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Modified comet assay as a biomarker of sodium dichromate-induced oxidative DNA damage: optimization and reproducibility

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Hexavalent chromium (Cr[VI]) is a genotoxic carcinogen that has been associated with an increased risk of nasal and respiratory tract cancers following occupational exposure. Although the precise mechanism(s) remain to be elucidated, there is evidence for a role of oxidative DNA damage in the genotoxicity of Cr(VI). In the current study, human white blood cells were treated *in vitro* with non-cytotoxic concentrations of sodium dichromate (1–100 μM) for 1 h. Analysis by immunocytochemistry indicated the presence of elevated levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine at concentrations of sodium dichromate greater than 10 μM. In contrast, the lowest concentration of dichromate that resulted in a statistically significant increase in levels of formamidopyrimidine DNA glycosylase (FPG)-dependent DNA strand breaks was 100 nM ($p < 0.05$). In addition, levels of both control and dichromate-induced FPG-dependent strand breaks from blood samples taken from the same individuals over 10 months proved remarkably reproducible in the individuals studied. The coefficients of variation over three different times of the year in control and dichromate-induced oxidative DNA damage for the four individuals were 54, 1, 37 and 4, and 45, 6, 21 and 18%, respectively. In summary, these results indicate that physiologically relevant, nanomolar concentrations of sodium dichromate cause DNA base oxidation in human white blood cells *in vitro* as assessed by the FPG-modified comet assay. Furthermore, comet assay data from an individual are reproducible over an extended period. This consistency is sufficient to suggest that the modified comet assay might prove to be a useful and sensitive biomonitoring tool for individuals occupationally exposed to hexavalent chromium.

Keywords: comet assay, sodium dichromate, DNA strand breaks, oxidative DNA damage.

Introduction

Hexavalent chromium (Cr[VI]) is genotoxic and has been classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (1990). It is used in a range of industrial processes, e.g. chrome plating, stainless steel welding, chromate manufacture and use, and ferrochromium production. Its use has been associated with an increased risk of respiratory tract cancer in a number of epidemiological studies of both chromate production workers (Alderson *et al.* 1981, Mancuso 1997, Gibb 2000) and chrome platers (Sorahan *et al.* 1998, Sorahan and Harrington 2000). Numerous *in vitro* studies have also demonstrated Cr(VI)-induced genotoxicity, including the generation of DNA strand breaks in a variety of human cell types (Gao *et al.* 1992, Pool-Zobel *et al.* 1994, Blasiak *et al.*

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1999, Martin *et al.* 1999, Blasiak and Kowalik 2000, Trzeciak *et al.* 2000, Hodges *et al.* 2001, Liu *et al.* 2001).

Biomonitoring studies have reported Cr(VI)-exposed workers with increased levels of genotoxic damage, including micronuclei (Vaglenov *et al.* 1999, Benova *et al.* 2002), sister chromatid exchange (Sarto *et al.* 1982, Lai *et al.* 1998, Werfel *et al.* 1998, Wu *et al.* 2000, 2001), chromosomal aberrations (Sarto *et al.* 1982) and DNA–protein cross-links (Popp *et al.* 1991, Werfel *et al.* 1998, Quievryn *et al.* 2001, Medeiros *et al.* 2003), in peripheral blood lymphocytes compared with matched control groups. Werfel *et al.* (1998) used alkaline elution to demonstrate increased levels of DNA strand breaks in welders exposed to Cr(VI). In contrast, Gao *et al.* (1994) failed to detect an increased level of DNA strand breaks in the peripheral blood cells of Cr-exposed workers using the fluorometric alkaline unwinding technique. Moreover, levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo dG) in lymphocytic DNA taken from Cr(VI)-exposed workers are not reported to be elevated above levels measured in unexposed individuals (Gao *et al.* 1994, Kim *et al.* 1999). There is a clear need for a sensitive assay for a relevant biomarker of Cr(VI) genotoxic exposure.

There is evidence that Cr(VI) can generate oxidative DNA damage. For example, the generation of sodium dichromate-induced oxidative DNA damage using the formamidopyrimidine DNA glycosylase (FPG)-modified version of the comet assay has been demonstrated, as has 8-oxo dG formation by immunocytochemistry in the A549 human lung carcinoma cell line (Hodges *et al.* 2001). Furthermore, there is mounting evidence that the generation of DNA strand breaks by Cr involves an oxidative mechanism. For example, the comet assay has also been used to demonstrate that antioxidants inhibit Cr(VI)-induced DNA damage (Blasiak and Kowalik 2000, Ueno *et al.* 2001).

