DNA–protein crosslinks in peripheral lymphocytes of individuals exposed to hexavalent chromium compounds

Anatoly Zhitkovich, Annekatrin Lukanova, Todor Popov, Emanuela Taioli, Henry Cohen, Max Costa and Paolo Toniolo

DNA–protein crosslinks were measured in peripheral blood lymphocytes of chrome-platers and controls from Bulgaria in order to evaluate a genotoxic effect of human exposure to carcinogenic Cr(VI) compounds. Chrome-platers and most of the unexposed controls were from the industrial city of Jambol; some additional controls were recruited from the seaside town of Burgas. The chrome-platers had significantly elevated levels of chromium in pre- and post-shift urine, erythrocytes and lymphocytes compared with the control subjects. The largest differences between the two groups were found in erythrocyte chromium concentrations which are considered to be indicative of Cr(VI) exposure. Despite the significant differences in internal chromium doses, levels of DNA–protein crosslinks were not significantly different between the combined controls and exposed workers. Individual DNA–protein crosslinks, however, correlated strongly with chromium in erythrocytes at low and moderate doses but at high exposures, such as among the majority of chrome-platers, these DNA adducts were saturated at maximum levels. The saturation of DNA–protein crosslinks seems to occur at 7–8 μg l^{-1} chromium in erythrocytes whereas a mean erythrocyte chromium among the chrome platers was as high as 22.8 μg l^{-1}. Occupationally unexposed subjects exhibited a significant variability with respect to the erythrocyte chromium concentration, however erythrocyte chromium levels correlated closely with DNA–protein crosslinks in lymphocytes. The controls from Jambol had higher chromium concentrations in erythrocytes and elevated levels of DNA–protein crosslinks compared with Burgas controls. Occupational exposure to formaldehyde among furniture factory workers did not change levels of DNA–protein crosslinks in peripheral lymphocytes. DNA–protein crosslink measurements showed a low intraindividual variability and their levels among both controls and exposed indivduals were not affected by smoking, age or weight.

Keywords: *chromium, formaldehyde, biomarkers, human exposure, DNA adducts.*

Anatoly Zhitkovich *(author for correspondence),* **Emanuela Taioli, Henry Cohen, Max Costa, Paolo Toniolo** *are at the Nelson Institute of Environmental Medicine, New York University Medical Center, 550 First Avenue, New York, New York 10016, USA;* **Annekatrin Lukanova** *and* **Todor Popov** *are at the National Institute of Hygiene and Medical Ecology, 15, bld. D. Nesterov 1431 Sofia, Bulgaria.*

Introduction

Hexavalent chromium compounds are well established human respiratory carcinogens (IARC 1990, Langardt 1990). Unlike the non-toxic Cr^{3+} form which is practically unable to cross cell membranes, Cr^{6+} exists as oxyanion at physiological pH and readily enters cells through anion channels (Buttner and Beyersmann 1985, DeFlora and Wetterhahn 1989). Inside the cell Cr^{6+} undergoes reductive metabolism which eventually leads to the production of a stable Cr^{3+} form but this reduction process is also associated with the formation of several reactive intermediates such as Cr^{5+}, Cr^{4+} and radical species (Snow 1992, Stearns and Wetterhahn 1994, Stearns *et al.* 1995). Once formed, Cr^{3+} reacts avidly with several cellular constituents producing kinetically inert complexes which can trap a significant portion of chromium intracellularly for the life of the cell (Lewalter *et al.* 1985). Exposure of cultured cells or animals to chromate was found to result in the induction of several types of DNA damage including chromosomal aberrations, DNA strand breaks, alkali-labile sites, DNA–protein and DNA–amino acids crosslinks (Sen and Costa 1986, Sugiyama *et al.* 1986, Snow 1992, Zhitkovich *et al.* 1995). All crosslinks were shown to be formed by Cr^{3+} acting as a bridge between DNA and a protein or amino acid (Costa 1990, Zhitkovich *et al.* 1995). Formation of reactive intermediates during the intracellular Cr^{6+} metabolism is thought to be responsible for the induction of other types of DNA damage (Shi *et al.* 1994, Casadevall and Kortenkamp 1995, Stearns *et al.* 1995).

Occupational exposure to carcinogenic forms of chromium occurs among workers in several professional groups with particularly high exposure being found among chrome-platers and stainless steel welders (IARC 1990). It is estimated that approximately 300 000 workers in the US are exposed to chromium compounds in the workplace (ATSD 1993). Biomonitoring studies involving measurements of chromium in different human specimens clearly demonstrated that despite significant improvements in industrial hygiene over recent decades welders and other groups of workers are still exposed to significant levels of chromium-containing compounds. In stainless steel welders, for example, elevated chromium concentrations were routinely found in urine, serum and blood (Kilburn *et al.* 1990, Bonde and Christensen 1991, Stridsklev *et al.* 1993). Several groups have also attempted to correlate internal dose measurements of chromium with cytogenetic damage in peripheral blood lymphocytes. Studies in welders have found an increased number of chromosomal aberrations and SCE (sister chromatid exchange) compared with unexposed controls (Popp *et al.* 1991, Knudsen *et al.* 1992, Jelmert *et al.* 1994). Welders, however, are concomitantly exposed to large amounts of nickel which is also clastogenic (Sen and Costa 1986) and both chromosomal aberrations and SCE were significantly confounded by smoking (Elias *et al.* 1989, Knudsen *et al.* 1992, Jelmert *et al.* 1994). Levels of cytogenetic damage generally showed rather poor correlation with chromium exposure indices in welders (Elias *et al.* 1989, Jelmert *et al.* 1994, 1995). No detectable increase in SCE was observed among chrome-

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platers who are exposed only to chromium compounds (Nagaya *et al.* 1989, 1991). Recently, measurements of 8-oxo dG and DNA single strand breaks which are oxidative forms of DNA damage associated with the metabolism of Cr(VI) to Cr(III), were carried out in workers employed in chromate production (Gao *et al.* 1994). Chromium concentrations in the whole blood, plasma and urine of exposed workers were significantly higher compared with controls, but there was no difference between the groups in the levels of strand breaks or 8-oxo dG in the DNA of white blood cells. The lack of difference in the oxidative types of DNA damage between chromate-exposed and control subjects can, at least in part, be due to rather high repair rates of oxidative lesions (Sugiyama *et al.* 1986).

