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Induction of DNA-strand breaks in human peripheral blood lymphocytes and A549 lung cells by sodium dichromate: association with 8-oxo-2-deoxyguanosine formation and inter-individual variability

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Hexavalent chromium [Cr(VI)] is a genotoxic carcinogen for which inhalation is a major potential route of exposure in occupational settings. In the present study, the ability of sodium dichromate to cause DNA strand breaks in three populations of cells, human whole blood cells, isolated human peripheral blood lymphocytes and cultured A549 lung epithelial cells, was investigated. Treatment with non-cytotoxic concentrations of sodium dichromate (for 1 h) resulted in a concentration-dependent increase in the number of DNA strand breaks as measured by the Comet assay. The lowest concentrations of sodium dichromate that resulted in a statistically significant ($P < 0.01$) increase in the number of DNA strand breaks were 500, 50 and 10 μM, respectively, in these cells. The use of formamido-pyrimidine glycosylase increased the sensitivity of detection of strand breaks in A549 cells 10-fold, suggesting a role for DNA base oxidation in the mechanism of dichromate-induced DNA strand breaks. In support of this hypothesis, immunocytochemistry indicated an elevation of 8-oxo-deoxyguanosine in A549 cells treated with 10 and 500 μM sodium dichromate for 1 h. We also demonstrated 2.11- and 2.5-fold ranges in the level of control and dichromate (500 μM)-induced DNA strand breaks, respectively, in cells of whole blood within a group of healthy volunteers ($n = 26$). A statistically significant ($P < 0.001$) positive Pearson's correlation ($r = 0.606$) was found between control and treated levels of DNA strand breaks, suggesting that factors responsible for relatively low levels of DNA strand breaks in untreated PBL may also offer protection against the formation of dichromate-induced DNA strand breaks.

Introduction

In nature, chromium exists in trivalent [Cr(III)] and hexavalent [Cr(VI)] forms. Cr(III) is an essential human micronutrient (1 μg/day) and is believed to mediate sensitivity of cells to the gluconeogenic effects of insulin (Brown *et al*., 1986). In contrast, epidemiological studies, supported by animal and *in vitro* data, suggest that many Cr(VI) compounds are carcinogenic and the International Agency for Research on Cancer has classified Cr(VI) as a group I carcinogen (International Agency for Research on Cancer, 1990). Cr(VI) compounds are used industrially as catalysts, corrosion inhibitors and as an alloying metal in making stainless steel and various superalloys. Subsequently, Cr(VI) is present in various welding fumes (Waterhouse, 1975; Alderson *et al.*, 1981; Cohen *et al*., 1993; Warfel *et al*., 1998). In the workplace, exposure to chromium can be oral and dermal, however, the respiratory tract is the major site of exposure and, consequently, there is an increased risk of cancers of the respiratory system in exposed individuals (Cohen *et al*., 1993).

Cr(VI) salts, including sodium dichromate, are genotoxic and cause DNA strand breaks (Gao *et al*., 1992), chromium–DNA adducts (Xu *et al.*, 1994) and DNA–DNA (Bridgewater *et al*., 1994) and DNA–protein crosslinks (Cupo and Wetterhahn, 1985) in a number of *in vitro* and *in vivo* experimental systems. Although the mechanism(s) involved remains to be fully elucidated (Cohen *et al*., 1993), intracellular reduction of Cr(VI) by cellular reductants such as reduced glutathione (GSH) and subsequent generation of partially reduced chromium species such as Cr(V) and Cr(IV) are believed to be important (Snow, 1991). Additionally, reduction of Cr(VI) may also result in concomitant generation of reactive oxygen species (ROS), including hydroxyl radicals (OH°), singlet oxygen (O°), superoxide ($O_2^{\circ-}$) and hydrogen peroxide (H_2O_2) (Aiyar *et al*., 1994; Shi *et al*., 1994; Ye *et al*., 1999; Wang *et al*., 2000). ROS are known to damage DNA, resulting in base modifications, including 8-oxo-2-deoxyguanosine (8-oxo-dG) (Tsou *et al*., 1999). Although Cr(VI) and Cr(III) have been demonstrated to cause 8-oxo-dG adducts in isolated DNA (Faux *et al*., 1992; Wenbo *et al*., 2000), there is no direct evidence for Cr-mediated formation of 8-oxo-dG in whole cell models. In addition to these directly genotoxic mechanisms, Cr(VI) has a number of other deleterious effects within the cell, including inhibition of cellular respiration, resulting in aberrant oxygen metabolism and subsequent indirect formation of ROS (Cohen *et al*., 1993). Recently, Cr(VI) has been demonstrated to modulate cell signalling pathways known to be involved in cell proliferation by activating JNK, p38 and ERK MAP kinase activity in CL3 human lung carcinoma cells (Chuang *et al*., 2000). Furthermore, the effects of Cr(VI) could be partially reversed by co-incubation with anti-oxidants, suggesting an intermediary role for the formation of ROS.