The comet assay (single-cell gel electrophoresis assay) is a sensitive method for the detection of DNA strand breaks at the single cell level. The alkaline version of the assay (Singh *et al.* 1998), which can detect both single- and double-strand breaks. Furthermore, a modified version of this assay (Collins *et al.* 1993) in which the bacterial repair enzyme FPG is incubated on the slides to produce DNA strand breaks at the position of oxidized purines. The comet assay is relatively inexpensive, fast, simple and only requires a small amount of biological material. Consequently, it has been suggested as a suitable method for biomonitoring studies (Møller *et al.* 2000). Gambelunghe *et al.* (2003) found a statistically significant increase in tail moment in workers occupationally exposed to Cr that also correlated with Cr lymphocyte concentrations. To the present authors' knowledge, this is the only study that has employed the comet assay to study DNA damage in occupationally Cr(VI)-exposed individuals and which highlights the potential of the comet assay as a biomarker of Cr(VI) genotoxicity.

The aim was to investigate the value of the FPG-modified version of the comet assay as a method for monitoring individuals exposed to nanomolar concentrations of Cr(VI). White blood cells (WBC) were chosen for the investigation due to their potential as surrogate tissue for biomonitoring studies. Furthermore, the reproducibility of the FPG-modified comet assay was tested by measuring levels of both

control and dichromate-induced FPG-dependent strand breaks from blood samples taken from the same individuals ($n = 4$) over 10 months.

Materials and methods

All chemicals were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

Sample preparation and cell treatment

Whole blood (10 ml) was collected by venupuncture into vacutainers (Haemograd EDTA, Becton Dickinson, Oxford, UK). The blood was diluted in an equal volume of Roswell Park Memorial Institute (RPMI)-1640 medium. WBC were separated from whole blood by centrifugation using Histopaque-1077 according to the manufacturer's instructions. The 'buffy coat' was aspirated and resuspended in RPMI-1640 medium.

Mononuclear WBC were cultured in T_{25} flasks at 37°C in a humidified 5% carbon dioxide atmosphere in RMPI-1640 medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin.

Blood samples (4 ml) for the study on intra-individual variation were obtained by venupuncture into Vacutainer tubes containing ethylenediamine tetra-acetic acid (EDTA) as an anticoagulant. Samples were taken from the same four individuals (one male, three female) on three separate occasions.

Tests for cytotoxicity and apoptosis

Levels of intracellular adenosine triphosphate (ATP) were determined using an ATP bioluminescent kit (Sigma-Aldrich; FL-AA) according to the manufacturer's instructions. WBC in suspension (3×10^4 cells ml^{-1}) were treated with sodium dichromate (0–100 μM) for 1 h in a six-well plate British Drug House (BDH). After treatment, the cells were pelleted by centrifugation at 13 000 rpm for 2 min, the supernatant was aspirated and the pellet resuspended in 300 μl 1 × somatic cell ATP releasing reagent (supplied in kit). ATP was determined using a luminometer (Tecan Spectrafluor) and ATP levels were expressed as a percentage of the control levels.

For the investigation of possible dichromate-induced apoptosis, a TiterTACS™ colorimetric apoptosis detection kit (Trevigen, Inc., Gaithersburg, MD, USA) was used and cells were pelleted by centrifugation (500 *g*, 5 min, room temperature) between each stage. Isolated WBC were cultured in 96-well plates (10^5 cells ml^{-1}). Cells were treated with 0–100 μM sodium dichromate for 1 h at 37°C. After treatment, cells were pelleted by centrifugation (200 *g*, 10 min), the media was discarded and the cells washed twice with phosphate-buffered saline (PBS). Cells were then fixed with 3.7% buffered formaldehyde for 7 min, washed twice in PBS, post-fixed in 100% methanol and then washed twice more in PBS.

Proteinase K (50 μl) was added to each well and incubated for 15 min at room temperature. It was discarded and cells were then washed with distilled water. As a positive control, cells were incubated for 50 min at 37°C in 50 μl TACS-nuclease solution. During this incubation period, the samples were incubated in an equal volume of PBS. After this incubation period, cells were washed for 2 min with PBS before endogenous peroxidase activity was quenched by the incubation with 3% H_2O_2 for 5 min at room temperature. Cells were washed with distilled water and incubated with terminal deoxynucleotidyl transferase (TdT) Labeling Buffer for 5 min. Cells were subsequently incubated for 1 h at 37°C with Labeling Reaction Mix (containing the TdT enzyme). Negative control cells were generated by incubating cells with the same volume of Labeling Reaction Mix in the absence of the TdT enzyme. After this period, the buffer was discarded and replaced with TdT Stop Buffer for 5 min. The buffer was then discarded and cells washed with PBS (2 × 2 min). Cells were then incubated with Strep-HRP solution (50 μl per well) for 10 min at room temperature and washed four times with PBS-0.01% Tween 20. The substrate, TACS-Sapphire (100 μl), was added to each sample and incubated in the dark for 30 min. The reaction was stopped by the addition of 100 μl 2 M HCl. Absorbance was measured at 450 nm using a Tecan Spectrafluor plate reader.