Another approach to the development of genetic biomarkers of Cr(VI) exposure can involve analysis of DNA–protein crosslinks (DPC), lesions known to be poorly repaired (Costa 1990). Although the mutagenic potential of DPC has not as yet been assessed directly, the formation of DPC interferes with DNA replication (Permana and Snapka 1994) which is thought to be responsible for a large proportion of deletion mutants in cells treated with known DPC-producing agents (Benyajiati *et al.* 1983, Klein *et al.* 1992, Yu *et al.* 1994). Utilization of DPC measurements as biomarkers, therefore, may not only represent an important index of biologically effective chromium dose but DPC levels may also be indicative of the extent of DNA damage with important biological consequences.

We have recently conducted a pilot study on mild steel welders from the USA and found that levels of DPC in their white blood cells were significantly higher than in unexposed controls (Costa *et al.* 1993). It is noteworthy that mild steel welders are considered to be a rather low chromium-exposure group. DPC measurements in this work, however, were not correlated with blood or urinary levels of chromium. The present study was conducted in order to address the question of the relationship between internal chromium dose indices and DPC, and to assess a utility of DPC measurements in highly Cr(VI)-exposed populations. We report the results of the DPC analysis in peripheral lymphocytes among highly exposed chrome-plating workers and two groups of population controls from Bulgaria.

MATERIALS AND METHODS

Study subjects

Study subjects were individuals residing in south-eastern Bulgaria. Exposed were 14 chrome-platers from a metallurgic plant in the industrial town of Jambol, Bulgaria, who had been continuously employed at the factory (8-h work shift) for a minimum of 1.5 to a maximum of 15 years (mean: 9.5 ± 4.0). In previous industrial hygiene measurements conducted above the plating baths, Cr(VI) ambient levels were 24 times higher than the maximum tolerated dose (0.02 mg m^{-3}) in 1990, eight times higher in 1991, and four times higher in 1993. Improvements in ambient exposure were the result in part of remedial actions and in part of reduced production. Controls were 12 Jambol residents not known to be exposed to chromium or other metals at work or elsewhere, and not living in the vicinity of the factory. Six additional unexposed controls were recruited from the relatively unpolluted coastal town of Burgas, approximately 100 km east of Jambol. A short questionnaire was administered to each participant at the time of recruitment in the study. The questionnaire was designed to collect demographic characteristics, self-reported height and weight, history of occupations, second jobs or hobbies, current use of medications, vitamins and minerals, and smoking and alcohol consumption.

Collection and preparation of biological samples

Blood and urine specimens of exposed and control subjects were collected simultaneously. Initial steps of sample preparation were performed immediately after collection and samples were frozen on site in dry ice. In order to ensure the confidentiality of the subjects' exposure status, all samples were assigned blind codes by an epidemiologist before being sent to the field laboratory. Prior to use, all plasticware for specimen collection was soaked for 24 h in 20% nitric acid and then rinsed five times with deionized distilled water. Chrome-platers were instructed to collect a small amount of midstream urine at the beginning and at the end of the work shift and not to urinate during the 2 h preceding the second sample collection. Controls were asked to collect a small amount of midstream urine at the time of recruitment.

Blood was drawn into metal-free sterile Vacutainer tubes containing EDTA as an anticoagulant (Beckton Dickinson, Rutherford, NJ). The initial 10 ml of blood was discarded to avoid chromium contamination from the needles. Among chrome-platers, blood collection was performed at the end of the work shift. Blood samples were left for 30–40 min at room temperature to obtain two fractions: a supernatant containing plasma and white blood cells, and the bottom fraction containing predominantly red blood cells. The supernatant fraction was used to isolate mononuclear cells by a standard Ficoll/sodium diatrizoate protocol using Histopaque-1077 (Sigma, St Louis, MO). Lymphocytes were twice washed in Ca, Mg-free PBS, counted and then lysed in 0.1 ml deionized distilled water. The second blood fraction was washed three times with 0.9% NaCl solution and the final erythrocyte pellet was diluted to the initial volume of the blood sample with Triton X-100 to give 0.1% final concentration of the detergent (Lewalter *et al.* 1985). Lymphocytes used in a DPC assay were isolated from freshly obtained whole blood by the Ficoll gradient centrifugation. Lymphocytes were twice washed in cold Ca, Mg-free PBS, counted and then resuspended in Ca, Mg-free PBS to give 20 million cells per ml.

Assessment of ambient exposure

For the duration of the one 8-h work shift, all chrome-platers were fitted with a battery-charged personal breathing air sampling pump (Casella/BGI Inc. AFC123), set at a sampling flow of 2 l min^{-1} using Millipore filters (cat. No. MAWP037A0) with cellulose ester membranes. Eleven of the workers were also fitted with medium-range flow pumps (1.2 l min^{-1}), equipped with 5 mm PVC filters (Higitest, Sofia, Bulgaria). All filters were coded by an epidemiologist and placed in airtight individual plastic bags until laboratory analysis.

Chromium measurements

The Millipore filters were digested in the presence of nitric acid and analysed by an atomic absorption flame method for total chromium (NIOSH 1984; method 7024). The 5 mm PVC filters were analysed by a visible absorption spectrophotometer for hexavalent chromium (NIOSH 1984; method 7600). A portion of each sample was analysed for total chromium by flame atomic absorption. Chromium concentrations in erythrocyte or lymphocyte lysates were measured by flameless atomic absorption spectrometry essentially as described by Lewalter *et al.* (1985) and Gao *et al.* (1994), respectively. All chromium measurements were performed with a Model 5100 atomic absorption spectrophotometer equipped with Zeeman background correction (Perkin-Elmer, Norwalk, CT). Accuracy of chromium measurements was verified by parallel analyses of biological reference materials.

The reference standards were SRM 2670, urine normal and spiked (National Bureau of Standards, USA), and Control blood for metals 1 (Behring Institute, Germany).

Assessment of intrasubject variability of DNA–protein crosslink measurements

Five healthy subjects (three male and two female) were recruited to participate in this study. All individuals were laboratory personnel from NYU. Blood was drawn three times with a 1 week interval between each blood sampling. Peripheral lymphocytes were purified from freshly obtained blood as described above. Half of the blood sample obtained at blood draw 2 was left at room temperature for 4 h before the cell separation was started.

