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Original

First Steps Towards an Understanding of a Mode of Carcinogenic Action for Vanadium Pentoxide

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Abstract: Inhalation of vanadium pentoxide clearly increases the incidence of alveolar/bronchiolar neoplasms in male and female B6C3F1 mice at all concentrations tested (1, 2 or 4 mg/m^3), whereas responses in F344/N rats was, at most, ambiguous. While vanadium pentoxide is mutagenic *in vitro* and possibly *in vivo* in mice, this does not explain the species or site specificity of the neoplastic response. A nose-only inhalation study was conducted in female B6C3F1 mice (0, 0.25, 1 and 4 mg/m^3, 6 h/day for 16 days) to explore histopathological, biochemical (α-tocopherol, glutathione and F2-isoprostane) and genetic (comet assays and 9 specific DNA-oxo-adducts) changes in the lungs. No treatment related histopathology was observed at 0.25 mg/m^3. At 1 and 4 mg/m^3, exposure-dependent increases were observed in lung weight, alveolar histiocytosis, sub-acute alveolitis and/or granulocytic infiltration and a generally time-dependent increased cell proliferation rate of histiocytes. Glutathione was slightly increased, whereas there were no consistent changes in α-tocopherol or 8-isoprostane F2α. There was no evidence for DNA strand breakage in lung or BAL cells, but there was an increase in 8-oxodGuo DNA lesions that could have been due to vanadium pentoxide induction of the lesions or inhibition of repair of spontaneous lesions. Thus, earlier reports of histopathological changes in the lungs after inhalation of vanadium pentoxide were confirmed, but no evidence has yet emerged for a genotoxic mode of action. Evidence is weak for oxidative stress playing any role in lung carcinogenesis at the lowest effective concentrations of vanadium pentoxide. (DOI: 10.1293/tox.24.149; J Toxicol Pathol 2011; 24: 149–162)

Key words: comet assay, DNA lesions, mouse inhalation, oxidative stress, vanadium pentoxide

Introduction

Although relatively common in the Earth's crust, vanadium is minable in very few regions. Its major commercial sources are the titanoferrous magnetites of South Africa, China and the former USSR. It is also found in some fossil fuels and is recoverable from oil, shale and coal residues[1]. It was "rediscovered" in 1830 in some iron ore in Sweden by Sefström, who named the element after Vanadis, the Scandinavian goddess of beauty, because of the intensity and variety of colours displayed by many of its compounds. Consequently, in addition to its use in the manufacture of certain alloys its compounds find use as pigments. Vanadium pentoxide specifically is used in the manufacture of ferrovanadium and as a catalyst in sulphuric acid manufacture, where it is used in the oxidation of SO_2 to SO_3. When used to produce steel its value is in forming V_4C_3 with any carbon that is present, thereby producing a fine-grained alloy with increased wear resistance and strength at higher temperatures, properties that are valued in the manufacture of (jet engine) turbine blades, high-speed tools and springs. When alloyed with titanium and aluminium it has a number of applications, e.g., its specification Ti-6%Al-4%V is used in many aerospace, industrial and medical applications (surgical implants). Vanadium compounds are present in various animal and plant species, sometimes at significant concentrations. In ascidians (sea squirts) vanadium serves a physiological function as a blood pigment in vanadocytes, or extracellularly in plasma as haemovanadin. The concentrations in certain of these organisms may be surprisingly high (e.g., 1900 mg/kg bw in *Phallusia mamillata*), indicat-

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ing a high potential for concentrating the element from the surrounding sea water[2,3]. In the general human population, food is the major source of vanadium, the higher concentrations being found in shellfish, mushrooms, parsley, dill and black pepper[4,5]. Vanadium compounds, especially insoluble and poorly soluble vanadium oxides, are hardly absorbed from the intestinal tract[6], whereas they are easily absorbed from the lung[7]; occupational exposure by inhalation of the oxides is probably the source of more concern regarding possible human effects of the element. Unlike some other oxides of vanadium, the pentoxide is slightly soluble in water (0.1–0.8 g/100 cm^3)[8,9].

Many aspects of vanadium pentoxide occurrence, use and toxicology have been addressed in the IARC (2006) monograph in which its carcinogenicity was evaluated[10]; a more truncated review was presented as part of the U.S. NTP technical report of the inhalation carcinogenicity studies in rats and mice[11,12]; and a look to the future has been presented in a review of toxicological concerns regarding vanadium pentoxide[13]. Conclusions reached by NTP regarding the carcinogenicity of vanadium pentoxide were: "… there was *some evidence of carcinogenic activity* of vanadium pentoxide in male F344/N rats and *equivocal evidence of carcinogenic activity* of vanadium pentoxide in female F344/N rats based on the occurrence of alveolar/bronchiolar neoplasms. There was *clear evidence of carcinogenic activity* of vanadium pentoxide in male and female B6C3F1 mice based on increased incidences of alveolar/bronchiolar neoplasms. Exposure to vanadium pentoxide caused a spectrum of non-neoplastic lesions in the respiratory tract (nose, larynx, and lung) including alveolar and bronchiolar epithelial hyperplasia, inflammation, fibrosis, and alveolar histiocytosis of the lung in male and female rats and mice and an unusual squamous metaplasia of the lung in male and female rats. Hyperplasia of the bronchial lymph node occurred in female mice"[11].

The NTP carcinogenic assessment was largely confirmed by the IARC (2006) evaluation: "In both male and female mice, the incidences of alveolar/bronchiolar neoplasms were significantly increased, and there were also increases in male rats. It was uncertain as to whether a marginal increase in alveolar/bronchiolar neoplasms in female rats was related to exposure to vanadium pentoxide"[10]. The results of the study, however, would not readily support a conclusion that there was any evidence for carcinogenicity in female rats (and even the results from male rats are questionable), if it was not for the clear data from mice (see Fig. 1). Some limitations of the NTP study have been discussed by Duffus (2007) who also speculated on the role of nascent ultrafine and nanoparticles that might have been produced during milling as sources of reactive oxygen species[14].

The pattern of carcinogenic responses in the lungs of mice and rats exposed by inhalation do not readily suggest a genotoxic mode of action and the published genetic toxicity and mutagenicity experiments with vanadium pentoxide have provided mixed responses *in vitro* and *in vivo* (reviewed in Assem and Levy, 2009)[13]. Consequently, the objective of the current experiments was to generate data that might suggest a mode of action for the clear carcinogenic response in mice. Since the exposure period used was short (16 days), it was recognised from the beginning that there are severely limited possibilities for identifying key events in the process. Nevertheless, should genotoxicity be a major factor in the process, this ought to be definable within this time frame. Female B6C3F1 mice were selected for the study since this was the strain used by NTP in their whole-body exposure study and the carcinogenic response over the control group was greater in females than in males. Observations and measurements made following nose-only exposure to a vanadium pentoxide aerosol 6h/day for 16 days included concentrations of V in lungs and blood, lung weight, histopathology of the airways, cell proliferation in lungs, concentrations in lungs of α-tocopherol, glutathione (reduced and total), 8-isoprostane F2α, as a representative of the isoprostanes, and 9 specific DNA-oxy-adducts and DNA damage (comet assays) in lung and bronchiolar-alveolar lavage (BAL) fluid cells. It was also planned to conduct a micronucleus assay on lung cells, but concerted and prolonged attempts to validate such an assay on cells from mouse lung were unsuccessful, so this part of the study design was eventually abandoned. While these efforts were in progress, a technique for conducting micronucleus studies on mouse lung cells was successfully devised and used to follow the effect of exposure to ethylene oxide[15].