Comet assay

The method used was a modification of the alkaline single-cell gel electrophoresis (comet) assay as developed by Singh *et al.* (1998). All stages were carried out under reduced light to prevent additional DNA damage.

Whole blood (10 μl) was suspended in Hank's balanced salt solution (calcium and magnesium free; 1 ml), and incubated in a water bath for 1 h at 37°C with sodium dichromate. WBC were isolated from whole blood and suspensions (8×10^4 cells ml^{-1}) were treated with sodium dichromate. After treatment,

lymphocyte suspensions were centrifuged at 3000 *g* for 5 min, the supernatants aspirated and the samples were placed on ice.

Normal melting point agarose (0.5%, 160 μl) was gently pipetted onto fully frosted microscope slides (Surgipath, Peterborough, UK), cover-slipped and placed on ice for at least 10 min to solidify. The cells prepared previously were resuspended in 110 μl low melting point agarose (0.5%) and pipetted on top of the normal melting point agarose layer. A coverslip was added to each slide, which was then placed on ice for a further 10 min so the agarose could solidify. The cover-slip was then removed and 160 μl 0.5% low melting point agarose was pipetted onto the existing agarose layers, spread and left on ice to solidify.

Once the final layer of agarose was solidified, the cover slips were removed and the slides lysed for 1 h at 4°C in lysis buffer. Slides were then transferred to a horizontal electrophoresis tank (Pharmacia Biotech, Buckinghamshire, UK), containing electrophoresis buffer (pH 12.0). After 20 min to allow DNA unwinding, they were subjected to electrophoresis (25 V, 300 mA) for 20 min using an electrophoresis power supply (Pharmacia LKB, Uppsala, Sweden). After electrophoresis, slides were neutralized by flooding with three changes of neutralization buffer for 5 min each wash. The slides were then stained with 50 μl ethidium bromide solution (20 μg ml^{-1}) and analysed.

The slides were examined at 320×magnification (32 ×/0.40 dry objective) using a fluorescence microscope (Zeiss Axiovert 10, Stuttgart, Baden-Wurttemberg, Germany), fitted with a 515–560 nm excitation filter and a barrier filter of 590 nm. A video camera (Kinetic Pulnix TM-765) received the images, which were analysed using a personal computer-based image analysis system Komet 3.0 Europe (Kinetic Imaging Ltd, Liverpool, UK). Images of 100 randomly selected nuclei were analysed per slide and the mean per cent tail DNA (TD, percentage of DNA in the tail) was determined to assess the extent of DNA damage.

FPG-modified comet assay

The comet assay was carried out as described above, except following lysis, slides were washed 3 ×5 min with FPG buffer (40 mM hydroxyethylpiperazine ethane sulfonate [HEPES], 100 mM KCl, 0.5 mM EDTA, 0.2 mg ml^{-1} bovine serum albumin, pH 8.0). After this time, the slides were incubated with 1 unit FPG enzyme (Trevigen) in 50 μl FPG buffer at 37°C for 1 h as described by Collins *et al.* (1993). One unit FPG is defined as the amount of enzyme required to cleave 1 pmole ^{32}P-labelled oligonucleotide probe containing 8-oxo dG within an oligonucleotide duplex in 1 h at 37°C. To minimize potential variation in FPG activity, the same batch number (3632LO, stored at −80°C in aliquots) was used in all experiments. Control slides were incubated with 50 μl FPG buffer only. DNA unwinding and electrophoresis were then completed as described above. The unmodified version of the comet assay was carried out in parallel so levels of FPG-dependent oxidative damage (exceeding levels of DNA strand breaks and alkaline-labile sites) could be determined.

Immunocytochemistry

Whole blood (4.5 ml) was collected from healthy volunteers into a Vacutainer tube containing EDTA as an anticoagulant (Becton Dickinson, Oxford, UK). WBC were isolated by density gradient centrifugation using Histopaque-1077, cultured at a density of 5×10^5 cells ml^{-1} and treated with sodium dichromate (control, 10 μM, 100 μM) for 1 h at 37°C. Following treatment, WBC were collected by centrifugation (10 min at 120 *g*, room temperature) and the supernatant removed. Cells were then smeared onto glass slides pretreated with adhesive (Vectabond, Vector Laboratories, Peterborough, UK). Cell smears were allowed to dry for 2 h at room temperature and fixed (10 min). Slides were air dried for a further 10 min and subsequently wrapped in foil and stored at −20°C.