DNA–protein crosslink assay

DPC were measured by the K-SDS precipitation method as previously described (Zhitkovich and Costa 1992) with some modifications. Two million freshly isolated lymphocytes were lysed in 0.5 ml of a 0.5% SDS, 20 mM Tris, pH 7.5 solution and samples were placed at –70 °C. Each batch of samples also included blank tubes which contained 0.2 mg BSA instead of cells. The SDS-lysed samples were thawed at 37 °C and the DNA was sheared by vigorous passing of the cell lysates four times through a 22-gauge needle. Then, 0.5 ml of 100 mM KCl, 20 mM Tris, pH 7.5 was added. The content was mixed by vortexing for 5 s at maximal speed and the tubes were then heated for 10 min at 65 °C. Samples were removed from the water bath, inverted three times then placed on ice for 5 min to form K-SDS precipitate. The precipitate was collected by centrifugation at 6000 × *g* for 5 min at 4 °C. The supernatant was saved and the pellet was resuspended in 100 mM KCl, 20 mM Tris–HCl (pH 7.5) by brief vortexing at the highest setting. It is important here and in all other steps to aspirate the supernatant using a pipette. The background is increased if tubes are inverted to remove the supernatant due to incomplete removal of the supernatant. The samples were again heated at 65 °C for 10 min and the washing and heating steps as described above, were repeated twice more. Protein-linked DNA was released from the final K-SDS precipitate by treatment with 0.2 mg ml^{-1} proteinase K in 0.5 ml solution containing 100 mM KCl, 20 mM Tris–HCl (pH 7.5) and 10 mM EDTA. The samples were then incubated at 50 °C overnight. To achieve more complete precipitation of residual SDS 50 µl of 4 mg ml^{-1} BSA was added to each tube and the samples were placed on ice for 30 min. The tubes were centrifuged at 12000 × *g* for 10 min at 4 °C and the supernatant was taken to determine the quantity of DNA. It is important at this point to avoid disturbing the K-SDS pellet while aspirating the supernatant since SDS interferes with the Hoechst 33258 assay. Total DNA in the combined supernatants and SDS-precipitable DNA were detected using Hoechst 33258 dye. Fluorescence was assessed by excitation at 365 nm and the emitted light was measured at 450–460 nm. A BSA tube was used as the standard blank. DPC values were expressed as a percentage of total cellular DNA crosslinked to proteins. Internal standards consisting of SDS-lysed human lymphocytes were analysed in each set of samples in order to correct for a possible batch effect. The internal standards were prepared by lysing a large number of lymphocytes obtained from one individual and these samples were stored at –70 °C. Storage of internal standards over a 9 month period did not affect DPC levels (DPC in freshly prepared standards—0.99%, after 3 months—1.00%, 6 months—1.04%, 9 months—1.03%). Each batch of samples included both controls and exposed individuals. The detection limit for DNA–protein crosslinks based on previous calculations is approximately one adduct per 1–2 × 10^7 bases in 10 µg DNA sample (Zhitkovich *et al.* 1995).

Statistical analysis

Student's *t*-test was utilized to evaluate difference in means between groups in normally distributed data, the Mann–Whitney test was used to evaluate difference in medians between groups in not-normally distributed data, and Pearson correlations were used to assess linear associations between variables. Linear and non-linear regression models were fit to study the relationship between chromium in erythrocytes and DPC. Log transformation of the data was performed, when necessary. Univariate analysis was used to determine whether specific host factors (age, weight and sex) and exposure variables (cigarette smoking, alcohol consumption, occupational exposure to chromium or formaldehyde and indices of internal dose) were associated independently with DPC.

Results

As a part of a field validation we first evaluated intraindividual variability of DPC measurements in peripheral lymphocytes (Figure 1). Blood was drawn from five healthy controls three times with a 1 week interval between each blood sampling. Lymphocytes were isolated from freshly obtained blood and in one case half of the blood sample was left on a bench-top for 4 h before the blood cell separation was initiated. The results show that individual DPC values are quite stable over this 3 week period (see Figure 1). Blood samples can also be stored for up to 4 h without any effect on DPC measurements.

Table 1 shows selected characteristics of the control and chromate-exposed populations. The exposed workers were slightly older and had fewer current and past smokers among them than the controls. All exposed were male and five (29.4%) of the controls were female. There was no difference in weight (Quetelet index) between the two groups. Exposed and controls were similar with respect to alcohol consumption and current use of medications, and reported no hobbies or second jobs with potential chromium exposure, nor consumption of vitamins or minerals of any kind.

The ambient air levels of total chromium for the 14 chrome-platers ranged between 0.009 and 0.327 mg m^{-3} (median = 0.041 mg m^{-3}) as measured with the Millipore filters. Eleven workers were also equipped with the Higitest filters and their

Figure 1. Intrasubject variability of DNA–protein crosslinks. DPC were determined in peripheral lymphocytes obtained from five healthy, unexposed individuals. Blood was drawn three times in three different weeks and lymphocytes were purified immediately after the blood draw. 'Aged'—lymphocytes were isolated 4 h after the blood draw.

Variable	Exposed (n = 14) (Jambol)	Controls (n = 17)	Controls (n = 11) (Jambol)	Controls (n = 6) (Burgas)
Age	41.1 ± 5.9	34.4 ± 8.6	35.2 ± 9.0	32.8 ± 8.4
Gender				
Male	14 (100%)	12 (71%)	6 (56%)	6 (100%)
Female	0	5 (29%)	5 (44%)	0
Smoking status				
Current smokers	11 (79%)	15 (88 %)	9 (82%)	6 (100%)
Never smoked	3 (21%)	1 (6%)	1 (9%)	0
Quetelet Index[a]	26.3 ± 3.4	24.4 ± 3.9	24.0 ± 4.1	25.1 ± 3.6

Table 1. Selected characteristics of the study population.

[a] Weight (kg) divided by the height squared (m^2).