Materials and Methods

Chemicals

The primary test material, vanadium pentoxide, a yellow-orange solid (batch no. H060391, purity 99.8% and containing 0.13% vanadium tetraoxide), was supplied by Stratcor Technology Center, Hot Springs, Arkanas, USA. Methylmethanesulphonate (stated purity 99%), the positive control material used in the genetic toxicity assays, was obtained from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany.

Mice and their exposure

This study was performed in an AAALAC-accredited laboratory in accordance with the Swiss Animal Protection Law under licence no. 45, "Subacute inhalation toxicity testing in rats and mice with an industrial chemical."

Female B6C3F1/Hsd mice were obtained from Harlan Laboratories Ltd., Dublin, Virginia, USA. The mice were 7–8 weeks of age at delivery and their body weights ranged from 16.9 g to 23.2 g at acclimatisation. 192 mice were allocated to 4 groups of 48 mice each. One additional group of six mice (group 5) was used as the positive control in a comet assay. The mice of each of the groups 1–4 were subdivided into eight sub-groups of six mice each to evaluate the range of toxicity on specific end-points in the lungs and to obtain data on the concentration of vanadium in blood and lungs. The mice of groups 1–4 were exposed to vanadium pentoxide for 6h/day for either 7 or 16 days at target

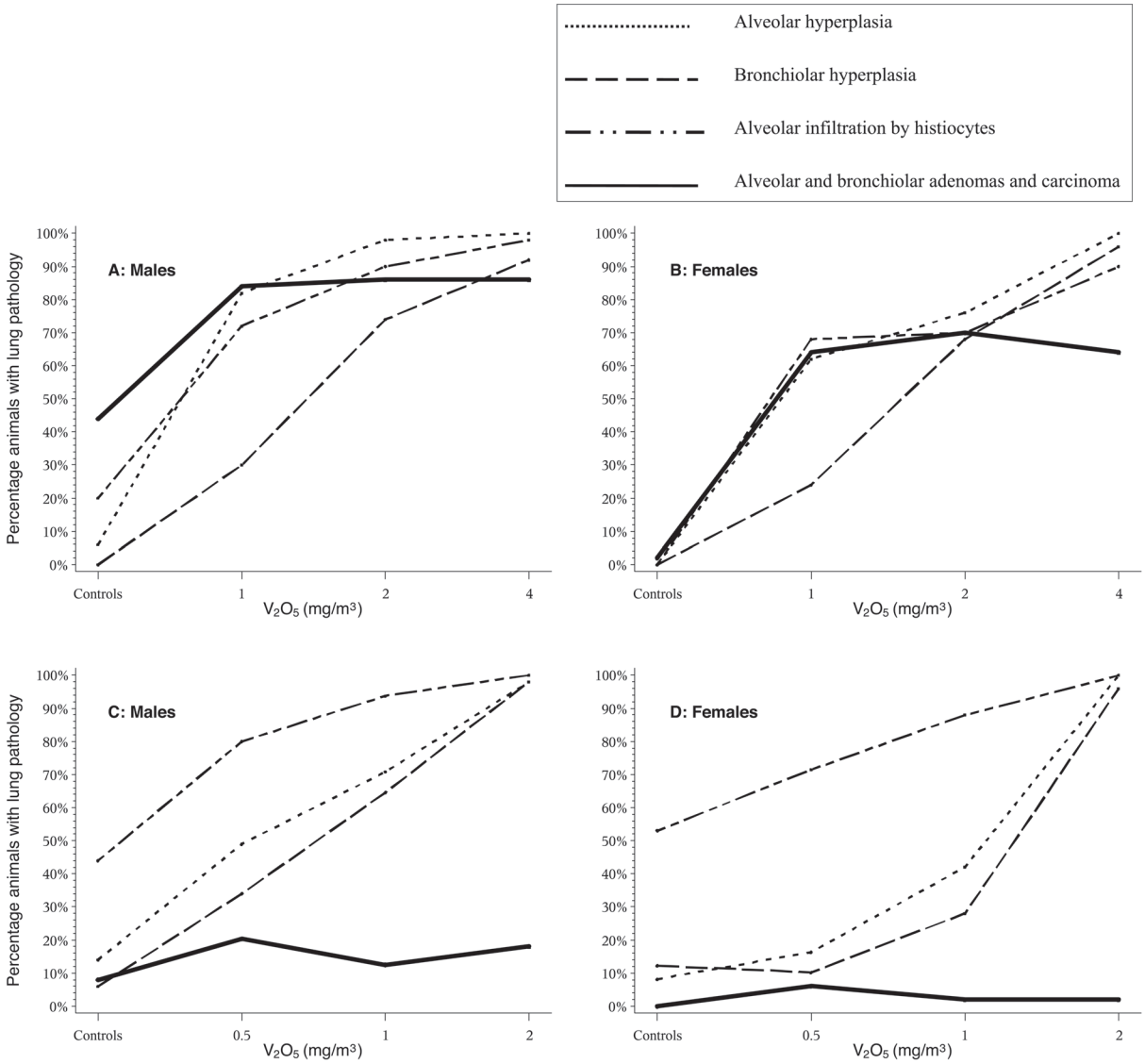

Fig. 1. Percentage incidences of pulmonary adenomas or carcinoma, and selected non-neoplastic pathology from the US NTP two-year inhalation studies in B6C3F1 mice (A, B) and F344/N rats (C, D) for vanadium pentoxide (NTP, 2002; Ress*et al.*, 2003).

aerosol concentrations of 0, 0.25, 1 or 4 mg/m³. During exposure, the mice were confined separately in restraint tubes positioned radially around a flow-past, nose-only exposure chamber (as described by Cannon *et al.*, 1983[16]). The flow of air at each tube was 0.5 L/min, which is more than twice the respiratory minute volume of a mouse, and therefore sufficient to minimise re-breathing of the aerosol.

Vanadium pentoxide dust aerosol was generated using a rotating brush aerosol generator connected to a micronizing jet mill. The highest exposure concentration aerosol generated was then electrostatically discharged into the exposure chamber through a ^{63}Ni charge neutraliser. An air-vacuum dilution system was used to achieve the lower target aerosol concentrations. Gravimetric determinations of the vanadium pentoxide aerosol concentrations were made daily using Millipore®-durapore filters, Type HVLP (pore size 0.45 µm) loaded in an in-line stainless steel filter sampling device (Gelman Science Inc., Ann Arbor, Michigan, U.S.A.). The distribution of particle size in the generated aerosol also was determined gravimetrically on two occasions during the 16-day treatment period for the 1 and 4 mg/m³ dose groups using a cascade impactor. In order to obtain sufficient material from the 1 mg/m³ concentration atmosphere to provide a result with reasonable precision, it was necessary to collect the sample over 4 consecutive days; but even this procedure would not have allowed estimates of particle size from the 0.25 mg/m³ atmospheres. Following these gravimetric determinations for concentration and particle size, chemical analyses of the weighed filters for vanadium were performed using an atomic absorption spectroscopy (AAS) method and the results reported as vanadium or as vanadium pentoxide after recalculation.