Although it is widely accepted that Cr(VI) is genotoxic, many previous mechanistic studies have focused on relatively high concentrations of Cr(VI) (mM range) which are not directly relevant to occupational exposure (Cr levels as high as 5 μM may be attained in the blood of chrome pigment production workers; Carson *et al.*, 1986; International Agency for Research on Cancer, 1990). The alkaline version of the single cell gel electrophoresis (SCGE) or Comet assay represents a sensitive technique for the detection of single- and double-strand DNA breaks and alkali-labile sites in cells exposed to genotoxic carcinogens (Singh *et al.*, 1988). The Comet assay has been used previously to investigate sodium dichromate-induced DNA damage both *in vivo* and *in vitro*

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(Pool-Zobel *et al*., 1994; Blasiak *et al.*, 1999; Martin *et al.*, 1999; Blasiak and Kowalik, 2000; Merk *et al*., 2000; Trzeciakl *et al*., 2000; Ueno *et al.*, 2001). However, in the few studies utilizing human cells, only relatively high (>0.2 mM) concentrations of Cr(VI) have been investigated (Pool-Zobel *et al*., 1994; Blasiak *et al*., 1999; Blasiak and Kowalik, 2000; Trzeciakl *et al.*, 2000) and the issue of cytotoxicity has not been adequately addressed. Furthermore, no studies have investigated cells derived from the lung, the principal biological target of Cr(VI).

The aim of the current study was to investigate the potential usefulness of the Comet assay as a tool for detecting DNA strand breaks caused by micromolar concentrations of Cr(VI). We chose to investigate this in three cell populations: whole blood cells, isolated peripheral blood lymphocytes (PBL), potentially useful for investigating biomarkers of exposure to Cr(VI) *in vivo*, and cultured A549 lung epithelial cells, which may represent a useful *in vitro* model of the lung, the primary biological target of Cr(VI). We also used a modified version of the Comet assay (Collins *et al.*, 1993) incorporating the repair endonuclease formamidopyrimidine glycosylase (Fpg) and immunocytochemistry to test the hypothesis that DNA strand breakage was related to formation of oxidised purines and 8-oxo-dG, respectively, in Cr(VI)-treated A549 cells. Additionally, we carried out a preliminary study to investigate potential inter-individual variability in levels of DNA strand breaks produced by Cr(VI) in lymphocytes derived from the whole blood of healthy individuals.

Materials and methods

All chemicals were purchased from Sigma-Aldrich, unless stated otherwise.

Cell culture

The A549 cell line (European Cell Culture Collection no. 86012804) is an epithelial-like human lung cell line derived through explant culture of lung carcinomatous tissue from a Caucasian 58-year-old male and was a gift from Dr S. Faux (MRC, Leicester, UK). Adherent cells were cultured in T_{25} flasks (Nunc) at 37°C in a humidified, 5% CO_2 atmosphere and maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were passaged twice weekly using a standard trypsinization/EDTA protocol.

Blood samples were collected by venipuncture into vacutainers (Hemograd EDTA; Becton Dickinson, Oxford, UK). Mononuclear white blood cells were separated from whole blood by centrifugation using Histopaque-1077 (Sigma) according to the manufacturer's instructions. The buffy-coat was aspirated and resuspended in RPMI 1640 medium. Cell viability was assessed using Trypan blue dye exclusion and was always >95%.

Treatment

To determine the concentration range of Cr(VI) that showed no evidence of cytotoxicity, post-confluent monolayers of A549 cells in 12-well plates (Nunc) and PBL in suspension (8×10^4 cells/ml) were treated with sodium dichromate (1–5 mM) at 37°C for 1 h. A549 cells were removed from culture flasks by trypsinization and resuspended in DMEM. Cell membrane integrity was assessed by exclusion of the dye Trypan blue. To investigate the possible effects of sodium dichromate (0–2 mM) on cellular ATP levels, A549 cells were grown to confluency and intracellular ATP levels were measured using an ATP bioluminescent kit (FL-AA) according to the manufacturer's instructions (Sigma). To investigate possible dichromate-induced apoptosis, A549 cells were cultured to confluency in microscope chamber slides (Lab-Tek II; Nunc). Following treatment with sodium dichromate (1–5 mM for 1 h) the frequency of apoptosis was assessed by the TUNEL method using a commercially available kit (Dead End; Promega).

For the investigation of Cr(VI) genotoxicity, A549 and PBL were treated with non-cytotoxic concentrations of sodium dichromate (0–1 mM) as described above, with the exception that A549 cells were removed from culture flasks using a plastic cell scraper to avoid trypsin-induced DNA damage. Whole blood (5 µl) was suspended in 1 ml of Hank's balanced salt solution (HBSS) containing the appropriate concentration of sodium dichromate and incubated at 37°C for 1 h. Following treatment, cells were pelleted by centrifugation (200 *g* for 10 min), the supernatant removed and the resulting pellet used for Comet analysis.

Comet assay

The alkaline version of the Comet assay was performed according to the method of Singh *et al.* (1988) with minor modifications. Briefly, fully-frosted microscope slides were pre-coated with 1 ml of 0.75% normal melting point agarose (NMA) and stored at 4°C. This layer was removed before use and 120 µl of 0.75% NMA was pipetted onto the slides, which were then covered with coverslips. Cell suspensions (~1×10^4 cells/5–10 µl) were mixed with 70 µl of 1% low melting point agarose (LMA) and pipetted over the first layer of agarose. NMA (80 µl) was used as a final protective layer. After each step the slides were incubated at 4°C for 10 min to allow the agarose to gel. Slides were placed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, and 1% sodium *N*-lauroyl sarcosinate to which 10% DMSO and 1% Triton X-100 were added immediately prior to use) for 1 h. After lysis, slides were placed in electrophoresis buffer (300 mM NaOH and 1 mM Na_2EDTA, pH 13) for 20 min to allow unwinding of DNA. Electrophoresis was conducted in the same buffer by applying an electric current of 0.8 V/cm (300 mA) for 20 min, using an electrophoresis power supply (Pharmacia LKB, Sweden). Finally, slides were washed in neutraliszation buffer (0.4 M Tris, pH 7.5) three times for 5 min each, dried and stained with 50 µl ethidium bromide solution (20 µg/ml).