Variable	Exposed (n = 14)		Controls[a] (n = 17)	
	Mean ± s.d	Median	Mean ± s.d.	Median
Chromium in lymphocytes (μg per 10^{10} cells)	1.16 ± 0.73	0.96	0.64 ± 0.16	0.57
Chromium in erythrocytes (μg l^{-1})	22.8 ± 12.1	19.0	2.5 ± 1.5	2.0
Pre-shift urine (μg g^{-1} creatinine)	4.3 ± 3.2	3.3	1.0 ± 0.7	0.8
Post-shift urine (μg g^{-1} creatinine)	9.0 ± 8.3	6.5		
DNA–protein crosslinks (%)	1.53 ± 0.33	1.65	1.45 ± 0.43	1.44

Table 2. DNA–protein crosslinks in peripheral lymphocytes and chromium levels in different biological specimens from chrome-platers and unexposed contros from Jambol and Burgas.

[a] Because of technical problems one of the control erythrocyte samples was not analysed.

exposure was found to range between 0.008 and 0.19 mg m^{-3} (median = 0.027 mg m^{-3}). The two methods gave very similar results (r = 0.97, $p < 0.0001$). The Cr(VI) ambient air concentrations ranged from 0.0005 to 0.13 mg m^{-3} (median = 0.003 mg m^{-3}). Chromium concentrations in blood and urine samples of exposed workers and controls are reported in Table 2. Among the chrome-platers, mean chromium levels were nearly twice as high in lymphocytes ($p < 0.01$), nine-fold in erythrocytes ($p < 0.0001$), four-fold in pre-shift urine ($p < 0.0001$), and eight-fold in post-shift urine, than among the controls. Chromium concentrations increased two-fold in post-shift urine as compared with pre-shift urine ($p < 0.05$). Despite the differences in internal chromium doses, levels of DPC in peripheral lymphocytes were comparable between the two groups (1.53 ± 0.33 in exposed vs 1.45 ± 0.43 in controls).

Next, we compared the controls from the industrial town of Jambol with those from the seaside town of Burgas (Table 3). Chromium levels in lymphocytes were similar between the two groups with urine levels being somewhat higher in the Burgas controls. However, urinary chromium measurements in environmentally exposed human populations, unlike occupational settings, may not be a very reliable indicator of toxicological chromium exposure since dietary chromium levels can significantly affect this parameter (Gargas *et al.* 1994). Mean chromium levels in erythrocytes were three times more elevated among the controls from Jambol than among those from Burgas ($p < 0.001$). The DPC levels were approximately 60% higher among the controls from Jambol than those from Burgas ($p < 0.01$). Male and female and controls from Jambol were not significantly different with respect to DPC and chromium in erythrocytes DPC averaged 1.71 ± 0.39 and 1.60 ± 0.23 whereas RBC chromium was 3.64 ± 1.80 and 2.86 ± 0.61 for male and female subjects, respectively.

Variable	Controls from Jambol (n = 11)	Controls from Burgas (n = 6)
Chromium in lymphocytes (μg per 10^{10} cells)	0.64 ± 0.16	0.64 ± 0.16
Chromium in erythrocytes (μg l^{-1})	3.2 ± 1.4**	1.14 ± 0.44
Chromium in urine (μg g^{-1} creatinine)	0.79 ± 0.4	1.45 ± 0.88
DNA–protein crosslinks (%)	1.66 ± 0.35*	1.04 ± 0.27

Table 3. DNA–protein crosslinks in peripheral lymphocytes and chromium levels in different biological samples from Jambol and Burgas controls (mean ± s.d.).

*$p < 0.01$; **$p < 0.001$.

Among chrome-platers, DPC correlated weakly with total ambient chromium ($r = 0.38$) and age ($r = 0.38$) and no correlation was observed with Cr(VI) in ambient air ($r = 0.18$), chromium in erythrocytes ($r = -0.10$), chromium in lymphocytes ($r = 0.12$), Quetelet Index ($r = -0.10$), and number of cigarettes per day ($r = 0.06$). Stronger positive correlations were observed between DPC and chromium in pre-shift urine ($r = 0.49$, $p < 0.1$), post-shift urine ($r = 0.52$, $p = 0.06$), and years of employment at the plant ($r = 0.49$, $p < 0.1$).

Among the controls, DPC correlated weakly with age ($r = 0.22$), number of cigarettes per day ($r = -0.23$), chromium levels in lymphocytes ($r = -0.20$), and with chromium in urine ($r = -0.39$), and very strongly with chromium in erythrocytes ($r = 0.62$, $p < 0.01$). Figure 2 illustrates a linear relationship between DPC in peripheral lymphocytes and chromium levels in red blood cells among the control subjects. When chromium concentrations in erythrocytes exceed 8 μg l^{-1}, as was found among the majority of chrome-platers, DPC levels do not increase further. We have also applied a non-linear model to analyse the relationship between DPC and erythrocyte chromium concentrations among all study subjects using log-transformed data (Figure 3). A function describing a growth curve was used for this non-linear model. The shape of the curve suggested that saturation of DPC begins to occur when levels of chromium in erythrocytes exceed 5 μg l^{-1} erythrocytes.

In addition to hexavalent chromium compounds, DPC can also be induced by some other chemicals. In *in vitro* experiments, for example, several aldehydes have been found to form DPC adducts (Kuykendall and Bogdanffy 1992). We therefore were interested in studying a possible effect of occupational exposure to aldehydes on levels of DPC in peripheral lymphocytes. This study would not only assess the utility of DPC measurements in aldehyde-exposed populations but in addition, it would also provide important information regarding potential confounders when DPC are used to evaluate human exposure to hexavalent chromium. Figure 4 shows the results of DPC determinations in peripheral lymphocytes of controls and formaldehyde-exposed workers. No differences were observed in DPC levels between these two groups. The exposed group consisted of furniture factory workers from Burgas who had been employed from 3.5 to 20 years (median = 16). All controls and cases were male and had similar demographic characteristics. DPC were also

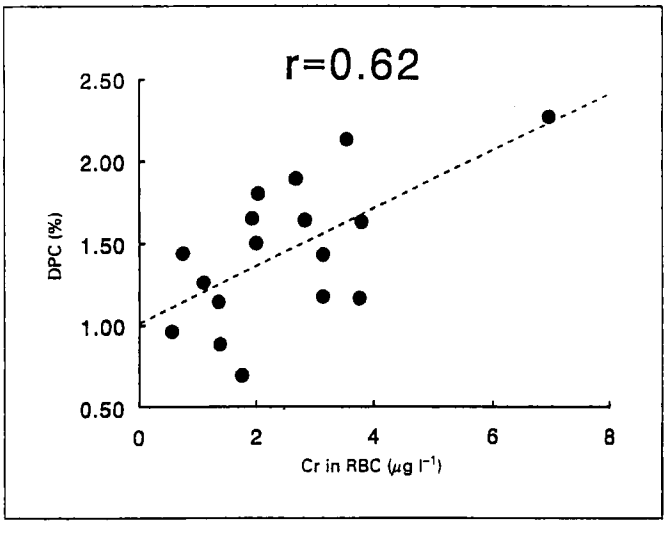

Figure 2. Correlation between DNA–protein crosslinks in peripheral lymphocytes and chromium concentrations in erythrocytes among control subjects. Shown are the results of DPC and chromium measurements for all control subjects recruited in Jambol and Burgas.