After 7 or 16 days exposure, mice were anaesthetized with 46% Ketamin (Ketavet 100, Pharmacia GmbH, 76139 Karlsruhe, Germany), 23% Xylazin (Rompun 2%, Bayer HealthCare, 51368 Leverkusen, Germany) and 31% Midazolan (Dormicum, Hoffmann LaRoche, 79639 Grenzach-Wyhlen, Germany) (approx. 2 mL/kg body weight). In mice allocated for histopathology, the lungs were instilled via the trachea with neutral buffered 4% formaldehyde solution. For biochemical and comet assay investigations the lungs were perfused through the right ventricle with saline, the lung lobes excised and, except for samples intended for the comet assay, they were shock-frozen in liquid nitrogen and shipped on dry ice to the appropriate analytical laboratory where they were stored at -80 °C pending analysis. For the comet assay, the freezing step was omitted and the lungs were lavaged with 20 mL mincing buffer (20 mM EDTA, 10% DMSO in HBSS pH 7.4–7.6) for collection of bronchiolar alveolar lavage (BAL) cells. Perfusion of the lungs was continued, then they were minced in 1 mL mincing buffer using fine scissors to obtain a suspension of single cells.

Observations and analyses

Vanadium concentrations were measured in blood and lung samples from exsanguinated mice by AAS.

In addition to standard histopathology of the lungs following staining with haematoxylin-eosin, lung tissue sections from two of the mouse allocations (one each after 7 and 16 days exposure) were stained for two endogenous proliferation markers: proliferating cell nuclear antigen (PCNA), that is expressed in the nuclei of cells during DNA synthesis and repair when it is clamped to DNA and assists in holding DNA polymerase δ in position, and Ki-67, which is a strictly nuclear protein during interphase, but relocates to the surface of chromosomes, where it is detectable during all phases of mitosis. Proliferation markers PCNA and Ki-67 were quantified by counting all marker-positive cells in 5 × 1 cm^2 microscope view fields/mouse at × 400 magnification. The grading system applied for the proliferation rate assessment was as follows: 1–15 positive cells = grade 1 minimal; 16–30 positive cells = grade 2 slight; 31–45 positive cells = grade 3 moderate; 46–60 positive cells = grade 4 marked; >60 positive cells = grade 5 severe.

Glutathione (reduced and total) and α-tocopherol concentrations were measured separately in the right and left lung lobes, respectively, of one set of 4 groups and 8-isoprostane F2α was measured in another.

Reduced (GSH) and oxidised (GSSG) glutathione: Frozen lung samples were homogenized in 10 volumes ice-cold 50 mM MES ([2-(N-morpholino) ethanesulphonic acid]) buffer, pH 6–7, containing 1 mM EDTA. Proteins were precipitated by the addition of an equal volume of 10% metaphosphoric acid and centrifugation at 2000 × g for 10 min. The supernatant fluid was diluted 20- to 100-fold with MES buffer and total glutathione (GSH plus GSSG) concentration in lung homogenates determined spectrophotometrically at 405 nm following reduction with glutathione reductase in the presence of NADPH and derivatisation with 5,5′-dithiobis-(2-nitrobenzoic acid) as described in the glutathione assay kit (Cayman Chemical Co., USA). Oxidized glutathione (GSSG) was quantified similarly following inactivation of GSH with 2-vinylpyridine at 2- to 10-fold dilutions with MES buffer[17]. For a determination of the recovery efficiency control lung samples were spiked with glutathione at concentrations of 1 and 250 µmol/g lung. Mean recovery efficiencies of 82.3 ± 15.3% (n = 3) and 76.1 ± 7.9% (n = 3), respectively, were obtained.

α-Tocopherol: Frozen lung samples were homogenized with 10 volumes 0.9% NaCl. Proteins were precipitated from 250 µL of the homogenate with an equal volume of ethanol and α-tocopherol was extracted with 1 mL hexane. Following lyophilisation of 0.5 mL of the hexane extract, the residue was reconstituted in 100 µL acetonitrile/methanol (85:15). An aliquot of 50 µL was analysed for α-tocopherol by HPLC with fluorescence detection (excitation at 295 nm, emission at 330 nm) on a Nucleosil 5 µm C18 column (125 × 4.6 mm) eluted with acetonitrile/methanol (85:15) at a flow of 1.5 mL/min[18]. For a determination of the recovery efficiency, control lung samples were spiked with α-tocopherol at a concentration of 57.3 µg/g lung. The mean recovery efficiency was 79.5 ± 7.6% (n = 2).

8-Isoprostane F2α: Frozen lung samples were homogenised in 3 volumes 0.9% (w/v) NaCl. [^{3}H]Prostaglandin F2α was added as internal standard and isoprostanes were extracted with 2 volumes ethanol from the homogenate. Following centrifugation for 10 min at 1500 × g the supernatant fluid was hydrolysed with an equal volume 15% (w/v) KOH for 1 h at 40 °C to release esterified isoprostanes. The hydrolysate was diluted with 2 volumes water, acidified with 2 M HCl to pH 2.0–3.5 and loaded on an Oasis HLB (30 mg) SPE column (Waters, USA) equilibrated with 1 mL methanol and 1 mL 0.02 M HCl. The column was washed subsequently with 2 mL 0.02 M HCl and with 2 mL 40% methanol in 0.02 M HCl. The column was dried with air and rinsed with 2 mL heptane. Isoprostanes were eluted from the column with 2 mL ethyl acetate. The final elute from the Oasis column was diluted with 2 mL heptane, loaded on a Sep-Pak Vac RC Silica (100 mg) column (Waters, USA) equilibrated with 1 mL methanol and 1 mL ethyl acetate/heptane (1:1 by volume), and eluted with 2 mL ethyl acetate/methanol (1:1 by volume). The eluate was dried in a Speed-Vac concentrator and reconstituted in 1 mL EIA (enzyme-immunoassay)-buffer. The recovery of the extraction was determined by scintillation counting of 100 µL of the reconstituted extract. Quantitative measurement of 8-isoprostane F2α was made using a commercially available ELISA (Cayman Chemical Co., USA) using different dilutions of the extract[19]. For a determination of the recovery efficiency control lung samples were spiked with 8-isoprostane F2α at a concentration of 5 ng/g lung. The mean recovery efficiency was 98.8 ± 34.7% (n = 4).

Comet assay: The BAL cell preparation was centrifuged at 300 × g for 10 min and resuspended in 0.7% agarose. The minced lung tissue cells were filtered through a 40 µm mesh cell strainer, centrifuged at 5000 × g for 1 min

and re-suspended in 0.7% agarose. Three slides per minced lung and one slide of BAL cell suspension per mouse were prepared by diluting 10% cell suspensions with 90% of 0.7% low melting point agarose solution and applying 0.1 mL per slide. Slides were cooled before submersion in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO) at 2–8 °C in the dark (for at least 1 h and up to 7 days). This was followed by 20 min of alkaline treatment in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) at pH ≥ 13 and then electrophoresis for 30 min at 25 V (0.72 V/cm), 300 mA. These operations were conducted at 2–8 °C in the dark. The preparations were neutralised with 0.4 M Tris, pH 7.5, dehydrated in 99% ethanol and finally air-dried before scoring. DNA was stained with ethidium bromide (20 µg/mL, 40 µL/slide) immediately before scoring tail intensity in 100 cells per preparation (BAL cells or minced lung tissue cells) from each mouse using a fluorescence microscope with a 40 × objective linked to an image analysis programme (Comet Assay IV, Perspective Instruments). The numbers of apoptotic and necrotic cells/500 cells per slide also were recorded in order to indicate the quality of slide preparation and exclude possible cytotoxic effects. Because the target cell populations were unusual for this type of assay, a small historical database was accumulated for both BAL and minced lung cells from untreated mice. The percentage tail intensities in this database were: BAL cells, mean 0.66 ± 1.26 SD, range, 0.04–6.34, n = 27; lung cells, mean 0.42 ± 0.60 SD, range, 0.04–3.29, n = 32. A positive control group of mice was dosed orally by gavage with methylmethanesulphonate (MMS), 200 mg/Kg body weight 4 h before they were killed. Data were analysed for statistical significance using the one-tailed Student's *t*-test.