Slides were washed 2 ×5 min in PBS and incubated for 10 min with proteinase K (10 μg ml^{-1} in PBS at room temperature). Slides were washed for 5 min in PBS and then incubated again for 1 h with RNase A (100 μg ml^{-1} in 10 mM Tris/1 mM EDTA/0.4 mM NaCl) at 37°C. After the incubation period, slides were washed (2 ×2 min each) in PBS and the DNA denatured by addition of 4 M HCl for 7 min at room temperature. Slides were neutralized (50 mM Tris, 5 min, room temperature), washed in PBS (2 ×2 min) and incubated for 4 h in 10 mM Tris-HCl, pH 7.5/10% FCS at 37°C to block any non-specific sites before being incubated with the monoclonal anti-8-oxo dG mouse primary antibody (MOG-100P; Japanese Institute for the Control of Ageing, Fukuroi City, Shizuoka, Japan) (5 μg ml^{-1} diluted in blocking buffer) overnight at 4°C. Previous studies have demonstrated the high specificity of this antibody towards 8-oxo dG compared with 21 analogues including 7,8-dihydro-2-deoxyguanosine (Toyokuni *et al.* 1997, 1999, Toyokuni 1999). Slides were then washed in PBS (2 ×2 min) and incubated with the goat anti-mouse secondary antibody (1:20 diluted in PBS, 1% bovine serum albumin). Slides were washed in PBS (2 ×2 min), incubated in 3% H_2O_2 in methanol (30 min at room temperature) to quench any endogenous peroxidase activity and then washed again in PBS (2 ×2 min). Slides were incubated with a streptavidin-horseradish peroxidase antibody (diluted 1:20 in PBS) for 30 min at room temperature, washed in 1% Triton X-100 in PBS (2 ×2 min) and then in PBS (2 ×2 min) before the addition of the AEC chromagen (AEC101; Sigma) for 8 min at room temperature. Slides were

then rinsed in distilled water, mounted using glycerol-gelatin (GG-1; Sigma) and photographed using a Zeiss photomicroscope.

Negative control slides were prepared as above with the exception that there was no incubation with the primary antibody. Instead, the slides were incubated overnight at 4°C with 10 mM Tris-HCl, pH 7.5, containing 10% FCS.

Statistical analyses

ATP and TiterTACS™ data were analysed by Kruskal–Wallis and one-way analysis of variance (ANOVA) tests, respectively. Comet tail DNA values were log-transformed (arcsine) and then means for each treatment group were compared using a repeated measures ANOVA with Bonferroni correction. As a measure of variability, the coefficient of variation (CV) ([mean/standard deviation] $\times 100\%$) was calculated from comet assay data generated from the four test individuals.

Results

Cytotoxicity

Human WBC were incubated with sodium dichromate (0–100 μM) for 1 h at 37°C and intracellular ATP levels assessed (figure 1A). No statistically significant decrease in intracellular ATP levels in the isolated WBC as a result of incubation with sodium dichromate was observed at any of the concentrations investigated ($p = 0.57$, Kruskal–Wallis ANOVA). A colorimetric assay was used to detect DNA fragmentation in the human WBC to investigate sodium dichromate-induced apoptosis (figure 1B). Cells were treated with sodium dichromate (0–100 μM) for 1 h at 37°C. There was no statistically significant increase in DNA fragmentation compared with controls at any of the concentrations of sodium dichromate tested ($p = 0.33$, one-way ANOVA). As a positive control, DNase treatment (50 min at 37°C) resulted in a 3.1-fold increase in A_{450} (2.88) compared with controls.

Immunocytochemistry

Faint brown nuclear staining was apparent in control slides indicating the presence of background levels of 8-oxo dG (figure 2A). However, nuclear staining in the cells treated with 10 μM sodium dichromate was darker than in control cells and staining was substantially increased in the cells treated with 100 μM dichromate (figure 2B, C). There was no cytoplasmic staining apparent in any of the treatments and there was no nuclear staining in the absence of primary antibody treatment (data not shown).