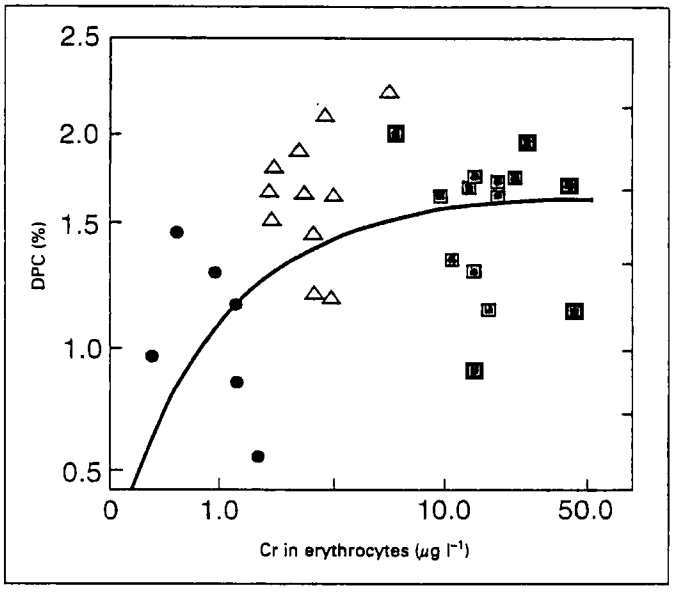

Figure 3. Correlation between DNA–protein crosslinks in lymphocytes and chromium levels in erythrocytes (all subjects). Shown are the results of chromium measurements and DNA–protein crosslinks for control subjects from Burgas, Jambol and chrome-platers. Data in the graph are log transformed. The equation describing the fit is the following: $y = 1.7\,(1-e^{1.4(\text{chromium in erythrocytes})})$, ●—Burgas, Δ—Jambol, ■—chrome-platers.

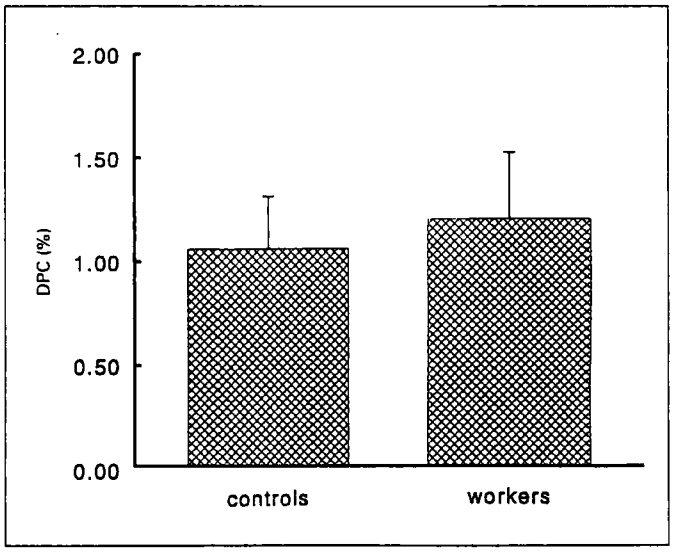

Figure 4. DNA–protein crosslinks in peripheral lymphocytes of controls and furniture factory workers. DPC were measured in 10 formaldehyde-exposed furniture workers and six local controls. All study subjects were males. Shown are means ± s.d.

measured in four female workers of the furniture factory and the values did not differ from the male participants (1.20 ± 0.32 and 1.24 ± 0.31 for male and female subjects, respectively). External exposure measurements conducted by the Bulgarian National Center of Hygiene and Medical Ecology indicated that formaldehyde concentrations in the working place were in the range 0.2–2 ppm. These are rather high doses but they still did not result in any significant changes in DPC levels in peripheral lymphocytes.

Discussion

Measurements of chromium in urine and serum (or plasma) are most frequently used to monitor occupational exposure to chromium compounds. However, Cr^{3+} is an essential element, and its dietary levels in these biological fluids might be enough to mask low level exposures and lead to a significant day-to-day variability (IARC 1990, Gargas *et al.* 1994). In addition, chromium can be readily excreted or redistributed from plasma and, therefore, chromium measurements in the human plasma or urine are indicative of relatively recent exposure and useful primarily for heavy exposure scenarios. The levels of chromium in the red blood cells may be more informative by being indicative of exposure to carcinogenic Cr(VI) compounds. *In vitro* and *in vivo* experiments indicate that unlike inorganic forms of Cr^{3+}, Cr^{6+} as a chromate anion can readily enter the red blood cells and once inside the cells, it is reduced and binds to haemoglobin (Lewalter *et al.* 1985, Merrit and Brown 1995). A significant portion of these Cr–haemoglobin complexes persist for a relatively long period of time; therefore, a single determination can potentially allow a Cr(VI) exposure assessment for an extended period of time in the past.