For the detection of oxidative DNA damage, Fpg, an endonuclease with specific activity towards oxidized purines, was used. After the lysis step, slides were washed (3×5 min) with enzyme reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTA, 0.2 mg/ml BSA, pH 10) and incubated with either buffer (50 µl) or Fpg in buffer (50 µl, 1 µg/ml Fpg) at 37°C for 30 min. DNA unwinding and electrophoresis was then completed as described above.

Image and data analysis

Image analysis was achieved using an Axiovert 10 fluorescent microscope. The SCGE Assay, Komet Analysis, v.3.0 Europe software package was used for digitalization and analysis of the fluorescent images. Nuclei were excited with green light and the emitted red spectrum was captured by a 32×/0.40 dry objective. Images of 50 randomly selected nuclei were analysed per duplicate slide (100 nuclei total). Tail DNA (TD, percentage of DNA in the tail) and tail length (TL, µm) were determined to assess the extent of DNA damage. Statistical analysis of the difference between means was carried out using a two-sample, one-tailed Student's *t*-test and results were considered statistically significant at the 5% level.

Inter-individual variability

Blood samples were obtained from the National Blood Service Birmingham Centre (Vincent Drive, Edgbaston, Birmingham) and placed immediately on ice. All samples ($n = 26$) were from healthy adults aged between 17 and 63 years. Blood samples were treated *in vitro* with 0.5 mM sodium dichromate for 1 h at 37°C. DNA damage was detected using the Comet assay as described above, with the exception that cells were resuspended in a single layer of LMA on commercially available comet slides (Trevigen, UK).

Immunohistochemistry

For immunocytochemistry A549 cells were grown to confluency on microscope chamber slides (Lab-Tek II; Nunc). Following treatment, the medium was removed and the slides washed in PBS. Cells were fixed with 70% ethanol (–20°C for 10 min) and washed with PBS (2×5 min). Fixed cells were incubated with proteinase K (10 µg/ml dissolved in PBS) for 15 min, washed with PBS (1×5 min) followed by treatment with RNase (100 µg/ml dissolved in 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.4 mM NaCl) for 1 h at 37°C. Following treatment, plates were washed with PBS (2×2 min) and denatured with 4 N HCl (7 min). Plates were then neutralized with 50 mM Tris base (5 min) and PBS (2×2 min).

Treated plates were then incubated with 10 mM Tris–HCl, pH 7.5, containing 10% FBS for 4 h (37°C) to block non-specific sites before being incubated overnight at 4°C with monoclonal mouse anti-8-oxo-dG (5 µg/ml, MOG-100P; Japanese Institute for the Control of Aging) dissolved in blocking buffer. The following day, plates were washed with PBS (2×2 min) and visualization of the primary antibody was carried out using ExtrAvidin peroxidase staining (EXTRA-2; Sigma) and AEC chromagen (AEC101; Sigma) kits according to the manufacturer's instructions. Slides were mounted using glycerol–gelatin (GG-1; Sigma) and photographed at a magnification of ×79 using a Zeiss photomicroscope.

Results

Chromium VI cytotoxicity

The results of Cr(VI) cytotoxicity show that viability as measured by Trypan blue exclusion was ~90% in A549 cells

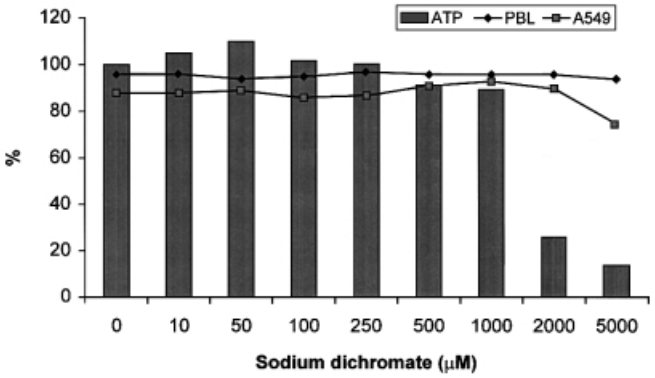


Fig. 1. Cytotoxicity data for A549 human lung cells and human peripheral lymphocytes. Lines represent per cent viability as measured by exclusion of the dye Trypan blue, bars represent intracellular ATP in A549 cells (% of control). Cells were treated with sodium dichromate at 37°C for 1 h. Values are the means of two experiments carried out in duplicate ($n = 2$).

and >95% in human peripheral lymphocytes treated with sodium dichromate (for 1 h) over the concentration range 0–2 mM (Figure 1). Furthermore, no significant decrease in intracellular ATP levels was observed following treatment with 0–1 mM sodium dichromate for 1 h in cultured A549 cells (Figure 1). There was also no evidence that sodium dichromate (1 h, 0–2 mM) induced apoptosis in confluent A549 cells as assessed by the TUNEL assay (<0.01% cells apoptotic). As a positive control, incubation of cells with DNase resulted in >95% labelling index (images not shown). Therefore, 0–1 mM sodium dichromate was chosen to investigate Cr(VI)-mediated DNA strand breaks in both cell types.

Chromium VI genotoxicity

A549 human lung cells

The background level of DNA strand breaks in untreated A549 cells was 13.13 and 106.18 as measured by TD and TL, respectively (Figure 2A). Treatment with sodium dichromate resulted in a concentration-dependent increase in the number of DNA strand breaks as measured by both parameters. The lowest concentration of sodium dichromate that resulted in a statistically significant ($P < 0.01$) increase in DNA strand breaks was 10 μM and the response was maximal at 1 mM dichromate (TD = 27.90, TL = 208.0).