DNA oxo-lesions:

1) DNA extraction

Nuclear DNA was isolated according to a method optimised for the study of oxidised nucleosides[20]. Thawed lung samples were minced with scissors and homogenised in 1.5 mL of ice cold lysis buffer A (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris, 0.1 mM deferoxamine pH 7.5, 1% Triton-X100) using a polytron homogeniser. The nuclei were recovered by centrifugation at 1500 × g for 10 min at 4 °C. The nuclear pellets were washed with 1.5 mL of buffer A and after re-centrifugation (1500 × g, 10 min) they were re-suspended in 600 µL of lysis solution B (10 mM Tris, 5 mM EDTA-di-Na, 0.15 mM deferoxamine, pH 8.0) and 35 µL of 10% SDS. Then, 30 µL of RNase A (1 mg/ml) and 8 µL of RNase T1 (1 U/µl) were added and the resulting solutions were incubated at 50 °C for 15 min. Subsequently, 30 µL of Qiagen protease (20 mg/ml) were added and the resulting solutions were incubated at 37 °C for 1 h after which they were centrifuged at 5000 × g for 15 min at 4 °C. The supernatant fractions were collected and mixed with 1.2 mL of sodium iodide solution (7.6 M NaI, 40 mM Tris, 20 mM EDTA-di-Na, 0.3 mM deferoxamine, pH 8.0) and 2 mL of cold (4 °C) 2-propanol. DNA precipitation was obtained by gently inverting the tube several times. DNA was recovered by centrifugation at 5000 × g for 10 min at 4 °C and washed with 1 mL of 40% 2-propanol. After re-centrifugation at 5000 × g for 10 min the DNA sample was washed again using 1 mL 70% ethanol. Finally, the DNA sample was again recovered by centrifugation and suspended into 50 µL of 0.1 mM deferoxamine and stored frozen at −80 °C until digestion.

2) DNA digestion

To 50 µL of the DNA solution was added 2.5 µL of buffer P1 10x (200 mM succinic acid, 100 mM $CaCl_2$, pH 6.0) 1 U of nuclease P1, 2.5 U of DNAse II and 0.025 U of phosphodiesterase II. The resulting solution was incubated for 2 h at 37 °C. Thereafter, 6 µL of alkaline phosphatase buffer 10× (500 mM Tris, 1 mM EDTA, pH 8) was added together with 0.015 U of phosphodiesterase I and 2 U of alkaline phosphatase. The samples were then incubated at 37 °C for 2 h. After enzymatic digestion, the solution was neutralized by addition of 3.5 µL of 0.1 M HCl. Then, the samples were centrifuged for 5 min at 5000 × g and transferred in HPLC-compatible vials prior to HPLC-MS/MS analysis. Traces of ethanol used for DNA precipitation were removed by putting the samples in a speed vac concentrator for 10 min (not more to avoid drying of samples). Directly after digestion samples were analysed by HPLC-MS/MS for which 20 µL of the solution was injected on to the HPLC column. HPLC-MS/MS measurements were performed using an Accela HPLC system coupled through electrospray to a Quantum Ultra triple quadrupole mass spectrometer. Separation was achieved on an octadecylsilyl silicagel Hypersyl (particle size: 3 µm, 150x2.0 mm I.D.) column obtained from Interchim (Montluçon, France). The mobile phase consisted of an increasing proportion of methanol (from 0 to 15% in 35 min) in 2 mM ammonium formate, adjusted to pH 4.8 with formic acid. The flow rate was 0.2 mL/min. Detection of the different DNA lesions was performed in the so-called multiple reactions monitoring (mrm) mode. Transitions used for detection of the different DNA lesions and the limits of their detection are given in Results *DNA lesions*. Results are expressed as the number of lesions relative to the number of normal nucleosides detected using an online UV detector at 260 nM.

Results

In vivo and post mortem observations

Measured aerosol concentrations were very close to the 0, 0.25, 1 and 4 mg/m^3 target concentrations (Table 1). The mass median aerodynamic diameters (MMAD) of the generated aerosols ranged between 1.22 and 1.43 µm and the

Table 1. Mean V_2O_5 Concentrations in Air during Exposure of Mice 6 h/day for 16 Days

Exposure group	V_2O_5 concentrations in air (mg/m^3)		
	Target	Actual	A/T
1	0	Not measured	
2	0.25	0.246 ± 0.026	98% ± 10%
3	1.00	0.993 ± 0.122	99 ± 12%
4	4.00	4.01 ± 0.55	100% ± 14%

Table 2. Lung Weights of Mice from Exposure Groups 1–4 in 5 Independently Processed Sub-groups

Allocation sub-groups (n=6)	V_2O_5 concentrations in air (mg/m³)			
	0	0.25	1	4
V concentration in blood and lungs	0.120 g	0.124 g	0.147 g[c]	0.186 g[c]
Lung cell proliferation (day 7)	0.158 g	0.155 g	0.153 g	0.177 g[b]
Lung cell proliferation and histopathology (day 16)	0.138 g	0.151 g	0.168 g[c]	0.199 g[c]
Lung tissue glutathione and α-tocopherol[a]	0.086 g	0.100 g[c]	0.107 g[c]	0.127 g[c]
	0.043 g	0.047 g	0.052 g[c]	0.062 g[c]
Lung tissue 8-isoprostane F2α	0.140 g	0.136 g	0.156 g[b]	0.197 g[c]

[a] Right (upper) and left (lower) lobes weighed separately. [b] $p \leq 0.05$; [c] $p \leq 0.01$.

Table 3. Vanadium Concentrations in Blood and Lungs after Exposure of Mice 6 h/day for 16 Days to Vanadium Pentoxide.

Exposure group	Vanadium concentration	
	Blood [µg/L]	Lung [µg/g]
0.25 mg/m³	–[a]	8.02 ± 0.84
1 mg/m³	51.15 ± 6.02	31.01 ± 2.22
4 mg/m³	160.13 ± 16.48	64.35 ± 7.45

[a]Below lowest calibration point (13 µg/L).

mean percentage of particles of < 3 µm diameter were 92 at 1 mg/m³ and 91 at 4 mg/m³. The MMAD at 0.25 mg/m³ was assumed to be similar to the other groups because the two lower target aerosol concentrations were achieved by air-vacuum dilution from a single aerosol that provided the 4 mg/m³ exposure concentration and the ranges of MMADs at 1 and 4 mg/m³ were similar.

One mouse in the 1 mg/m³ group was found dead shortly after the end of exposure during the second week of treatment, although it was most likely not a treatment-related event since no clinical signs of toxicity were observed during the study. During the first week of exposure, marginal body weight losses were noted in the 1 and 4 mg/m³ groups that were statistically significant in the higher group, but had resolved during the second week. Lung weights showed a dose-related increase in these same groups. In the groups killed after 7 days, there were no effects on the lung weights in the 1 mg/m³ group and the statistically significant increased lung weight in the 4 mg/m³ group was less pronounced (Table 2). The increases in lung weights were not attributable to deposition of vanadium pentoxide, although vanadium concentrations showed clear, dose related increases in both lung and blood (Table 3). It is more likely the lung weight increases were due to an influx of fluid and cells in an inflammatory response.