Internal dose measurements based on chromium levels in erythrocytes can provide rather strong evidence of exposure to carcinogenic Cr(VI) compounds; however, they do not prove that toxicological damage has occurred. It is known that Cr(III) can form very stable inert complexes with many cytoplasmic molecules precluding damage to DNA (Denniston and Uyeki 1987, Hneihen *et al.* 1993). In addition, intracellular metabolism of Cr(VI) and, subsequently, chromium accumulation kinetics in RBC and nucleated cells is likely to be quite different since ascorbate plays a dominant role in Cr(VI) reduction in WBC and tissue-targets (Bergsten *et al.* 1989, Suzuki and Fukuda 1990, Standeven and Wetterhahn 1991) whereas in RBC glutathione is the most abundant reductant (Aaseth *et al.* 1982, Vinson *et al.* 1989). A measurement of Cr–DNA adducts would be of significant importance, because they are direct products of damage to a critical genetic target. Previous studies have suggested that DNA–protein crosslinks could be used as biomarkers of human exposure to chromium (Popp *et al.* 1991, Costa *et al.* 1993) but the relationship between internal chromium dose and this molecular biomarker, however, has not been adequately addressed. In this study we have used red blood cell chromium concentrations as a parameter of internal dose and found a very good correlation with DPC in peripheral lymphocytes at low and intermediate exposures. Interestingly, DPC values in Caucasian controls from the New York area (Taioli *et al.* 1995) are significantly lower than those in Burgas or Jambol controls (0.68 ± 0.06, 1.06 ± 0.27 and 1.66 ± 0.35%, respectively). At high chromium exposures, as exemplified by a group of heavily exposed chrome-platers, DPC levels do not increase above the values found at the intermediate chromium burden. The reasons for the saturation of DPC values in chrome-platers is not known but it may, at least partially, be due to enhanced repair capabilities and possible elimination of lymphocytes with a particularly heavy burden of DPC. Changes in lymphocyte composition of chromium-exposed workers were indeed documented (Tanigawa *et al.* 1991) and a reported decrease in the number of chromosomal aberrations in certain groups of welders suggests enhancement of DNA repair capabilities at occupational chromium exposures (Jelmert *et al.* 1995). It should be noted that at the high dose range weak correlations between a particular biomarker and exposure are not uncommon (Hulka and Margolin 1992).

Results of this and some other studies (Angerer *et al.* 1987, Minoia and Cavalleri 1988) show that erythrocyte chromium concentration represents a good marker of exposure providing a measure of internal dose. A general premise for use of chromium concentrations in erythrocytes or lymphocytes as cumulative index of Cr(VI) exposure over an extended period of time is effective penetration of Cr^{6+} anions into any type of cells with the subsequent intracellular trapping of the final Cr^{3+} form. It was somewhat surprising that despite a lymphocyte life span of several years, chromium measurements in these cells did not provide a sensitive measure of chromium exposure. Gao *et al.* (1994) made the same conclusion based on their biomonitoring studies of chromate workers. When calculated per number of cells, chromium concentrations in lymphocytes from control subjects were more than two orders of magnitude higher than corresponding values in erythrocytes (640 ng vs 2.4 ng per 10^{10} cells). This high lymphocytic level of chromium is likely to be a reflection of the very long life of peripheral lymphocytes. Relative insensitivity of chromium measurements in lymphocytes even at high occupational exposures is not well understood but it may involve, for example, some excretion of intracellular chromium and a lower membrane permeability. Long-term exposure of cells to Cr(VI) has indeed been shown to result in dramatically decreased intracellular uptake associated with modifications of anion transport system (Lu and Yang 1995). Marginal penetration of dietary Cr^{3+} into lymphocytes over an extended period of time may also make a significant contribution to masking actual Cr^{6+} exposure. Unlike inorganic Cr^{3+}-containing compounds, complexes of Cr^{3+} with several amino acids are known to enter cells (Kortenkamp *et al.* 1987).

Formaldehyde and glutaraldehyde are two other chemicals of occupational and environmental significance that are also capable of inducing DPC adducts in exposed cells (D'A Heck and Casanova 1990, St Clair *et al.* 1991). Several facts, however, makes us think that elevation in DNA–protein crosslinks in human lymphocytes may largely be due to exposure to hexavalent chromium compounds and not confounded by a possible aldehyde exposure. Aldehyde-induced DPC, unlike those formed by chromium, are generally quite thermolabile (Kuykendall and Bogdanffy 1992) and most

of them are not likely to survive under our DPC assay conditions, i.e. four cycles of heating at 65 °C. Glutaraldehyde exposure is found almost exclusively among health care workers using a 'cold' sterilization technique (Beauchamp *et al.* 1992) and this agent, therefore, can be excluded as an inducer of DPC in peripheral lymphocytes in our studies. Inhalation exposure of both experimental animals and humans to formaldehyde did not result in any detectable elevation of this agent in the bloodstream (D'A Heck and Casanova 1990) presumably because formaldehyde is chemically very active and it reacts primarily with respiratory tissues. No correlation was found between DNA–protein crosslink levels in human lymphocytes and either smoking status or the number of cigarettes smoked per day (Costa *et al.* 1993, Oesch *et al.* 1994, Taioli *et al.* 1995, this study). However, it is well known that cigarette smoke contains a number of bifunctional agents and reactive aldehydes including formaldehyde. Finally, no increase in DPC levels was observed in lymphocytes of furniture workers who were highly exposed to formaldehyde.

Results of this work revealed quite strong correlation between DPC in peripheral lymphocytes and chromium concentration in erythrocytes which is a marker of Cr^{6+} exposure. A linear relationship between these parameters was observed at low and intermediate doses which were defined as those in the range of 0.5–8 μg chromium per litre. Although such chromium exposures were found among occupationally unexposed controls in Bulgaria, the measured values are high and in western countries they can be found only in occupational settings (Angerer *et al.* 1987, Minoia and Cavalleri 1988, Stridsklev *et al.* 1993, Jelmert *et al.* 1994). Unfortunately, the exact sources of chromium pollution in the studied Bulgarian cities were not identified but several industrial processes such as oil and coal combustion, steel production, chemical manufacturing, chrome-plating, and cooling towers can release significant quantities of chromium in the environment (IARC 1990, ATSD 1993). The observed sensitivity of the DPC assay at the relatively low chromium exposures can make it useful in assessing DNA damage in the USA among several professional groups as well as in populations being at risk of environmental exposure to chromium.

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References

Aaseth, J., Alexander, J. and Norseth, T. (1982) Uptake of ^{51}Cr-chromate by human erythrocytes—a role of glutathione. *Acta Pharmacology and Toxicology*, **50**, 310–315.

Angerer, J., Amin, W., Heinrich-Ramm, R., Szadkowski, D. and Lehnert, G. (1987) Occupational chronic exposure to metals: chromium exposure of stainless steel welders—biological monitoring. *International Archives of Occupational and Environmental Health*, **59**, 503–512.

