 ± 5	15# ± 4	5# ± 6	20 ± 3
10	25 ± 3	30 ± 2	26 ± 5	21.5 ± 2	17 ± 5	25 ± 2
Water (g)						
0	35 ± 7	31 ± 6	30 ± 7	32 ± 4	37 ± 9	38 ± 9
4	37 ± 6	14# ± 5	38 ± 4	35 ± 7	18# ± 10	35 ± 6
10	38 ± 5	36.5 ± 5	37 ± 4	35 ± 9	32 ± 6	36 ± 12

Note. The values are given as mean ± SE, $n > 3$.

$p < 0.05$ between the exposed groups and the control group.

sulphuric acid) or organic reagent (1 ml solution of 4 mM butylated hydroxytoluene and 125 µM xylenol orange in 90% methanol and 10 µl of 25 mM ferrous ammonium sulfate in 2.5 M sulphuric acid). The hydrogen peroxide H₂O₂ levels in aqueous solutions and lipid hydroperoxide levels in organic solvents were measured by absorbance at 560 nm.

RESULTS

General Health Parameters

The general health parameters are shown in Table 3 for the different groups and for some relevant days post-exposure. The control, AcUO2-1, and RepUO2 rat groups had similar weight curves. However, the mean weight of the rats of the AcUO2-2, AcUO2-3, and AcUO4 groups decreased after acute inhalation during the first 10 days. The mean weight then stabilized and afterwards increased in the same way as the mean weight of the other groups but nevertheless remaining lower. The food consumption of rats was similar for the control, AcUO2-1, and RepUO2 groups but was momentarily lower (Day 4) for the AcUO2-2, AcUO2-3, and AcUO4 groups. The decrease in rat weights and food consumption for the AcUO2-2 group was lower than the decrease for the AcUO2-3 group. The water consumption followed the same pattern as food consumption, except for the AcUO2-2 group where no change during time was noticed. These results showed that the general health parameters of rats exposed to uranium compounds by inhalation were dependant of the dose, solubility, and type of inhalation (acute or repeated).

Analysis of the biochemical parameters in serum of the control, AcUO4, and RepUO2 groups (Table 4) revealed stable levels of ALT and AST, sign of no liver failure. Kidney failure was observed only for the AcUO4 group, with an increase in concentrations of both creatinine and urea at 3 and 8 days post-exposure. It is due to the UO₄ solubility.

DNA Damage

The number of DNA strand breaks in epithelial nasal cells was unaffected by the different exposures at the different days post-exposure tested (data not shown) with an OTM chi2 ranging from 2 to 3 in both control and exposed groups. This heterogeneity in the response could mask a slight effect.

The OTMchi2 values, after comet assay under alkaline conditions in BAL cells, were presented at the different days post-exposure (Fig. 1). A positive induction of DNA damage was observed only after the highest single inhalation of UO₂

TABLE 4
Biochemical Parameters in Serum for the Groups Control
($n = 4$), AcUO4 ($n = 3$), and RepUO2 ($n = 6$)

Days post-exposure	Control	AcUO4	RepUO2
CREAT µmol.L ⁻¹			
1	49 ± 5	44 ± 6	48 ± 3
3	52 ± 6	73# ± 8	48 ± 3
8	49 ± 6	264# ± 168	49 ± 1
UREA mmol.L ⁻¹			
1	6 ± 0.8	6.9 ± 1	6.1 ± 0.7
3	6.1 ± 0.4	11# ± 2	6 ± 0.7
8	6.2 ± 0.6	34# ± 22	6.1 ± 0.4
ALT U.L ⁻¹			
1	39 ± 15	35 ± 14	30 ± 4
3	35 ± 9	34 ± 14	34 ± 14
8	31 ± 9	28 ± 16	27 ± 6
AST U.L ⁻¹			
1	126 ± 61	86 ± 29	132 ± 23
3	115 ± 55	72 ± 15	133 ± 32
8	152 ± 87	127 ± 32	122 ± 21

Note. The values are given as mean ± SE.

$p < 0.05$ between the exposed groups and the control group.