In the mice killed after 16 days exposure, the following histopathological diagnoses were made:

• ultifocal/diffuse alveolar histiocytosis that was to some degree dose-dependent in all 6 mice of the 1 and 4 mg/m³ groups;

• ultifocal subacute alveolitis that was clearly dose-dependent in 5 mice each of the 1 and 4 mg/m³ groups; and

• ultifocal granulocytic infiltration at similar severity in 4 mice at 1 mg/m³ and 5 mice at 4 mg/m³.

No treatment-related pathology was noted in mice exposed to 0.25 mg/m³ for 16 days. Examples of these findings are shown in Fig. 2.

Cell proliferation

The mean cell proliferation rate grades, as indicated by immunohistochemical staining for Ki-67 protein after 7 and 16 days, were time-dependently and dose-relatedly increased in the 1 and 4 mg/m³ groups. There were also dose-related increases in immunohistochemical staining for PCNA in the 1 and 4 mg/m³ groups after 7 days, but only in the 4 mg/m³ group after 16 days, the score for the 1 mg/m³ group at this time being similar to the controls. Thus, a time-related increase was observed only in the 4 mg/m³ group when staining for PCNA (Table 4). With neither method was there evidence for cell proliferation following exposure to 0.25 mg/m³.

Glutathione

Reduced glutathione is the most abundant water-soluble cellular scavenger for reactive oxygen species. Induction of oxidative stress might result in a depletion of GSH with an accompanying increase of oxidized glutathione, GSSG. The respective tissue concentrations, therefore, are generally considered to be measures of intracellular oxidative stress[21].

Mean GSH concentrations in the lungs of mice exposed to 0.25 mg/m³ were slightly but statistically significantly lower (17.4%) than the control value (Table 5), whereas there were no significant differences from the control in mean GSH concentrations in the lungs of the 1 and 4 mg/m³ groups. However, there were slightly but significantly increased concentrations of GSSG in the lungs of mice from all treatment groups, although indications of a concentration-related effect were weak, the percentage increases being 159%, 223% and 215% in the 0.25, 1 and 4 mg/m³ groups, respectively.

As a result of the increased GSSG concentrations the ratio of reduced to oxidized glutathione (GSH/2 × GSSG) decreased slightly in all dose groups to 53%, 55% and 53% of the control group ratio in the 0.25, 1 and 4 mg/m³ groups, respectively.

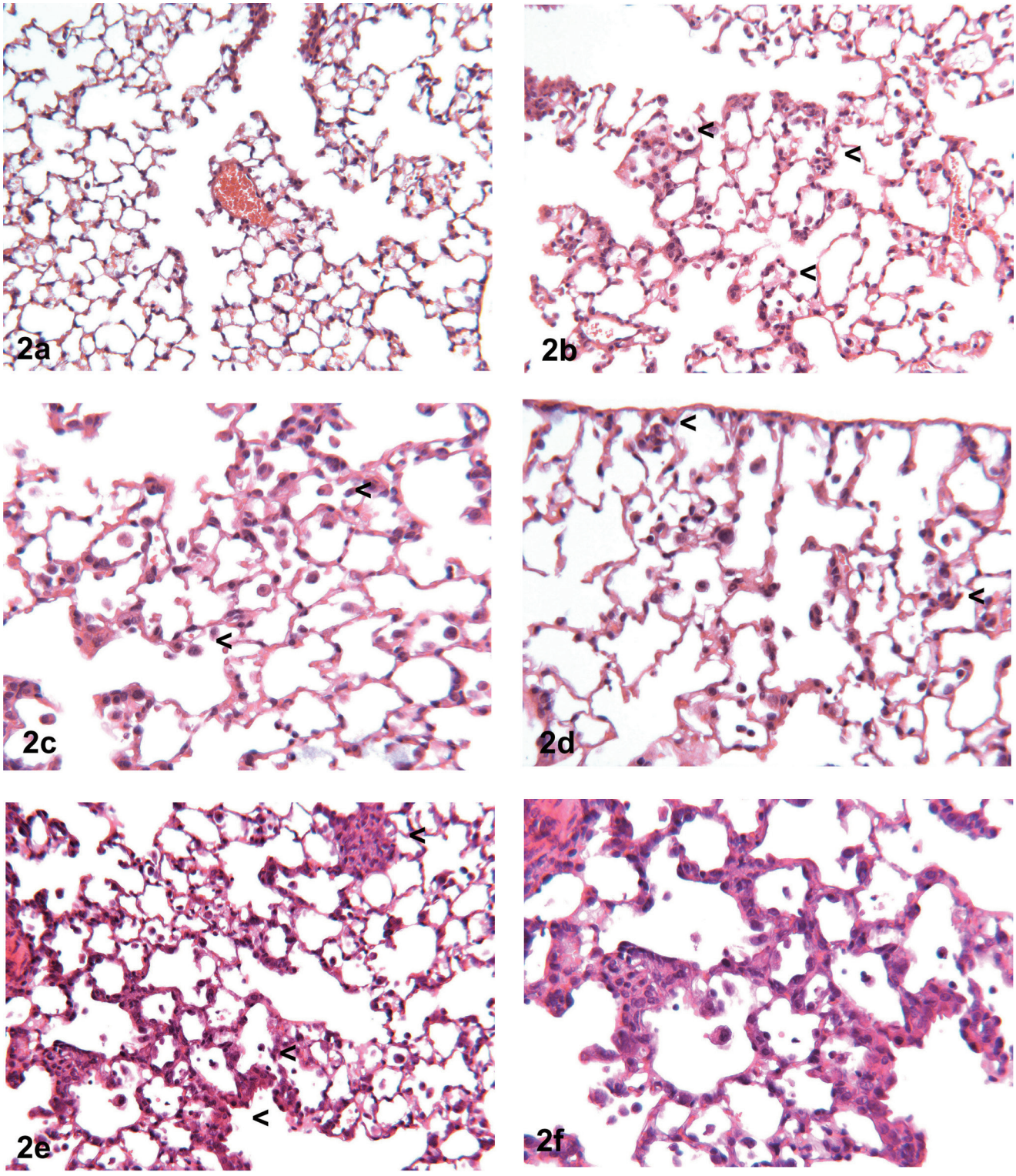

Fig. 2. Photomicrographs of lungs from female B6C3F1 mice showing focal and diffuse alveolar histiocytosis after exposure to vanadium pentoxide 4 mg/m^3 6h/day for 16 days (b–f) or air control (a). Magnifications ×160 (a, b, e) or ×250 (c, d, f). All staining was with haematoxylin and eosin. a: control, b: diffuse histiocytosis in entire left and right cranial lobe, c: as b, d: histiocytosis and granulocytosis, e: alveolitis and histiocytosis, f: as e.

Table 4. Cell Proliferation in Lungs of Mice Exposed to Vanadium Pentoxide for 7 or 16 Days

Indicator and sample time	V_2O_5 concentrations in air (mg/m³)			
	0	0.25	1	4
Ki-67-positive cells after 7 days	10.1 (1)	11.4 (1)	18.1 (2)	24.7 (2)
Ki-67-positive cells after 16 days	10.0 (1)	9.2 (1)	27.1 (2)	87.8 (5)
PCNA-positive cells after 7 days	25.1 (2)	25.0 (2)	41.4 (3)	47.8 (4)
PCNA-positive cells after 16 days	33.0 (3)	26.3 (2)	34.9 (3)	68.3 (5)

Figures in parentheses indicate the severity grades as described in the Materials and Methods section.