ATSD (1993) Toxicological Profile for Chromium. US Dept. of Health and Human Services.

Beauchamp, R. O., Jr, St Clair, M. B., Fennell, T. R., Clarke, D. O. and Morgan, K. T. (1992) A critical review of the toxicology of glutaraldehyde. *Critical Reviews in Toxicology*, **22**, 143–174.

Benyajiati, C., Place, A. R. and Sofer, W. (1983) Formaldehyde mutagenesis in *Drosophila*. Molecular analysis of ADH-negative mutants. *Mutation Research*, **111**, 1–7.

Bergsten, P., Amitai, G., Kehrl, J., Dhariwal, K. R., Klein, H. G. and Levine, M. (1989) Millimolar concentrations of ascorbic acid in purified human mononuclear leucocytes. *Journal of Biological Chemistry*, **265**, 25–84.

Bonde, J. P. and Christensen, J. M. (1991) Chromium in biological samples from low-level exposed stainless steel and mild steel welders. *Archives of Environmental Health*, **46**, 225–229.

Buttner, B. and Beyersmann, D. (1985) Modification of the erythrocyte anion carrier by chromate. *Xenobiotica*, **15**, 735–741.

Casadevall, M. and Kortenkamp, A. (1995) The formation of both apurinic/apyrimidinic sites and single strand breaks by chromate and glutathione arises from attack by the same single reactive species and is dependent on molecular oxygen. *Carcinogenesis*, **16**, 805–809.

Costa, M. (1990) Analysis of DNA–protein complexes induced by chemical carcinogens. *Journal of Cellular Biochemistry*, **44**, 127–135.

Costa, M., Zhitkovich, A. and Toniolo, P. (1993) DNA-protein crosslinks in welders: molecular implications. *Cancer Research*, **53**, 460–463.

D'A Heck, H. and Casanova, M. (1990) Formaldehyde toxicity—new understanding. *Critical Reviews in Toxicology*, **20**, 397–426.

DeFlora, S. and Wetterhahn, K. E. (1989) Mechanisms of chromium metabolism and genotoxicity. *Life Chemistry Reports*, **7**, 169–244.

Denniston, M. L. and Uyeki, E. M. (1987) Distribution and HPLC study of chromium-51 binding sites in Chinese hamster ovary cells. *Journal of Toxicology and Environmental Health*, **21**, 375–386.

Elias, Z., Mur, J.-M., Pierre, F., Gilgenkrantz, S., Schneider, O., Baruthio, F., Daniere, M.-C. and Fontana, J.-M. (1989) Chromosome aberrations in peripheral blood lymphocytes of welders and characterization of their exposure by biological samples analysis. *Journal of Occupational Medicine*, **31**, 477–483.

Gao, M., Levy, L. S., Faux, S. P., Aw, C. T., Braithwaite, R. A. and Brown, S. S. (1994) Use of molecular epidemiological techniques in a pilot study on workers exposed to chromium. *Occupational and Environmental Medicine*, **51**, 663–668.

Gargas, M. L., Norton, R. L., Paustenbach, D. J. and Finley, B. L. (1994) Urinary excretion of chromium by humans following ingestion of chromium picolinate. *Drug Metabolism Disposition*, **22**, 522–529.

Hneihen, A., Standeven, A. M. and Wetterhahn, K. E. (1993) Differential binding of chromium(VI) and chromium(III) complexes to salmon sperm nuclei and nuclear DNA and isolated calf thymus DNA. *Carcinogenesis*, **14**, 1795–1803.

Hulka, B. S. and Margolin, B. H. (1992) Methodological issues in epidemiologic studies using biologic markers. *American Journal of Epidemiology*, **135**, 200–209.

IARC (1990) *Monograph on the Evaluation of Carcinogenic Risk to Humans. Chromium, Nickel and Welding*, Volume 48 (IARC, Lyon, France), pp. 49–508.

Jelmert, O., Hansteen, I.-L. and Langard, S. (1994) Chromosome damage in lymphocytes of stainless steel welders related to past and current exposure to manual arc welding fumes. *Mutation Research*, **320**, 223–233.

Jelmert, O., Hansteen, I. L. and Langard, S. (1995) Cytogenetic studies of stainless steel welders using the tungsten inert gas and metal inert gas methods for welding. *Mutation Research*, **342**, 77–85.

Kilburn, K. H., Warshaw, R., Boylen, T., Thornton, J. C., Hopfer, S. M., Sunderman, W. F., Jr and Finklea, J. (1990) Cross-shift and chronic effects of stainless steel welding related to internal dosimetry of chromium and nickel. *American Journal of Industrial Medicine*, **17**, 607–615.

Klein, C. B., Su, L. and Snow, L. (1992) Chromium mutagenesis in transgenic *gpt*$^+$ Chinese hamster cell lines. *Environmental and Molecular Mutagenesis*, **19(S20)**, 29a.

KNUDSEN, L. E., BOISEN, T., CHRISTENSEN, J. M., JELNES, J. R., JENSEN, G. E., JENSEN, J. C., LUNDGREN, K., LUNDSTEEN, C., PEDERSEN, B., WASSERMANN, K., WILHARDT, P., WULF, H. C. AND ZEBITZ, U. (1992) Biomonitoring of genotoxic exposure among stainless steel welders. *Mutation Research*, **279**, 129–143.

KORTENKAMP, A., BEYERSMANN, D. AND O'BRIEN, P. (1987) Uptake of Cr(III) complexes by erythrocytes. *Toxicology and Environmental Chemistry*, **14**, 23–32.

KUYKENDALL, J. R. AND BOGDANFFY, M. S. (1992) Efficiency of DNA–histone crosslinking induced by saturated and unsaturated aldehydes *in vitro*. *Mutation Research*, **283**, 131–136.

LANGARDT, S. (1990) One hundred years of chromium and cancer: a review of epidemiological evidence and selected case reports. *American Journal of Industrial Medicine*, **17**, 189–215.

LEWALTER, J., KORALLUS, R. AND WEIDEMANN, H. (1985) Chromium bond detection in isolated erythrocytes: a new principle of biological monitoring of exposure to hexavalent chromium. *International Archives of Occupational and Environmental Health*, **55**, 305–318.