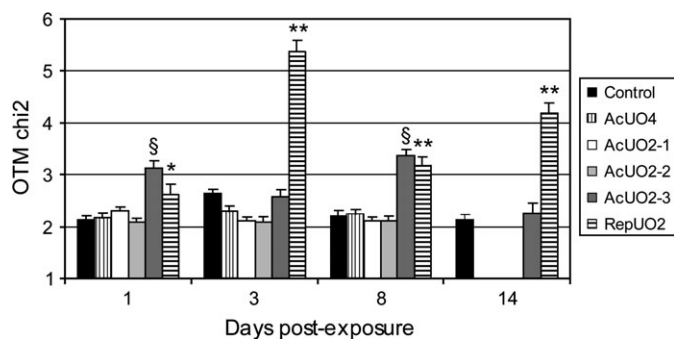


FIG. 1. Comet assay under alkaline conditions in BAL cells. Mean of OTMchi2 values are presented in relation to days post-exposure. * $p < 0.01$ and ** $p < 0.001$ between control and RepUO2. § $p < 0.001$ between control and AcUO2-3.

($600 \mu\text{g.g}^{-1}$ Lung in AcUO2-3 group) at 1 and 8 days post-exposure. No DNA damage was observed at the tested dose ($40 \mu\text{g.g}^{-1}$ Lung) in the AcUO2-1 and AcUO4 groups. BAL cells from the RepUO2 group showed DNA damage at all days post-exposure. Compared to the AcUO2-3 group, repeated inhalations induced longer responses. The AcUO2-3 and RepUO2 groups, with a genotoxic response under alkaline conditions, also showed DNA damage after comet assay under neutral conditions (Fig. 2), except for the AcUO2-3 group at 8 days post-exposure. UO_2 inhalations induced both single and double DNA strand breaks in BAL cells.

The results of comet assay under alkaline conditions in kidney cells (Fig. 3) only showed a positive response in the RepUO2 group with DNA damages at 3 and 8 days post-exposure.

Inflammation Cytokines

The expression of cytokines TNF- α , MIP-2, IL8, IFN- γ (data no shown), and IL-10, involving inflammatory responses to particles, were investigated in lung tissue from Control, AcUO2-3, and RepUO2 groups at 1, 3, and 14 days post-exposure (Fig. 4). Single inhalation exposure to UO_2 (AcUO2-3) significantly increased the mRNA levels of TNF- α , IL8, and

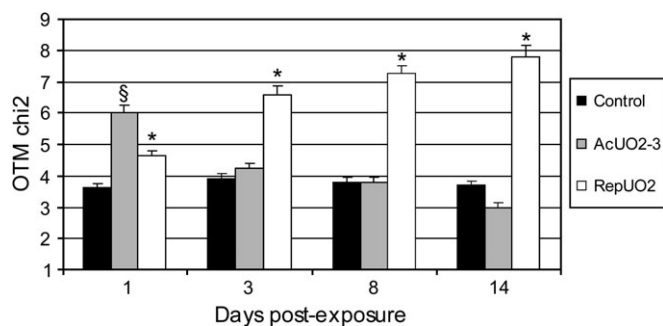


FIG. 2. Comet assay under neutral conditions in BAL cells. Mean of OTMchi2 values are presented in relation to days post-exposure. * $p < 0.001$ between control and RepUO2. § $p < 0.001$ between control and AcUO2-3.

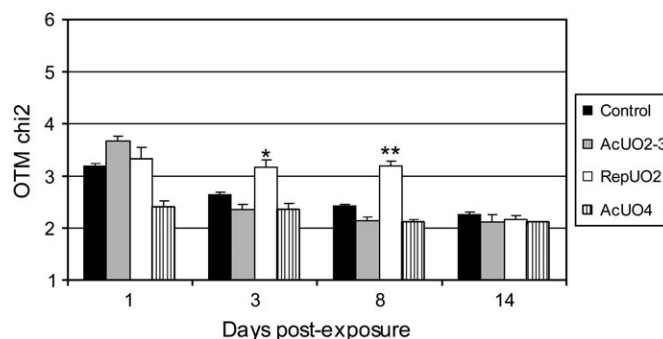


FIG. 3. Comet assay under alkaline conditions in kidney cells. Mean of OTMchi2 values are presented in relation to days post-exposure. * $p < 0.05$ and ** $p < 0.001$ between control and RepUO2.

IL-10 but not IFN- γ , only one day post-exposure. Repeated inhalation exposure to UO_2 (RepUO2) increased significantly the mRNA levels of TNF- α and IL8 and reached a significance for MIP-2 but not IFN- γ and IL-10 at three days post-exposure and reached a significance for TNF- α at 14 days post-exposure.

Hydroperoxide Level

The measurement of peroxide levels in lungs made it possible to evaluate the level of free radicals (Fig. 5). The levels of aqueous and lipid hydroperoxides in lung from the AcUO2-3 group were significantly higher as compared to the control group at only 14 days post-exposure while, in the RepUO2 group, the levels of aqueous hydroperoxides were significantly higher at 1, 3, and 14 days post-exposure and the levels of lipid hydroperoxides were significantly higher at 3 and 14 days post-exposure.