Comet assay

Intra-individual variability. Experiments were carried out on WBC isolated from four separate individuals on three occasions (January, March and either August or October). Levels of FPG-dependent oxidative DNA damage were calculated from samples before (control) and after treatment with 100 μM sodium dichromate for 1 h at 37°C. Mean levels of background per cent tail DNA (indicative of oxidative damage) for the four individuals were $2.16\% \pm 1.16\%$, $8.31\% \pm 0.81\%$, $6.44\% \pm 2.41\%$ and $6.31\% \pm 0.28\%$, respectively (figure 3). The CV was calculated for each individual to assess variability between the three time points (intra-individual variability), i.e. 54, 1, 37 and 4%, respectively (figure 3). The mean levels of per cent tail DNA representing sodium dichromate-dependent oxidative damage for

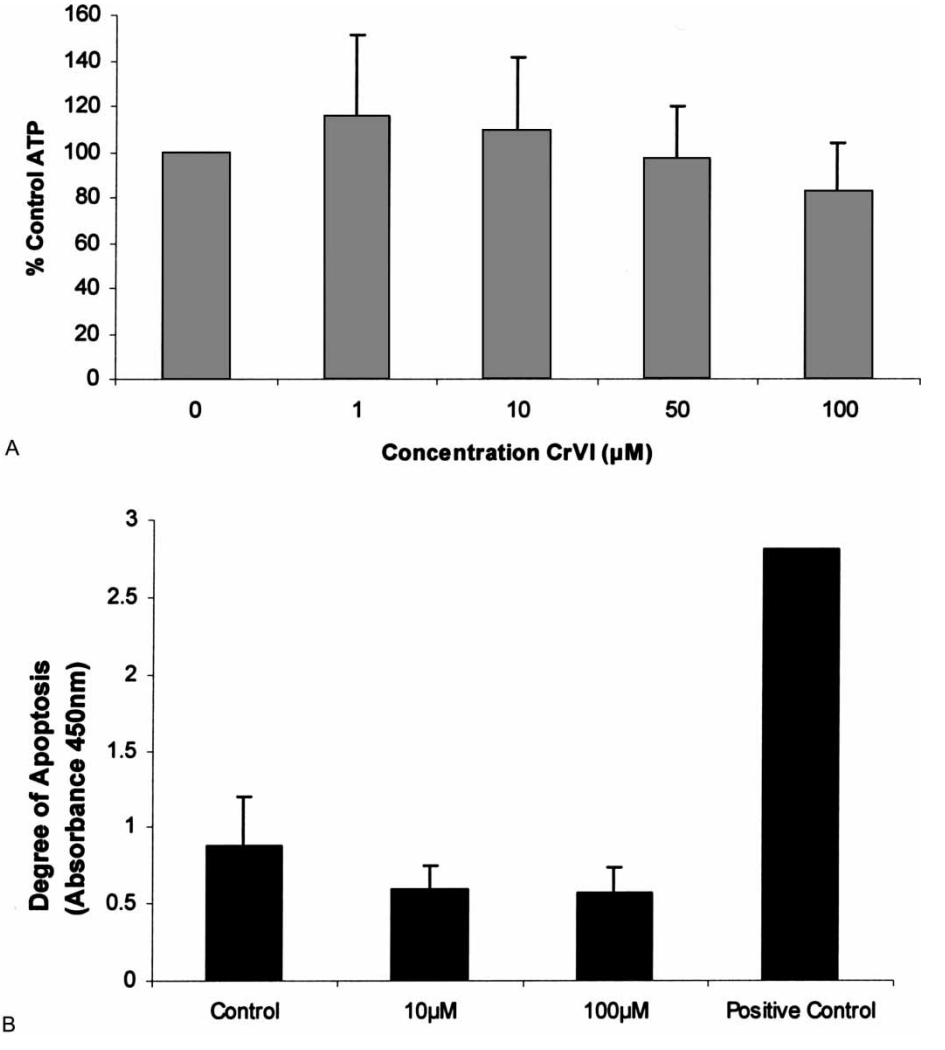


Figure 1. (A) Intracellular ATP (per cent of control) in human peripheral blood lymphocytes treated with sodium dichromate for 1 h at 37°C. Values are the means of three experiments+SD ($n=3$). No statistically significant difference in ATP levels between any of the treatments ($p=0.57$; Kruskal–Wallis ANOVA). (B) TiterTACS™ colorimetric apoptosis detection kit for apoptosis in human peripheral blood lymphocytes incubated with sodium dichromate for 1 h at 37°C. Positive control was generated by incubating cells with DNase for 50 min at 37°C. Values are the means of three experiments+SD ($n=3$). No significant increase in DNA fragmentation in any of the treatments compared with controls ($p=0.33$, one-way ANOVA). DNase treatment resulted in a 3.1-fold increase in A_{450} compared with controls.

the four individuals were 5.91%±2.68%, 9.52%±0.56, 9.27%±1.99% and 12.14%±2.14%, respectively. The respective coefficients of variation were 45, 6, 21 and 18% (figure 3).