Human peripheral lymphocytes

The background level of DNA strand breaks in untreated human peripheral lymphocytes was low when compared with A549 cells (TD = 7.66, TL = 50.63; Figure 2B). Treatment with sodium dichromate resulted in a concentration-dependent increase in the number of DNA strand breaks as measured by both parameters. The lowest concentration of dichromate that resulted in a statistically significant (TL, $P < 0.05$, TD, $P < 0.01$) increase in DNA strand breaks was 50 μM. A maximal response was observed at 500 μM dichromate (TD = 17.01, TL = 95.0), no further increase was detected with higher concentrations up to 1 mM. Interestingly, compared with A549 cells, dichromate treatment resulted in the formation of relatively short comet tails in this cell type.

Whole blood cells

The background level of DNA strand breaks in untreated human whole blood cells was higher than the values for PBLs but lower than those for A549 cells (TD = 10.92, TL = 61.36; Figure 3). Treatment with sodium dichromate resulted

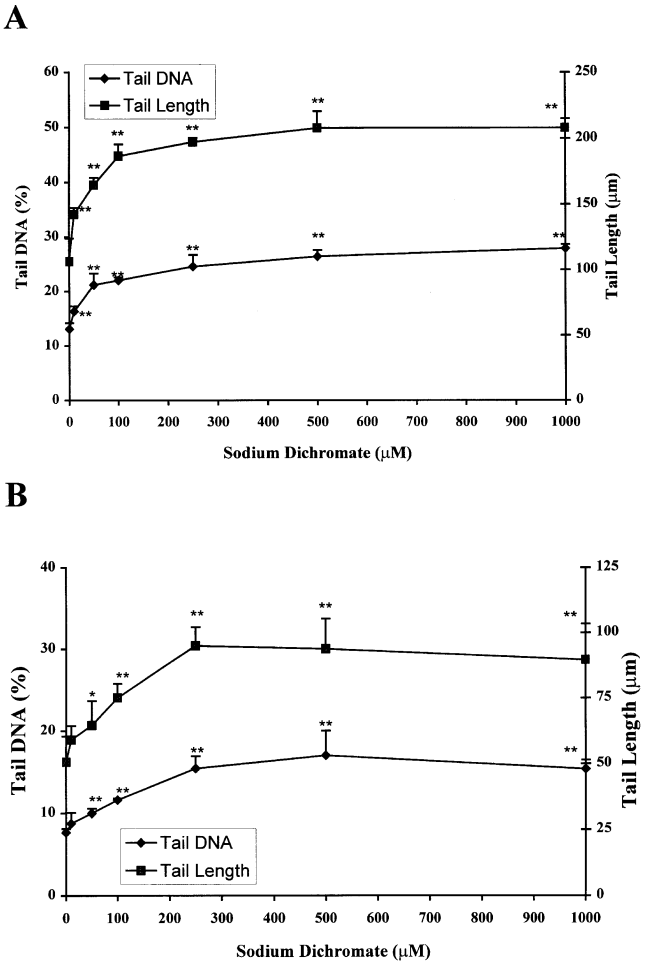


Fig. 2. TD and TL (μm) values representing DNA damage induced by Cr(VI) in A549 human lung cells (**A**) and human peripheral blood lymphocytes (**B**). Cells were treated with sodium dichromate at 37°C for 1 h. Values are the means ± SD of three experiments carried out in duplicate ($n = 3$). *, **, Significantly different from control at $P < 0.05$ and $P < 0.01$, respectively (*t*-test).

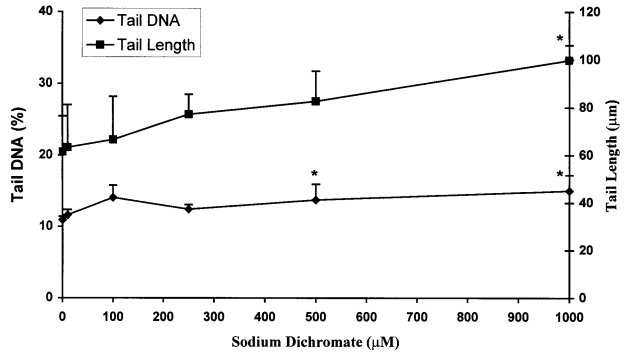


Fig. 3. TD and TL (μm) values representing DNA damage induced by Cr(VI) in lymphocytes derived from human whole blood. Cells were treated with sodium dichromate at 37°C for 1 h. Values are the means ± SD of three experiments carried out in duplicate ($n = 3$). *, Significantly different from control at $P < 0.05$ (*t*-test).

in a concentration-dependent increase in the number of DNA strand breaks as measured by both parameters. The lowest concentration of dichromate that resulted in a statistically significant ($P < 0.05$) increase in DNA strand breaks was 500 μM as assessed by TD, but significance was only achieved at 1 mM and above as measured by TL.