LU, Y. Y. AND YANG, J. L. (1995) Long-term exposure to chromium (VI) oxide leads to defects in sulfate transport system in Chinese hamster ovary cells. *Journal of Cellular Biochemistry*, **57**, 655–665.

MERRITT, K. AND BROWN, S. A. (1995) Release of hexavalent chromium from corrosion of stainless steel and cobalt–chromium alloys. *Journal of Biomedical Materials Research*, **29**, 627–633.

MINOIA, C. AND CAVALLERI, A. (1988) Chromium in urine, serum and red blood cells in the biological monitoring of workers exposed to different chromium valency states. *Science of the Total Environment*, **71**, 323–327.

NAGAYA, T., ISHIKAWA, N. AND HATA, H. (1989) Sister-chromatid exchange analysis in lymphocytes of workers exposed to hexavalent chromium. *British Journal of Industrial Medicine*, **46**, 48–51.

NAGAYA, T., ISHIKAWA, N., HATA, H. AND OTOBE, T. (1991) Sister-chromatid exchanges in lymphocytes from 12 chromium platers: a 5-year follow-up study. *Toxicology Letters*, **58**, 329–335.

NIOSH (1984) *Manual of Analytical Methods*, 3rd edition, Volume 1, P. M. Eller, ed. US Department of Health and Human Services, Cincinnati, OH.

OESCH, F., HENGSTLER, J. G. AND FUCHS, J. (1994) Cigarette smoking protects mononuclear blood cells of carcinogen-exposed workers from additional work exposure-induced DNA single strand breaks. *Mutation Research*, **321**, 175–185.

PERMANA, P. A. AND SNAPKA, R. M. (1994) Aldehyde-induced DNA–protein crosslinks disrupt specific stages of SV 40 DNA replication. *Carcinogenesis*, **15**, 1031–1036.

POPP, W., VARENHOLZ, C., SCHMIEDING, W., KREWET, E. AND NORPORTH, K. (1991) Investigations of the frequency of DNA strand breakage and cross-linking and of sister chromatid exchange in the lymphocytes of electric welders exposed to chromium- and nickel-containing fumes. *International Archives of Occupational and Environmental Health*, **63**, 115–120.

SEN, P. AND COSTA, M. (1986) Incidence and localization of sister chromatid exchanges induced by nickel and chromium compounds. *Carcinogenesis*, **7**, 1527–1533.

SHI, X., MAO, Y., KNAPTON, A. D., DING, M., ROJANASAKUL, Y., GANNETT, P. M., DALAL, N. AND LIU, K. (1994) Reaction of Cr(VI) with ascorbate and hydrogen peroxide generates hydroxyl radicals and causes DNA damage: role of a Cr(IV)-mediated Fenton-like reaction. *Carcinogenesis*, **15**, 2475–2478.

SNOW, E. (1992) Metal carcinogenesis: mechanistic implications. *Pharmacology and Therapeutics*, **53**, 31–65.

STANDEVEN, A. M. AND WETTERHAHN, K. E. (1991) Ascorbate is a principal reductant of Cr(VI) in rat liver and kidney ultrafiltrates. *Carcinogenesis*, **12**, 1733–1737.

ST CLAIR, M. B., BERMUREZ, E., GROSS, E. A., BUTTERWORTH, B. E. AND LESLIE, R. (1991) Evaluation of the genotoxic potential of glutaraldehyde. *Environmental and Molecular Mutagenesis*, **18**, 113–119.

STEARNS, D. M. AND WETTERHAHN, K. E. (1994) Reaction of Cr(VI) with ascorbate produces chromium(V), chromium(IV), and carbon-based radicals. *Chemical Research in Toxicology*, **7**, 219–230.

STEARNS, D. M., KENNEDY, L. J., COURTNEY, K. D., GIANGRANDE, P. H., PHIEFFER, L. S. AND WETTERHAHN, K. E. (1995) Reduction of chromium(VI) by ascorbate leads to chromium-DNA binding and DNA strand breaks *in vitro*. *Biochemistry*, **34**, 910–919.

STRIDSKLEV, I. C., HEMMINGSEN, B., KARLSEN, J. T., SCHALLER, K. H., RAITHEL, H. J. AND LANGARD, S. (1993) Biologic monitoring of chromium and nickel among stainless steel welders using the manual metal arc method. *International Archives of Occupational and Environmental Health*, **65**, 209–219.

SUGIYAMA, M., WANG, X.-W. AND COSTA, M. (1986) Comparison of DNA lesions and cytotoxicity induced by calcium chromate in human, mouse, and hamster cell lines. *Cancer Research*, **46**, 4547–4551.

SUZUKI, Y. AND FUKUDA, K. (1990) Reduction of hexavalent chromium by ascorbic acid and glutathione with special reference to the rat lung. *Archives of Toxicology*, **64**, 169–176.

TAIOLI, E., ZHITKOVICH, A., TONIOLO, P. AND COSTA, M. (1995) Normal values of DNA–protein crosslinks in mononuclear cells of a population of healthy controls. *The Cancer Journal*, **8**, 76–78.

TANIGAWA, T., ARAKI, S., ARAKI, T. AND MINATO, N. (1991) A decrease in Leu-11a negative lymphocytes in relation to natural killer cell activity in chromate workers. *British Journal of Industrial Medicine*, **48**, 211–213.

VINSON, J. A., STARETZ, M. E., BOSE, P., KASSM, H. M. AND BASALYGA, M. E. (1989) *In vitro* and *in vivo* reduction of erythrocyte sorbitol by ascorbic acid. *Diabetes*, **38**, 1036–1041.

YU, Y., XU, Z. AND HSIE, A. W. (1994) Adriamycin induces large deletions as a major type of mutation of CHO cells. *Mutation Research*, **325**, 91–98.

ZHITKOVICH, A. AND COSTA, M. (1992) A simple, sensitive assay to detect DNA–protein-crosslinks in intact cells and *in vivo*. *Carcinogenesis*, **13**, 1485–1489.

ZHITKOVICH, A., VOITKUN, V. AND COSTA, M. (1995) Glutathione and free amino acids form stable complexes with DNA following exposure of intact mammalian cells to chromate. *Carcinogenesis*, **16**, 907–913.

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