DISCUSSION

Very few studies have been carried out to assess the processes associated with the effects of inhalation exposure to U or DU, despite the knowledge that most U intoxication in workers occurs via this route. The molecular consequences of DU inhalation are poorly known *in vivo*, particularly in the case of repeated exposure. The studies on military personnel concern principally the case of exposure by embedded DU shrapnel fragments (McDiarmid *et al.*, 2004; Pellmar *et al.*, 1999). However, inhalation is another important route of contamination and finally the estimation of the health effect of DU inhalations by soldiers are based on the knowledge acquired on U miners, nuclear workers, and animal studies of U inhalations (The Royal Society, 2001). In this study, some of the events in terms of genotoxicity and inflammation were investigated after DU exposures to inhalation in rats.

The comet assay was tested recently for human biomonitoring studies (Kassie *et al.*, 2000; Moller, 2005) including

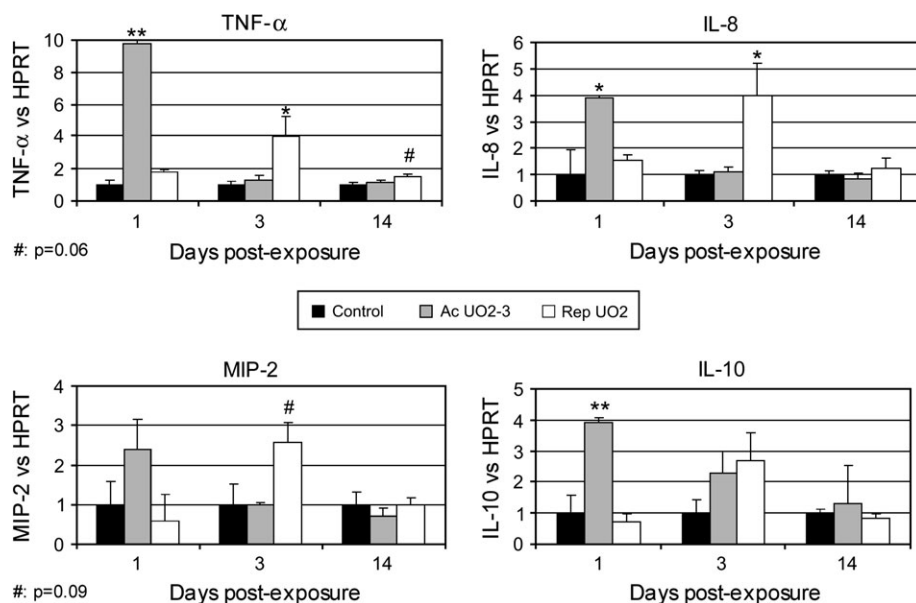


FIG. 4. Relative mRNA expression of the cytokines TNF- α , MIP-2, IL-8, and IL-10 in lung tissues at 1, 3, and 14 days post-exposure for the groups control ($N = 5$), AcUO₂-3 ($N = 3$), and RepUO₂ ($N = 3$). ** $p < 0.01$, * $p < 0.05$ and # reached a significance indicated on the graph between the exposed and control group. The results are expressed as a ratio to the mRNA levels of the reference gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Data are the means \pm SEM.

radiobiology (Olive, 1999; Tice and Strauss, 1995) and inhalation exposure (Knudsen *et al.*, 2005; Sul *et al.*, 2003). Different extents of DNA damage, measured by comet assay under alkaline conditions, were observed, in the present study, after DU inhalations for different target organs as a function of the exposure and the time post-exposure. Comparison of the different acute UO₂ inhalations showed that the genotoxic effect appeared only at the highest tested dose suggesting that a threshold dose could exist in BAL cells. No effect on the compound solubility could be observed at the tested dose. And at the same cumulative dose as AcUO₂-3, given as 12 lower doses, DNA damage in RepUO₂ group was induced in BAL cells at all days post-exposure tested. After lung deposition, U is absorbed into the blood plasma and lymph as a function of this solubility and distributed to the different organs. The kidney is the site of U accumulation and the critical target organ for U toxicity (ATSDR, 1999; Gilman *et al.*, 1998). Induction of DNA damage in kidney cells was observed only after repeated UO₂ exposure. When no DNA damage was observed, it has been hypothesized that endogenous antioxidant levels may influence the susceptibility to some toxic effects of metal which would explain why we did not detect induction of DNA damage in acute exposures (Gochfeld, 1997). A threshold for *in vivo* genotoxicity is often a consequence of adequate DNA repair and/or antioxidant status (Kirsch-Volders *et al.*, 2000). The consequences of repeated inhalation compared to acute exposure could be opposite in function of the compound. For example, it was reported that pretreatment of cadmium produces adaptative tolerance (Hart *et al.*, 1996; Valverde *et al.*, 2000) and, on the other hand, repeated inhalations of lead

induced a genotoxic response in more organs than after a single inhalation (Valverde *et al.*, 2002). In this study, DU particles after repeated inhalation seemed to induce a potentiation effect of U toxicity in BAL and kidneys cells.