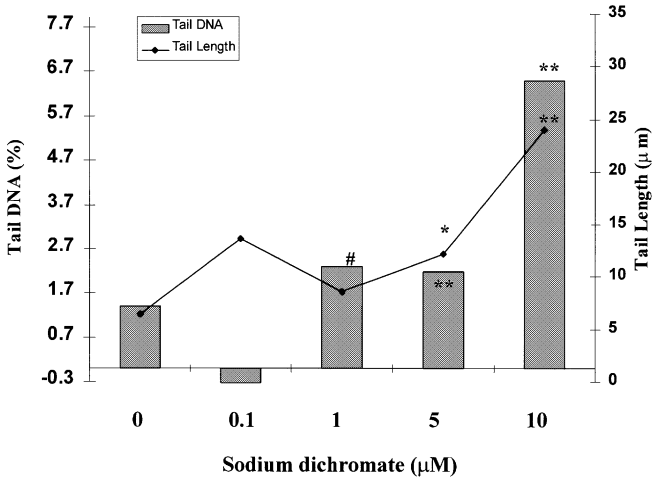


Fig. 4. Formation of oxidative DNA damage by hexavalent chromium in cultured A549 cells. The line and bars represent additional damage detected as measured by TL and TD, respectively, following incubation with Fpg as described in Materials and methods. Cells were treated with sodium dichromate at 37°C for 1 h. Values are the means ± SD of three experiments carried out in duplicate ($n = 3$). *, **, Significantly different from control at $P < 0.05$ and $P < 0.01$, respectively (*t*-test). #, Significantly different from control when assessed by tail moment ($P < 0.05$, *t*-test) data not shown.

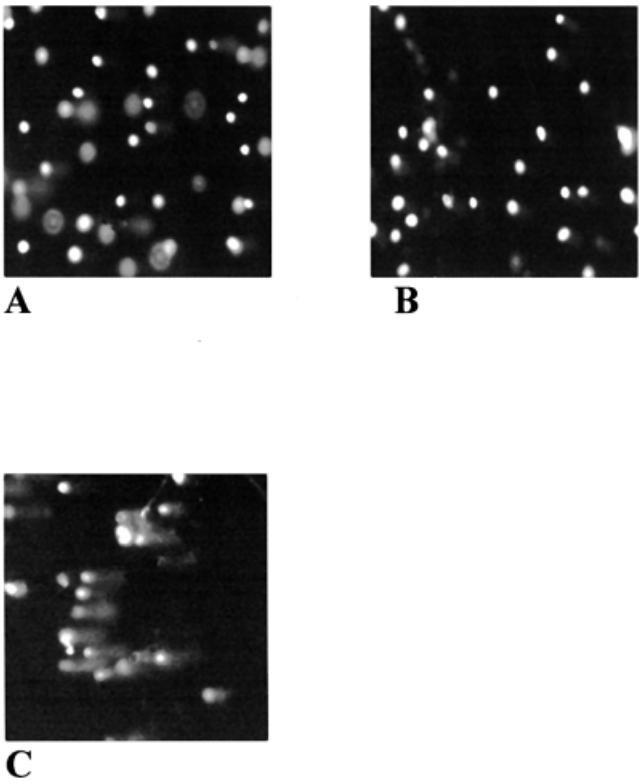


Fig. 5. Effect of Fpg treatment on the length of DNA comets in lung A549 cells treated with 10 μM sodium dichromate for 1 h at 37°C. (**A**) Fpg alone; (**B**) 10 μM sodium dichromate; (**C**) Fpg + 10 μM sodium dichromate. Note that some nuclei appear out of focus because of their depth in the agarose sandwich.

Detection of oxidative DNA damage

Modified Comet assay

There was no significant Fpg-dependent oxidative DNA damage in control A549 cells, TD being 11.41 and 12.49 and TL 119.1 and 125.7 in the absence and presence of Fpg treatment, respectively (Figure 4). Treatment with sodium dichromate resulted in a concentration-dependent increase in the levels of Fpg-dependent DNA strand breaks (Figure 4). The lowest concentration of dichromate which resulted in a statistically significant ($P < 0.05$) increase in Fpg-dependent DNA strand breaks was 1 μM when measured by tail moment (data not shown). Treatment with 5 μM dichromate resulted in a statistically significant increase ($P < 0.01$) when measured by both TD and TL (Figure 4). The additional DNA damage induced by Fpg was 6.5-fold greater in A549 cells treated with sodium dichromate (10 μM for 1 h) compared with controls, indicating that dichromate is capable of causing considerable levels of Fpg-dependent DNA strand breaks in this cell type (Figure 5).

Immunocytochemistry

Faint nuclear staining was apparent in control slides, indicating the presence of background levels of 8-oxo-dG (Figure 6A). However, following treatment with sodium dichromate (10 and 500 μM) for 1 h, the level of nuclear staining was considerably enhanced (Figure 6B and C), indicating that sodium dichromate treatment results in formation of elevated 8-oxo-dG in A549 lung cells. Cytosolic staining was not apparent in any of the treatments. Additionally, as a negative control, slides were processed in the absence of primary antibody. Neither nuclear nor cytosolic staining was apparent in the negative controls (images not shown).

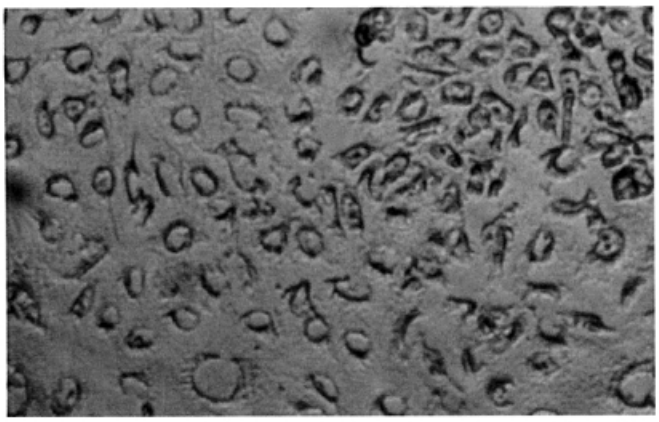
Inter-individual variability

The mean age of the study population was 39.2 ± 15.0 years and it comprised 12 females (46%) and 14 males (54%). Mean TD was calculated for the population before (control) and after treatment with 0.5 mM sodium dichromate for 1 h at 37°C (Figure 7) and were 13.38 ± 2.31 and 22.26 ± 5.03, respectively. Analysis using a one-tailed paired Student's *t*-test demonstrated a statistically significant ($P < 0.001$) increase in TD following treatment with sodium dichromate.