Table 5. Concentrations of Indicators of Oxidative Stress (Glutathione, α-tocopherol, 8-isoprostane F2α) in the Lungs of Female B6C3F1 Mice Exposed by Inhalation to Vanadium Pentoxide for 16 Days

Parameter	V_2O_5 concentrations in air (mg/m³)			
	0	0.25	1	4
GSH µmol/g Lung ± SD	1.95 ± 0.30	1.61 ± 0.31[a]	2.26 ± 0.27	2.21 ± 0.28
GSSG µmol/g Lung ± SD	0.168 ± 0.036	0.268 ± 0.073[b]	0.376 ± 0.161[b]	0.363 ± 0.103[b]
Ratio GSH/2 × GSSG	5.95 ± 1.18	3.15 ± 0.79[b]	3.29 ± 0.85[b]	3.12 ± 0.75[b]
α-tocopherol µg/g Lung ± SD	8.24 ± 1.60	6.72 ± 1.24[a]	8.37 ± 0.97	10.42 ± 0.74[b]
8-isoprostane F2α ng/g Lung ± SD	39.8 ± 23.9	24.7 ± 11.1	31.5 ± 25.5	30.4 ± 7.2

[a] $p \leq 0.05$; [b] $p \leq 0.01$.

Table 6. Comet Assay Mean Percentage Tail Intensities in BAL and Lung Cells of Female B6C3F1 Mice Exposed by Inhalation to Vanadium Pentoxide for 16 Days or 4 h after 200 mg MMS/Kg Body Weight Orally by Gavage

Cell type scored	V_2O_5 concentrations in air (mg/m³)				MMS
	0	0.25	1	4	200 mg/Kg
BAL cells	0.97 ± 0.94	0.31 ± 0.32	2.34 ± 2.23	0.28 ± 0.12	65.10 ± 9.95
Lung cells	0.53 ± 0.43	0.62 ± 1.00	0.21 ± 0.14	0.43 ± 0.40	48.34 ± 11.88

α-Tocopherol

α-Tocopherol was measured as a representative of lipid soluble cellular scavengers for reactive oxygen species that might be depleted under conditions of intracellular oxidative stress[21], but there were no consistent effects of treatment upon the mean α-tocopherol concentrations in lungs (Table 5). While there was a small, statistically significant reduction in the 0.25 mg/m³ group there was no effect at 1 mg/m³ and a small, statistically significant increase at 4 mg/m³.

F2-isoprostane

F2-isoprostanes are derived from polyunsaturated fatty acids, e.g., arachidonic acid, in cell membrane lipids by non-enzymic, free radical oxidation, so an increase in their concentration can be another indication of treatment-related oxidising activity. 8-Isoprostane F2α was measured as a representative of this group. There were no significant changes in its concentration in the lungs of the mice exposed to vanadium pentoxide, the mean values being less than in the controls in all groups, but the variation within the data was relatively large (Table 5).

Comet assay

In BAL cells, there was a small, non-significant increase in percentage tail intensity in the 1 mg/m³ group ($p = 0.234$), but there was no indication of an exposure concentration-related response (Table 6). In the lung cell preparations there were no increases in percentage tail intensity in any group exposed to vanadium pentoxide. The positive control, MMS, produced large increases in tail intensity in both BAL ($p < 0.001$) and lung ($p = 0.004$) cells.

DNA lesions

The quantities of some for the DNA lesions analysed (Fig. 3) were below the limit of detection by the methods used. These lesions included 8-oxodAdo, 5-OH-dCyd, ThdGly, 5-hmdUrd, 5-FodUrd, EdGuo and EdAdo. Therefore, it cannot be concluded that treatment of mice with vanadium pentoxide was without effect on the generation of these lesions in lung cells, only that their natural and any induced occurrences were below the detection limits indicated in Table 7. Levels of 8-oxodGuo and dCyd341 were high enough to enable their quantification by HPLC-MS/MS in all the analysed mouse lung samples. The reported levels of these DNA lesions, expressed as the numbers of lesions per million normal nucleosides are reported in Table 8. In conclusion, statistically significant, concentration-related increases in 8-oxodGuo were observed in the 1 and 4 mg/m³ groups in comparison with the control group, whereas there was no increase in the 0.25 mg/m³ group. In the case of dCyd341, no significant variation from the background level was observed in any group.

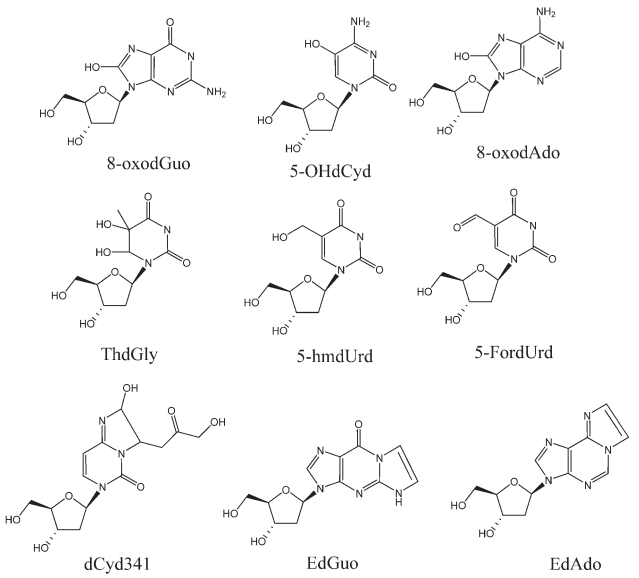

Fig. 3. Structures of purine and pyrimidine nucleosides specifically analysed by the techniques described in the Materials and Methods section.

Discussion

The original objective of the experiment described here was in the nature of a hypothesis-generating study investigating the species difference in tumorigenic response to inhaled vanadium pentoxide. The U.S. NTP (2002) had clearly shown that B6C3F1 mice were susceptible to tumours of the bronchi and alveoli when exposed to atmospheric concentrations of vanadium pentoxide of 1, 2 or 4 mg/m^3 and that within this concentration range there was little indication of concentration dependence in the response[11]. Thus, the overall rates for adenomas or carcinomas of the alveoli and bronchi at these concentrations, respectively, were 84%, 86% and 86% for male mice and 64%, 70% and 64% for female mice[11,12]. Female mice were selected for study here because the induced response was greater than in males amongst which control group overall tumour rates were 44% whereas they were only 2% in females. The corresponding overall tumour rates for rats exposed to 0, 0.5, 1 or 2 mg/m^3, were 8%, 20%, 13% and 18% for males and 0%, 6%, 2% and 2% for females. Since there is only a four-fold difference in concentrations it could have been that there was no real difference in received dose at the target tissue for each species. Also, the difference in inhalation characteristics of rat versus mouse could have resulted in a far larger dose to the target tissue in mouse compared to rat. Being fully aware of these complexities, the NTP incorporated kinetic studies[11,27] in parallel with the rat and mouse studies of carcinogenicity. These kinetic studies were reported to show that the lung burdens of vanadium appeared to reach steady-state with time at the lowest administered concentrations (0.5 mg/m^3 for rats and 1 mg/m^3 for mice), but at higher concentrations the lung burdens in both species declined with time after reaching maximum values at about 6 months for rats and 1–2 months for mice. The tabulated data in Dill et al. (2004) also suggest that, if the only determinant for lung tumours was the lung burden of vanadium (μg V/g lung) then one might predict the tumour incidences in female rats exposed to 1 or 2 mg/m^3 to be very similar to the incidences in female mice exposed to the same atmospheric concentrations of vanadium pentoxide[27]. This is because the lung burdens at these concentrations in the two species were very similar *throughout* the exposure period, with eight measurements taken, beginning on day 1 and ending on day 535. It is also noted that these lung weight-specific vanadium burdens were approximately three-fold higher in the