Nanomolar concentrations of dichromate cause DNA base oxidation. Whole human blood was treated with sodium dichromate (0–500 nM) for 1 h at 37°C (figure 4). The lowest concentration of sodium dichromate that resulted in a statistically

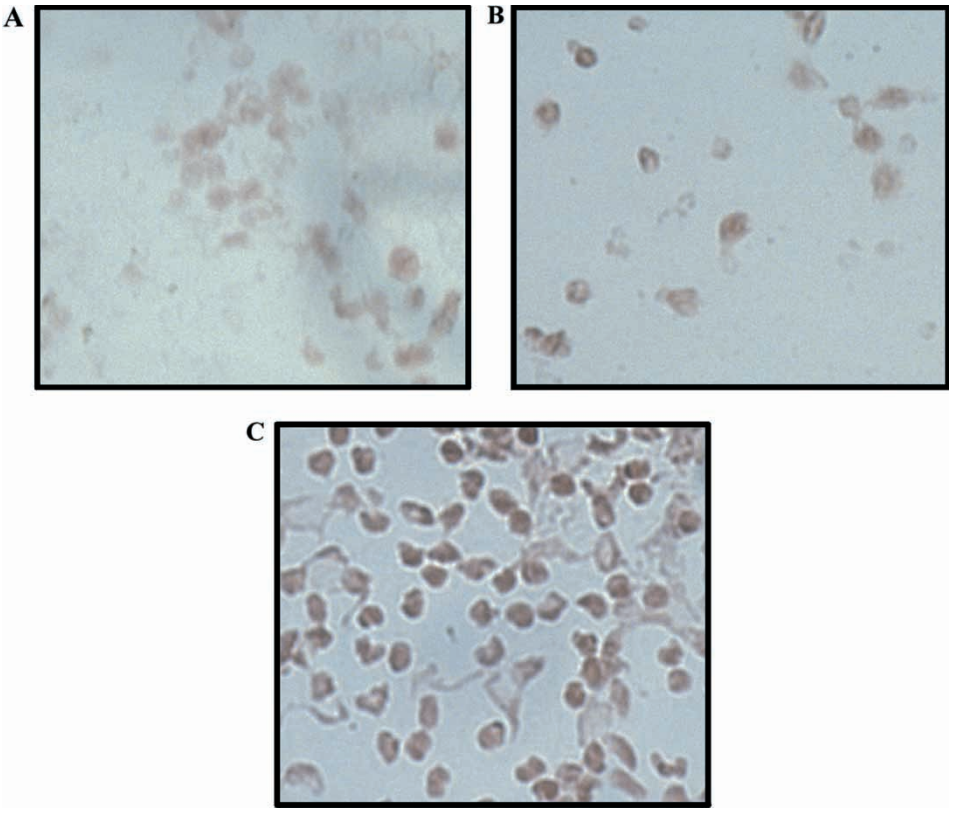


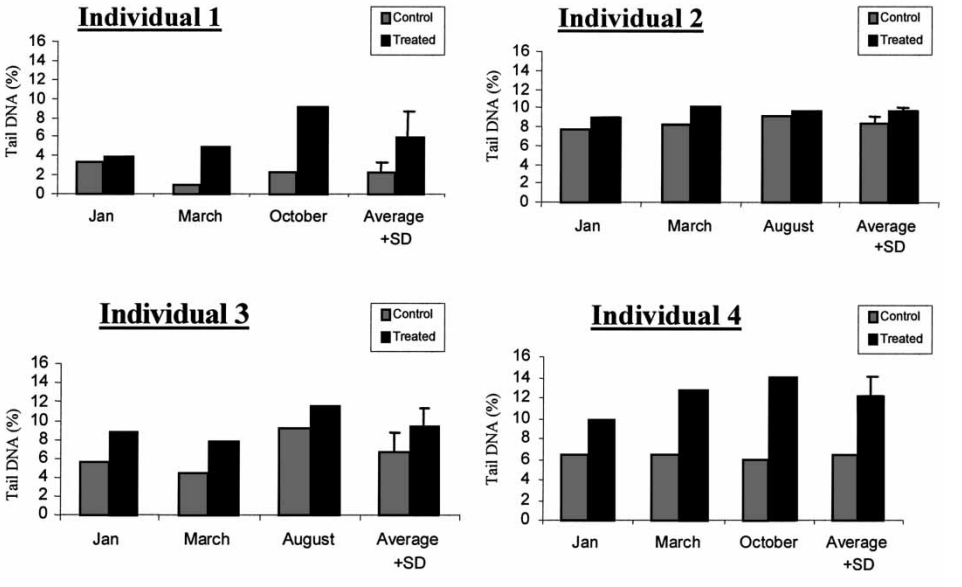
Figure 2. Formation of 8-oxo dGuo in cultured human white blood cells treated with sodium dichromate as assessed by immunocytochemistry. Cells were cultured for 1 h at 37°C. (A) Control, (B) 10 μM, (C) 100 μM sodium dichromate. Magnification 400 ×.