The inter-individual ranges of DNA strand breaks before and after treatment were 8.54–18.07 and 13.4–33.14% of DNA in the tail, respectively, indicating the existence of substantial variations in levels of both background and dichromate-mediated DNA strand breaks. Additionally, when the data were analysed using Pearson's correlation test it revealed a statistically significant ($P < 0.05$) positive correlation ($r = 0.307$) between control and treated levels of DNA strand breaks in the lymphocytes of the study population. Further analysis revealed that expression of the data as tail length resulted in a stronger correlation ($r = 0.606$, $P < 0.001$) between the control and treated levels of DNA damage (Figure 8).

Discussion

Cr(VI) has previously been reported to cause DNA strand breaks *in vitro* in human PBL using techniques such as the fluorometric analysis of DNA unwinding assay (Gao *et al.*, 1992). However, this and similar studies have been limited by the sensitivity of the assays employed and have been restricted to investigating DNA damage caused by high concentrations of Cr(VI) (>1 mM), which are not relevant to occupational exposure to Cr(VI). The Comet assay represents a sensitive method for the detection of frank DNA strand breaks and alkali-labile (e.g. apurinic) sites in cells exposed to genotoxic carcinogens (Singh *et al.*, 1988). Few studies (Pool-Zobel



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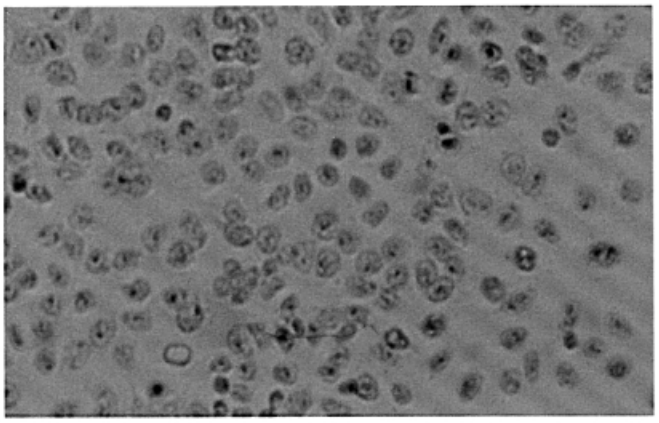

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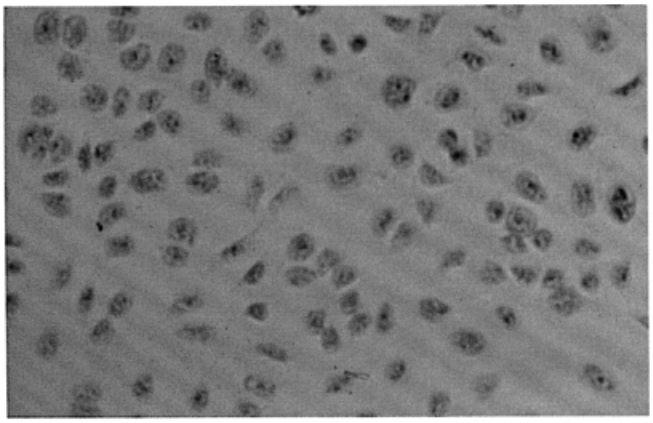

C

Fig. 6. Formation of 8-oxo-dG in cultured A549 lung cells treated with sodium dichromate as assessed by immunocytochemistry. Cells were cultured for 1 h at 37°C. (**A**) Control; (**B**) 10 µM sodium dichromate; (**C**) 500 µM sodium dichromate.

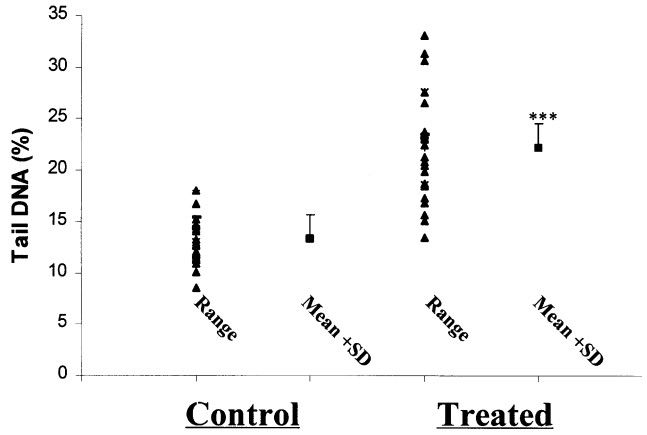

Fig 7. Inter-individual range, mean and SD of comet tail DNA (%) in human peripheral blood lymphocytes from individuals before and after treatment with 0.5 mM sodium dichromate for 1 h at 37°C ($n = 26$). ***, Significantly different from control ($P < 0.001$, one-tailed paired t-test).