Table 7. Transitions Used for Detection of the Different DNA Lesions, Together with The Limits of Detection

Nucleoside oxidation product	Transition	Limit of detection (lesions per 10^6 nucleosides)	Reference
8-oxodGuo	284 → 168	0.05	Ravanat *et al.*, 1998[22]; Frelon *et al.*, 2000[23];
8-oxodAdo	268 → 152	0.8	Frelon *et al.*, 2000[23];
5-OH-dCyd	244 → 128	0.1	Riviere *et al.*, 2006[24];
ThdGly	275 → 116	0.3	Frelon *et al.*, 2000[23];
5-hmdUrd	257 → 126	0.1	Frelon *et al.*, 2000[23];
5-FodUrd	255 → 212	0.2	Frelon *et al.*, 2000[23];
dCyd341	342 → 226	0.02	Regulus *et al.*, 2007[25];
EdGuo	292 → 176	0.02	Douki *et al.*, 2004[26];
EdAdo	276 → 160	0.01	Douki *et al.*, 2004[26];

Table 8. Levels of 8-oxodGuo and dCyd341 Measured in Mouse Lung Samples Following Exposure to Vanadium Pentoxide

Nucleoside oxidation product	V_2O_5 concentrations in air (mg/m^3)			
	0	0.25	1	4
8-oxodGuo mean ± SD	0.24 ± 0.04	0.26 ± 0.07	0.43 ± 0.14[a]	0.49 ± 0.11[b]
dCyd341 mean ± SD	0.067 ± 0.007	0.061 ± 0.028	0.075 ± 0.022	0.068 ± 0.010

Values are expressed as the numbers of lesions/10^6 nucleosides. [a] $p < 0.01$; [b] $p < 0.001$

highest exposure group compared with the lowest exposure group for both female rats and female mice; consequently lung burden parity cannot be invoked to explain the lack of exposure-related increases in tumorigenic responses, unless it is suggested that a plateau has been reached in pathology development even at the lowest vanadium pentoxide exposure concentration. This also appears unlikely, however, because the non-neoplastic pathology in the same organs does show exposure concentration-related incidence increases in male and particularly in female rats and mice (see Fig. 1). Furthermore, the profound species differences in tumorigenic responses in the NTP study[11,12] do not have their counterparts in the non-neoplastic lung pathology, such as the incidences of hyperplasia of the alveolar epithelium or the bronchiolar epithelium and the incidences of histiocytic infiltration of the alveoli.

The NTP[11] found no evidence for genetic toxicity from use of the *in vitro Salmonella*/microsomal assay (*S. typhimurium* strains TA1535, TA100, TA97, TA98 and TA102) and in contrast to a number of other studies, which were reviewed in Assem and Levy (2009)[13], there was no evidence for micronucleus induction in an *in vivo* assay of peripheral blood cells from mice that had been exposed to vanadium pentoxide by inhalation at five concentrations ranging from 1 to 16 mg/m^3 for three months. Now, pentavalent vanadium compounds form vanadates and vanadate derivatives in aqueous media and there are many examples in publications from the 1980's and 1990's that rapid reduction of vanadium(V) to vanadium (IV) compounds occurs in the presence of thiols such as reduced glutathione, cysteine, 2-mercaptoethanol and dithiothreitol (DTT) under physiological conditions[28]. Should these assertions be correct, it is possible that the NTP's peripheral blood micronucleus test may not reflect activity occurring at the site of first contact of vanadium pentoxide. Reduced glutathione forms weak complexes with tetravalent vanadium (V^{IV}) and even weaker ones with V^{V}, because it will undergo oxidation while V^{V} is reduced[29]. However, it has been demonstrated that 2-mercaptoethanol forms complexes with both V^{V} and V^{IV}, and recent studies have found that, at pH 7, the amount of V^{V} compared with the amount of V^{IV} that is bound to the ligand is 10^6 greater at 1 mM and 500-fold greater at 1μM, suggesting not only a greater stability of vanadate-2-mercaptoethanolate complex but also that vanadate can coexist in solution with 2-mercaptoethanol[28]. In addition to reduction, therefore, vanadate complexes might be stable in the presence of reduced glutathione. Consequently, the NTP's peripheral blood micronucleus assay could be adequate for the assessment of genotoxic effects with relevance to lung carcinogenesis. Verification of this suggestion requires study of vanadium speciation in mouse-derived blood. Such studies have been conducted in ascidians, using vanadium K-edge X-ray absorption spectroscopy[30], but apparently not in mammalian blood. In the absence of such information, it was considered preferable to include as part of the current study an examination of possible genetic toxicity as well as other possible components of a mode of action within the known target tissue for carcinogenesis.

Administration of vanadium pentoxide at actual mean concentrations very close to the targets of 0.25, 1 or 4 mg/m^3 air for at least 16 days to mice by nose-only, flow-past inhalation exposure resulted mainly in pulmonary effects (increased lung weight, inflammatory lesions, DNA lesions and increased cell proliferation rate) at the two higher exposure levels only, with increasing dose-dependent severity. Dose-related increases in lung weight that occurred in the 1 and 4 mg/m^3 groups were not the simple result of vanadium pentoxide deposition: the quantities deposited over 16 days were insufficient to account for the observed lung weight increases. A much more likely explanation is that the weight increases were due to the recruitment of inflammatory cells and an accumulation of fluid within the pulmonary tissues. Deposition of vanadium pentoxide particles in the lungs was considered to result in histopathological changes in the 1 and 4 mg/m^3 groups that may have been the result of incomplete particle clearance from the lung as well as a direct toxic action on the pulmonary epithelium induced by the oxide. It is plausible that macrophages involved in the clearance process were unable to destroy the phagocytised particles, died and released pro-inflammatory cytokines which would have resulted in the attraction of yet more inflammatory cells and in a sustained oxidative stress. Indeed, Rondini *et al.* (2010)[31] have demonstrated increases in keratinocyte-derived chemokine(KC), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), NFκB and c-FOS binding activity as well as sustained phosphorylated ERK1/2 binding activity in lung homogenates from mice of strains susceptible to pulmonary inflammation that were treated with vanadium pentoxide (5 weekly aspiration of 4 mg/kg body weight) after a single intraperitoneal injection of 3-methylcholanthrene (10 mg/kg body weight). This treatment regime also resulted in pulmonary tumour promotion with an increased tumour multiplicity that followed the susceptibility to inflammation, i.e., A/J > BALB/cJ while C57BL/6 J was refractory to tumour promotion and showed the weakest inflammatory response. Rondini *et al.* (2010) suggest that the tumorigenic activity of particulate vanadium pentoxide in mouse lung is a strain-specific augmentation of lung carcinogenesis in susceptible individuals through oxidative stress mediated pathways[31].