Comet assay under neutral conditions makes us possible to underlined that, after acute or repeated UO₂ inhalations (around 500 $\mu\text{gUO}_2\cdot\text{g}^{-1}$ Lung that is 7 $\text{Bq}\cdot\text{g}^{-1}$ Lung), the observed DNA damage in BAL cells was in part double DNA strand breaks. Double DNA strand breaks could be double strand breaks or two nearby single strand breaks. Miller *et al.* have suggested that radiation can play a role in DU-induced biological effect *in vitro*. They have shown that DU induced a significant elevation in the dicentric frequency in human osteosarcoma cells (HOS) cells in contrast to incubation with other heavy metals and an increase in neoplastic transformation process dependant to the specific activity (Miller *et al.*, 2002b). The mechanisms by which radiation can contribute to the DU effects are unknown. A hypothesis is that the radiation and chemical effects could be synergistic.

We investigated the inflammatory gene expression in lungs because genotoxicity can be a consequence of the inflammatory response and a relationship between inflammation and tumorigenesis has been shown with poorly soluble dusts (Driscoll *et al.*, 1996). TNF- α is one of the pre-eminent pleiotropic cytokines. It reflects the release of cytokines from macrophages and neutrophils, as well as the cytokine activity of various lung cell types, such as bronchial epithelial cells (Ermer *et al.*, 2003). It acts as an initiator of the respiratory tract inflammatory response to particles (Driscoll, 2000) by the orchestration of cytokine networking, the induction of

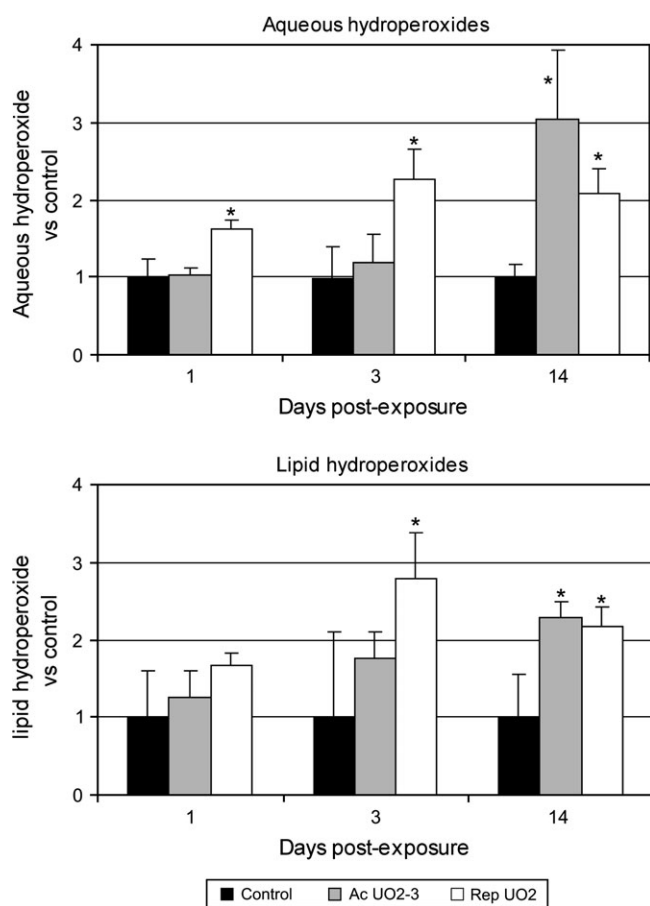


FIG. 5. Hydroperoxide levels in lung tissues at 1, 3, and 14 days post-exposure for the groups control ($N = 5$), AcUO₂-3 ($N = 3$), and RepUO₂ ($N = 3$). * $p < 0.05$ between exposed and control group.