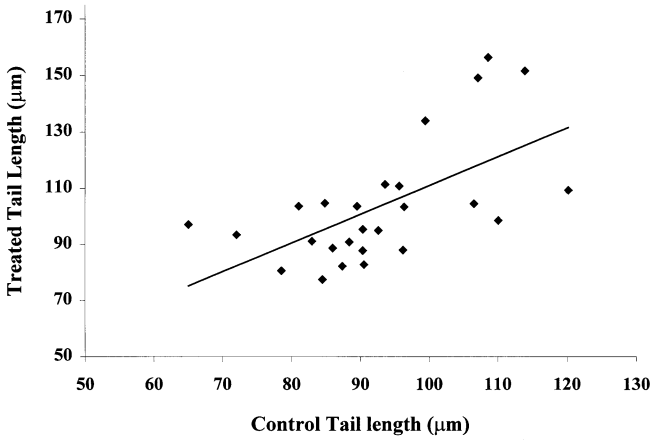

Fig. 8. Positive correlation between levels of DNA strand breaks in human lymphocytes before and after treatment with 0.5 mM sodium dichromate for 1 h at 37°C ($n = 26$). The best-fit line is shown. The two-tailed Pearson correlation coefficient (r) of 0.607 is statistically significant ($P < 0.001$).

et al., 1994; Blasiak *et al*., 1999; Blasiak and Kowalik, 2000; Trzeciakl *et al.*, 2000) have utilized the Comet assay to investigate the formation of Cr(VI)-mediated DNA strand breaks in human cells and only relatively high concentrations (>0.2 mM) of Cr(VI) have been investigated. No studies have investigated Cr(VI)-mediated DNA damage in cells derived from the lung (the principal biological target).

In the current study we have utilized the Comet assay to further investigate Cr(VI)-mediated DNA damage and have determined the concentration–response characteristics of Cr(VI)-mediated DNA strand breaks in three cell populations: human whole blood cells, isolated human PBL and cultured A549 lung cells. Compared with isolated PBL, cells in whole blood proved to be relatively insensitive to dichromate-induced DNA strand breaks, which only achieved significance at concentrations of ⩾500 µM. The relative insensitivity of whole blood to Cr(VI)-mediated DNA strand breaks may be a result of sequestration and reduction of Cr(VI) by red blood cells, a process known to be important *in vivo* (De Flora *et al.*, 1997), reducing the biologically effective dose of Cr(VI). Alternatively, it may be a result of the higher levels of background DNA damage observed in untreated whole blood cells (TD = 10.92) compared with PBL (TD = 7.5). One

possible explanation for high background levels of DNA strand breaks in whole blood is the generation of OH° radicals by the Fenton reaction due to the release of free iron from haemoglobin during processing of comet slides.

In contrast to whole blood cells, isolated human PBL are a sensitive *in vitro* model for the detection of hexavalent chromium-induced DNA strand breaks. The lowest concentration of sodium dichromate that resulted in a statistically significant ($P < 0.01$) increase in DNA strand breaks was 50 μM. At the lower concentration of 10 μM DNA strand breaks were elevated by 35% compared with controls, but were not statistically significant ($P = 0.14$). These observations suggest that the Comet assay may be sensitive enough to detect Cr(VI)-mediated strand breaks in PBL isolated from individuals occupationally exposed to Cr(VI), where it has been estimated that Cr levels up to 5 μM may be attained in the blood of chrome pigment production workers (Carson *et al*., 1986; International Agency for Research on Cancer, 1990). Our data also support previous observations of increased levels of DNA strand breaks measured by alkaline elution (Warfel *et al.*, 1998), DNA–protein crosslinks (Costa *et al.*, 1993) and sister chromatid exchange (Jelmert *et al*., 1994) in occupationally exposed workers.

The lung represents the principal *in vivo* target for the carcinogenic effects of Cr(VI) following occupational exposure. In the current study we have demonstrated the formation of Cr(VI)-mediated DNA strand breaks in cultured human A549 lung cells. The lowest concentration of sodium dichromate that resulted in a significant increase in DNA strand breaks was 10 μM in this cell type. Furthermore, the addition of Fpg increased the sensitivity of the Comet assay 10-fold, being able to detect statistically significant ($P < 0.05$) levels of DNA damage in cells exposed to concentrations as low as 1 μM sodium dichromate. As such, A549 cells represent a sensitive and biologically relevant *in vitro* model for further investigations into the mechanism(s) of Cr(VI)-mediated strand breaks. The explanation for the difference in sensitivity between PBL and A549 cells is unclear, but it may reflect differences in reductive activation of Cr(VI) or DNA repair between the two cell types. For example, the levels of GSH in PBL and A549 cells have been reported to be 4–10 and 40–100 nmol/10^6 cells, respectively (Cantin *et al.*, 2000).

The observed plateau of the dose–response curve at concentrations >500 μM and 1 mM in PBL and A549 cells, respectively, is a consistent finding. One possible explanation of this observation is that treatment with higher (>500 μM) concentrations of sodium dichromate may result in the formation of DNA–DNA and DNA–protein crosslinks which would impede DNA migration. In support of this hypothesis, Wedrychowski *et al*. (1994) reported formation of DNA–protein crosslinks in PBL only at concentrations >200 μM sodium dichromate and a maximal response was observed at 5 mM. In addition, Xu *et al*. (1996) have reported the formation of G-G inter-strand DNA crosslinks as measured by DNA polymerase arrest following treatment of human lung fibroblasts with hexavalent chromium (>200 μM). Other possible explanations include saturation of pathways involved in activation of sodium dichromate (e.g. reduction by GSH) or modulation of DNA repair mechanisms.