Such an explanation is in accordance with the small, statistically significant increase in 8-oxodGuo levels in the 1 and 4 mg/m^3 groups, although there are alternative explanations for this oxo-adduct lesion change. Any increases in seven of the specific DNA lesions investigated would have been at very low frequencies below the limits of detection listed (Table 7). Vanadium pentoxide might have been reasonably predicted to produce a broader spectrum of damage if, indeed, oxidation was the mechanism leading to the increase in 8-oxodGuo. It was once believed that the steady-state amount of DNA oxidation were very large in comparison with non-oxidative adducts[32,33], although it is not clear how much of this apparent difference was due to unrecognised inadequacies in DNA processing. Oxidation

lesions of DNA occur at high daily frequencies under non-stress conditions and this lesion in particular is normally rapidly and efficiently repaired[34,35]. It has been estimated that in liver of homozygous *ogg1*$^{-/-}$ (i.e., oxoguanine glycosylase repair enzyme deficient) mice the level of 8-oxodG accumulates at a rate of 12,000 modifications per diploid cell per week, based on measurements at 14 weeks of age compared with 9 weeks[36]. Hydroxyl radicals (HO•) is one of the reactive oxygen species involved in oxidative stress and is held responsible for the formation of both 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) and, to a minor extent, 8-oxo-7,8-dihydro-2′-deoxyadenosine (8-oxodAdo)[37]. 8-oxodGuo can also be produced by singlet oxygen ($^1O_2$), this being a highly specific reaction in that guanine is the exclusive target in DNA[38,39]. Experiments in which $^1O_2$ was the only reactive oxygen species generated have shown that it produces predominantly Fpg-sensitive sites that are detectable with the comet assay and attributable in the main to 8-oxodGuo, as confirmed by HPLC-MS/MS measurements[20,40]. Endo III-sensitive sites (attributed to oxidized pyrimidines) were not generated, thereby demonstrating an absence of oxidised pyrimidine lesions[39]. Amongst several other mechanisms by which 8-oxodGuo can be produced is the formation of tandem lesions. Peroxyl radicals of a pyrimidine base can react with a vicinal DNA base (i.e., where 2 functional groups are bonded by 2 adjacent atoms) by addition of the pyrimidine peroxyl radical onto the C8 of a purine is capable of producing 8-oxodGuo that is involved in a tandem lesion on a neighbouring formylamine[41,42]. Although this specific lesion may account for no more than 10% of 8-oxodGuo, there may be other 8-oxodGuo containing tandem lesions produced by similar mechanisms. These tandem lesions generated by hydroxyl radicals are at least partly refractory to excision repair by DNA glycosylases[43]. In addition, one-electron oxidation of DNA would generate mostly 8-oxodGuo[44] and thus Fpg sensitive sites without creating a large number of direct strand breaks[45], as observed in potassium bromate treated cells[46]. Consequently, an alternative to lesion induction as the reason for the increase in 8-oxodGuo is the inhibition of repair enzyme activity by vanadium ions or complexes. However, the rate of accumulation is reported to be tissue-specific and lung is not one of the organs examined[47] so this hypothesis remains to be tested although inhibitory activity of vanadium towards specific enzymes is well-documented[29]. Furthermore, vanadium pentoxide might have been reasonably predicted to produce a broader spectrum of damage if, indeed, oxidation was the mechanism leading to the increase in 8-oxodGuo.

The comet assay result did not support an involvement of genetic toxicity in a mode of carcinogenic action for vanadium pentoxide, although the concentrations of vanadium measured in lungs of the mice after 16 days exposure (Table 3) suggest that, if this had been an *in vitro* study a significant response would have been possible. Vanadium in lung measurements found during the current study (Table 3) would translate into concentrations between 8 and 64 µg V/mL culture medium, which is comfortably within the effective vanadium pentoxide concentration range for significant comet assay responses *in vitro*[48–50]. This assay is dependent on the formation of DNA strand breaks. If, as has been suggested[51], vanadium pentoxide interferes with tubulin polymerisation and thereby causes aneuploidy then this would not have been detected with the comet assay, but could have been in a micronucleus test. Such assays have been conducted, but unfortunately not in the target tissues for carcinogenesis. The soluble V^V salts sodium orthovanadate (Na_3VO_4) and ammonium metavanadate (NH_4VO_3) as well as the V^{IV} compound, vanadyl sulphate ($VOSO_4$), increased the frequencies of cells with micronuclei, aneuploidy and (but only for vanadyl sulphate) structural chromosomal aberrations in bone marrow cells of mice treated orally by gavage[52], although the micronucleus test positive reported for sodium orthovanadate in this study was not confirmed either in a more recent study after i.p. injection of 25 mg/Kg body weight[53] or in a drinking water study of vanadyl sulphate at exposures up to 1000 mg/L for 5 weeks[54]. In this last study, the concentration of vanadium measured in the target tissue (femur) was 13.03 µg V/g. No strictly comparable measurements of vanadium in the target organ are available following 16 weeks exposure to vanadium pentoxide (16 mg/m^3 6h/day, 5 days/week) by inhalation in the study of micronucleus induction conducted by NTP[11]. The closest to an appropriate vanadium measurement for comparison in female mice comes from the 2 year segment of that study in which the highest exposure was to 4 mg/m^3 and the highest blood concentration of vanadium was only 1.1 µg/g blood at 26 days[11]. Thus, the absence of an effect can be ascribed to the low concentration of vanadium V^V at the target cells or to selection of resistant cell populations in the marrow during the three months inhalation exposure in the NTP study, but not because of possible valence changes V^V to V^{IV} upon inhalation.

Other indicators of oxidation status measured in the study included α-tocopherol, but alterations in the levels of this antioxidant (a decrease at 0.25 mg/m^3 and an increase at 4 mg/m^3) were considered to be of no biological significance. GSSG, as the oxidation product of GSH, was slightly increased with a weak dose-relationship, possibly as a result of either scavenging reactive oxygen by GSH or because of its interaction with vanadium pentoxide. While an accompanying decrease in GSH might then be expected, GSH is much less affected due to its 10-fold constitutive excess (see the control groups in Table 5). In addition, the *de novo* syntheses of GSH and glutathione reductase are known to be induced very quickly by oxidative stress, thereby compensating for any depletion of GSH. Nevertheless, the increase in GSSG was marginal in this experiment. This weak response finds support in the absence of a significant change in isoprostane levels, indicating that the natural defence mechanisms of the lung against oxidative stress were sufficient to protect the tissues in all groups against a weak oxidative stress that might be induced by vanadium pentoxide. It is noted, however, that a different pattern of responses has been observed in the livers of streptozotocin-induced

diabetic rats treated with vanadate (as Na_3VO_4), where reduced glutathione levels decreased while GSSG levels and glutathione reductase activity remained unchanged[55].

Inflammatory lesions in the lungs consisting of alveolar histiocytosis, alveolitis and granulocytic infiltration were noted in the 1 and 4 mg/m^3 groups and confirm the observations made in the NTP study over a similar exposure period at concentrations down to 2 mg/m^3[3,11,12]. A corresponding increase in the proliferation rate of histiocytes was demonstrated in mice of the current study exposed to 1 mg/m^3 for either 7 or 16 days that confirmed the increases in bromodeoxyuridine-labelled lung nuclei reported in the terminal bronchi after exposure of female mice to 2 mg/m^3 and female rats to 1 mg/m^3 for either 6 or 13 days in the NTP (2002) study[11]. These pathological findings were concordant with the increased lung weights and were probably related to an incomplete lung clearance of particulates. This response may also have contributed to the marginal body weight loss during the first week of this 16-day study.

In conclusion, several of the pathological findings made in the short-term whole-body exposure studies conducted by the NTP (2002)[11] have been confirmed and the absence of evidence from that study for a genotoxic mode of (carcinogenic) action extended to the target tissues, at least as observable with the comet assay. It is not yet clear whether the increase in 8-oxodGuo DNA lesions was due to induction by vanadium pentoxide or inhibition of repair of spontaneous lesions and evidence is weak for oxidative stress playing any role in lung carcinogenesis at the lowest effective concentrations of vanadium pentoxide.

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