significant increase in DNA strand breaks over control levels was 100 nM ($p < 0.05$, repeated measures ANOVA with Bonferroni correction). The background level per cent tail DNA indicative of FPG-dependent oxidative damage was 1.81% ± 0.96% and this increased twofold to 3.6% ± 0.33% following 1-h treatment with 100 nM sodium dichromate (figure 4).

Discussion

Despite extensive study, the precise mechanism of Cr(VI)-induced DNA damage remains to be elucidated. In particular, there is much controversy in the literature over a possible role of reactive oxygen species. Cr(VI)-mediated 8-oxo dG formation has been demonstrated in isolated DNA (Aiyer *et al.* 1991, Faux *et al.* 1992, Qi *et al.* 2000) and in cells *in vitro* (Hodges *et al.* 2001). In addition to DNA base oxidation, there is evidence for several other types of Cr(VI)-mediated DNA damage, including Cr–DNA adducts, DNA–DNA and DNA–protein cross-links, chromosomal aberrations and DNA strand breaks.

The 8-oxo dG lesion is mutagenic and, through mispairing with adenine during DNA replication, results in formation of G → T transversions, a commonly



	Coefficient of variation (%)	
	Control	Treated
Intra-individual variability:		
Individual 1	54	45
Individual 2	1	6
Individual 3	37	21
Individual 4	4	18
Mean ($n=4$)	*24*	*23*

Individual 1 = female, white Caucasian, 27 years, never smoked, non-vegetarian.
Individual 2 = male, white Caucasian, 32 years, never smoked, non-vegetarian.
Individual 3 = female, white Caucasian, 43 years, current smoker, non-vegetarian.
Individual 4 = female, white Caucasian, 26 years, never smoked, non-vegetarian.

Figure 3. Formation of oxidative DNA damage and intra-individual variability (measured using the FPG-modified comet assay) before and following treatment with sodium dichromate (100 μM, 1 h, 37°C) in the white blood cells of four individuals (three separate incubations repeated in duplicate).

observed mutation in human cancers (e.g. in p53), 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 can also cause G → T transversions, it is likely that formation of 8-oxo dG by low concentrations of sodium dichromate contributes to its genotoxicity and carcinogenicity.

In vitro, many of the effects of Cr(VI) appear to be dependent upon the generation of intracellular oxidative stress. For example, both Cr(VI)-dependent activation of mitogen-activated protein kinase activity and binding of p53 to its promoter are inhibited by co-incubation with antioxidants and radical scavengers (Chuang *et al.* 2000, Wang *et al.* 2000). Additionally, the use of fluorescent probes has demonstrated directly that intracellular levels of H_2O_2 and $O_2^{\bullet -}$ are elevated

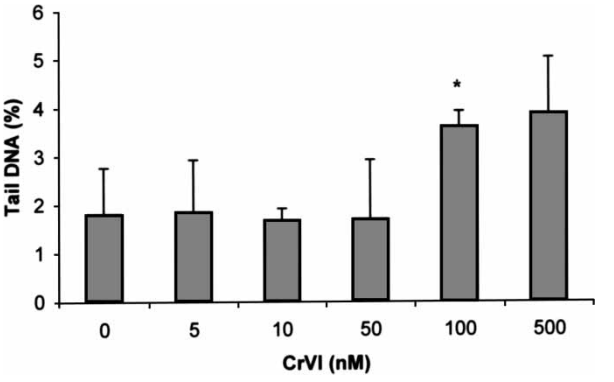


Figure 4. Formation of oxidative DNA damage (measured using the FPG-modified comet assay) by sodium dichromate in cultured human peripheral blood lymphocytes derived from whole blood. Cells were incubated with concentrations of sodium dichromate 0–500 nM for 1 h at 37°C. Values are the means of three experiments ($n = 3$) in both cases. *Significantly different from control at $p < 0.05$ (repeated measures ANOVA with Bonferroni correction).

following treatment with Cr(VI) (Wang *et al.* 2000) and it is believed that $^{\bullet}OH$ radicals are generated from H_2O_2 by reduction reactions catalysed by partially reduced Cr species in processes analogous to the Fenton reaction. It is recognized, however, that Cr(V) can also directly oxidize the fluorescent dye 2′,7′-dichlorofluorescein (Martin *et al.* 1998) and may contribute to DNA oxidation directly (Sugden and Wetterhahn 1997). Furthermore, the addition of various antioxidants has been demonstrated to reduce levels of Cr(VI)-induced DNA strand breaks as measured by the comet assay (Blasiak and Kowalik 2000).