The increase in DNA strand breaks was seen in the absence of loss of membrane integrity and, as demonstrated in A549 cells, under conditions in which ATP concentrations were not reduced compared with controls. The effects are therefore not secondary to mitochondrial damage or cytotoxicity. It is known, however, that concentrations of ~20 μM and above can effect mitogen-activated signal transduction pathways and clonogenicity in lung epithelial cells (Chuang *et al.*, 2000; Martin *et al*., 1998)

Although Cr(VI)-induced DNA damage has been extensively studied, there remain many questions concerning the precise mechanism(s) involved. One particular area of uncertainty is the possible role played by ROS. Mechanistic studies utilizing isolated single-stranded and double-stranded DNA have demonstrated that stabilized Cr(V) species such as bis-(2-ethyl-2-hydroxy-butanato)oxochromate(V) are able to bind directly to phosphate groups in the DNA backbone. Once bound, these species abstract hydrogen atoms from either the C1 or C5 position of the ribose moeity of DNA, leading to formation of an abasic site and subsequent strand scission (Bose *et al.*, 1998, 1999). Interestingly, in these systems the predominant site of strand breaks is adjacent to guanine and these processes do not require or generate ROS (Bose *et al.*, 1998, 1999). In contrast, in other isolated DNA systems, induction of DNA strand breaks by Cr(VI) and GSH has been shown to be dependent upon ROS (Molyneux and Davis, 1995). In these models it is possible that strand breaks occur as a result of hydrogen abstraction from the ribose moiety by hydroxyl radicals generated during the reduction of Cr(VI) by glutathione (Casedeval and Kortenkamp, 1994, 1995).

In whole cell *in vitro* models many of the effects of Cr(VI) appear to be dependent upon the generation of intracellular oxidative stress. For example, Cr(VI)-dependent activation of MAP kinase activity and p53 binding to its promoter can be inhibited by co-incubation with anti-oxidants and radical scavengers (Chuang *et al.*, 2000; Wang *et al.*, 2000). Additionally, the use of fluorescent probes has directly demonstrated that intracellular levels of H_2O_2 and $O_2^{\circ -}$ are elevated following treatment with Cr(VI) (Wang *et al.*, 2000) and it is believed that OH° radicals are generated from H_2O_2 by reduction reactions catalysed by partially reduced chromium species in processes analogous to the Fenton reaction. It is recognized, however, that Cr(V) can also directly oxidize the fluorescent dye 2,7-dichlorofluorescin (Martin *et al.*, 1998) and may also contribute to DNA oxidation directly (Sugden and Wetterhahn, 1997). Furthermore, various anti-oxidants have been demonstrated to inhibit Cr(VI)-induced DNA damage (Blasiak and Kowalik, 2000).

In support of a role for DNA base oxidation in the biological effects of Cr(VI), we have demonstrated use of a modified version of the Comet assay (Collins *et al.*, 1993) incorporating Fpg, an endonuclease specific for oxidized purines (e.g. 8-oxo-dG, 5-oxodeoxycytosine and FaPyG), that in A549 cells sodium dichromate (10 μM) results in a statistically significant ($P < 0.01$) 6.5-fold increase in Fpg-dependent oxidative DNA damage compared with controls. In addition, using immunocytochemistry we have directly demonstrated for the first time formation of 8-oxo-dG in an *in vitro* cell culture model following treatment with sodium dichromate (10 and 500 μM). The 8-oxo-dG lesion is highly mutagenic and, through mispairing with adenine during DNA replication, results in formation of G→T transversions, a commonly observed mutation in the gene of the tumour suppressor p53 in human cancers, including lung cancer (Takahshi *et al.*, 1989; Iggo *et al.*, 1990; Lehman *et al.*, 1991; D'Amico *et al.*, 1992). Although other types of DNA damage are also able to cause G→T transversions, these observations suggest that

formation of 8-oxo-dG may represent an important mechanism by which Cr(VI) can cause human cancers.

Humans are continually exposed to a range of DNA-damaging agents in the environment. For example, solar UV radiation, ionizing radiation (cosmic rays) and mutagenic compounds present in food, as well as occupational exposure to carcinogens. It is extremely likely that inter-individual variations in susceptibility to these genotoxic insults exist within human populations. In the current study we have demonstrated a 2.5-fold range in individual response to sodium dichromate (500 μM)-induced single-strand breaks in human PBL within a group of healthy volunteers. In addition, we also observed a 2.1-fold range in background levels of DNA damage in the same population ($n = 26$). Anderson *et al*. (1994) observed a similar range in the control levels of DNA strand breaks as measured by the Comet assay in a group of healthy volunteers and related this to variability in anti-oxidant status. Furthermore, Warfel *et al*. (1998) observed a similar variation in DNA strand breaks measured by alkaline elution in both control populations and welders exposed to nickel and hexavalent chromium. However, to our knowledge this is the first time that DNA strand breaks have been measured in the same population before and after *in vitro* treatment of whole blood cells with hexavalent chromium. Further analysis of the data revealed a statistically significant ($P < 0.001$) positive Pearson's correlation ($r = 0.606$) between control and sodium dichromate-induced levels of DNA strand breaks. This suggests that factors responsible for low levels of DNA strand breaks in untreated PBL may also offer protection against dichromate-induced DNA strand breaks.

There are a number of potential factors that could explain these observed inter-individual variations, including genetic polymorphisms in DNA repair enzymes as well as enzymes that offer protection from oxidative stress (e.g. glutathione *S*-transferases π and θ) (Yin *et al.*, 2000). In addition, environmental factors such as dietary consumption of antoxidants and smoking habit may also play an important role. It has been demonstrated that DNA strand breaks in lymphocytes are increased by smoking (Betti *et al*., 1994; Frenzilla *et al.*, 1997), however, the influence of smoking on response to hexavalent chromium is not known. These hypotheses are currently under investigation in our laboratory.

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