production of ROS, and the recruitment of inflammatory cells (Vassalli, 1992). But TNF- α can mediate tissue injury such as fibrosis (Vassalli, 1992). IFN- γ is produced by immune effector cells in response to a variety of stimuli. It modulates the fibrotic response in the lung by inhibiting the growth of fibroblasts (Narayanan *et al.*, 1992) and suppressing collagen synthesis and histamine content (Gurujeyalakshmi and Giri, 1995). IFN- γ also modulates the inflammatory response. IL-8 is a leukocyte chemotactic activating cytokine (chemokine) and is produced by various types of cells upon stimulation with inflammatory stimuli, while exerting a variety of functions on leukocytes in particular (Harada *et al.*, 1994; Mukaida *et al.*, 1998). MIP-2 is a heparin-binding protein that exhibits a number of inflammatory and immunoregulatory activities. MIP-2 plays a major role in mediating the neutrophilic inflammatory response of the rodent lung to particles such as quartz and crocidolite asbestos (Driscoll, 2000). TNF- α and MIP-2 seems to play a major role in particle-induced inflammation and regulation by oxidative stress (Driscoll, 2000; Long *et al.*, 2004). IL-10 is an anti-inflammatory cytokine that inhibits the production of other cytokines and indirectly the innate immune

responses, such as the macrophage function and activation (Moore *et al.*, 2001). In the present study, the acute inhalation exposure to UO₂ induced a rapid and brief inflammatory gene expression in rat lungs with a rapid increase of both pro-inflammatory cytokines (IL-8 and particularly TNF- α but not MIP-2 and IFN- γ) and anti-inflammatory cytokine (IL-10). The repeated inhalation exposure to UO₂ also seemed to induce an inflammatory gene expression but time-delayed compared to the single exposure with comparable lung deposit. This inflammatory gene expression was characterized by an increase in mRNA levels of pro-inflammatory cytokines TNF- α , IL-8, and MIP-2 but not IFN- γ at 3 days post-exposure and possibly at 14 days post-exposure for TNF- α , which seems to suggest a slight persistent expression of TNF- α . On the other hand, anti-inflammatory cytokine IL-10 mRNA was not increased, suggesting an imbalance in the inflammatory gene expression. Our results were in accordance with an *in vitro* study which showed that U exposure of macrophage cell line induces accumulation of TNF- α mRNA and induction of TNF- α secretion but not of IL-10 (Gazin *et al.*, 2004). An increased prevalence of both fibronectin and TNF- α has also been found in the BAL fluid of U miners (Popp *et al.*, 2000).

As previously described, the production of ROS was linked to the effect of particles and the inflammatory response. The most important cellular effects of ROS in the lung may include damage to cell membranes by means of lipid peroxidation processes, oxidation of proteins, and damage to the DNA of target cells (Schins and Borm, 1999). Excessive and persistent formation of ROS from inflammatory cells is considered as the characteristic of the indirect genotoxicity of particles following exposure to relatively high concentrations of poorly soluble dust (Schins, 2002). An increase in peroxides levels were revealed in lung tissue, after repeated exposure, for several days contrary to the acute exposure. This leads credence to the notion that repeated inhalation exposure to UO₂ does not produce an adaptative tolerance. A previous *in vitro* study has shown that DU can catalyze biochemical reactions, involving ROS like hydroxyl radicals, singlet oxygen and/or superoxide radicals, inducing oxidative DNA damage without significant radioactive decay (Miller *et al.*, 2002a). SOD, catalase, and GPx constitute the main components of the antioxidant defense system and modification in their expression reflects a potential oxidative stress. A study observed that ingested U induces, in mice kidneys, a dose-dependent production of H₂O₂ and an increase in SOD and GPx mRNA levels (Taulan *et al.*, 2004). Such different data suggest that DU can induce oxidative stress.

In summary, the *in vivo* results showed firstly that DU inhalations could induce DNA damage in different rat cell types. In BAL cells, DNA lesions were linked to the dose, independent of the solubility of U compounds while correlating with the type of inhalation and were composed partly by double strand breaks suggesting that radiation could contribute to DU genotoxic effects *in vivo*. In kidney cells, only repeated exposure was able to induce DNA strand breaks. Secondly,

insoluble DU particles induced time-dependent increases in mRNA levels of different cytokines and in hydroperoxide production in rat lungs; the pattern differs between acute and repeated inhalation exposures. These results suggest that the DNA damage was partly a consequence of the inflammatory processes and ROS production and that repeated exposure of insoluble DU particles could induce a potentiation effect. All these observations contribute to better understand the pathologies associated with U inhalation, the mechanism of DU toxicity, and may have a significant impact on risk assessment.

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