The current study supports the hypothesis that DNA base oxidation is important in the genotoxicity of Cr(VI). Treatment of WBC with sodium dichromate (>10 μM) resulted in elevated nuclear levels of 8-oxo dG in WBC as assessed by immunocytochemistry. Furthermore, the FPG-modified version of the comet assay was extremely sensitive in the detection of dichromate-dependent DNA base oxidation and a statistically significant ($p < 0.05$) twofold increase in FPG-sensitive oxidative damage was observed after treatment (1 h) with 100 nM sodium dichromate. In contrast, concentrations of sodium dichromate below 100 nM (0–50 nM) had no statistically significant effect on FPG-dependent oxidative damage ($p > 0.05$). Interestingly, FPG-sensitive sites are more predominant than frank DNA strand breaks at these concentrations, which are below the threshold of detection (data not shown). It is possible, therefore, that reactive oxygen or Cr species other than, or in addition to, $OH^{\bullet}$ may be responsible for the DNA oxidation.

The concentrations of sodium dichromate used in the present study are physiologically relevant, as it has been estimated that low micromolar plasma concentrations of Cr(VI) may be obtained in occupationally exposed individuals (Carson *et al.* 1986, International Agency for Research on Cancer 1990). It is apparent that treatment with FPG greatly enhances the sensitivity of the comet assay to detect sodium dichromate-mediated DNA. For example, significantly increased levels of DNA strand breaks only following *in vitro* treatment of WBC with concentrations of sodium dichromate >100 μM using the conventional comet

assay were reported (Hodges *et al.* 2001). Similarly, Blasiak *et al.* (1999) reported statistically significantly ($p < 0.001$) increases in comet tail moment in human WBC treated with concentrations of dichromate > 500 μM for 1 h using the conventional comet assay. These observations suggest that at low concentrations of sodium dichromate, the measurement of oxidative DNA damage using the FPG-modified comet assay may be a more sensitive marker than the measurement of the level of frank DNA strand breaks induced by Cr(VI) or as a result of the generation of alkali-labile sites.

However, a recent study by Gambelunghe *et al.* (2003) used the unmodified version of the comet assay as a biomonitoring tool to detect DNA strand breaks in the peripheral blood lymphocytes of 19 chrome platers. Despite exposure to relatively low levels of ambient Cr (< 5.6 μg m^{-3}), the group of platers demonstrated significantly higher levels of DNA strand breaks in peripheral WBC than a matched control group, and this correlated with Cr concentrations measured in the WBC.

Inter-experimental variability is a potential problem in the comet assay due to the number of protocol-related parameters that influence the extent of DNA migration (De Boeck *et al.* 1998). The current study investigated intra-individual variation in oxidative DNA damage in four healthy volunteers. Individuals showed relatively little intra-individual variability both in control (CV mean = 24%) and dichromate-induced (23%) levels of oxidative DNA damage over the 10 months the three measurements were taken. It is recognized, however, that the CV varied between individuals and was as low as 1% (control) and 6% (treated) for one individual. In the case in which the background per cent tail DNA was relatively high, the Cr(VI)-induced effect appears to be minimal. Increased numbers of subjects are needed to substantiate this preliminary finding. Possible causes of intra-individual variation over time include diet (e.g. fruit, vegetable, meat and fish consumption), exercise, infection, smoking and alcohol consumption. Interestingly, when using a general linear model, GLM-ANOVA model, Lee *et al.* (2004) observed that these factors in isolation had no statistically significant effect on inter-individual oxidative DNA damage in either control or dichromate-treated WBC in a population of 72 individuals. In agreement with these findings, previous studies have also observed no significant association between these factors and levels of DNA damage (Singh *et al.* 1990, Betti *et al.* 1995, Frenzilli *et al.* 1997, Møller *et al.* 1998, Štrám 1998, Wojewódzka *et al.* 1998, Van Zeeland *et al.* 1999, Lodovici *et al.* 2000, Giovannelli *et al.* 2002).

In conclusion, detection of oxidative DNA damage after treatment of WBC with nanomolar concentrations of sodium dichromate and a relative lack on interindividual variation over 10 months suggests that the FPG-modified comet assay might be a suitable technique for the long-term biomonitoring of DNA base oxidation in individuals occupationally exposed to Cr(VI).

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