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Enhanced Production of Micronuclei by Hexavalent Chromium in Cultured CHO Cells

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Many Studies have indicated that hexavalent chromium compounds enhance carcinogenesis. For instance these compounds increased the rates of chromosome aberrations (Tsuda and Kato 1977; Umeda and Nishimura 1979; Howard et al. 1991) in cultured mammalian cells. A study conducted recently in this laboratory (Howard et al. 1991) also revealed an increase in sister-chromatid exchange (SCE) by CrO_3 and $K_2Cr_2O_7$ in cultured CHO cells. Furthermore, it has been noticed that $K_2Cr_2O_7$ enhanced the morphological transformation rates of hamster cells in short term colony assay (Tsuda and Kato 1977), offering additional evidence of carcinogenic potential of hexavalent chromium.

In recent years, several investigators have used the micronucleus assay to detect cytological damage in cells in addition to the traditional aberration and SCE assays. Since the appearance of micronuclei represents the loss of a chromosome fragment from one of the daughter cells during cell division, these can be used as an index of cytogenetic damage in mammalian cell culture systems (Raj and Heddle 1980). Like traditional metaphase analyses of aberrations and SCE, in vitro micronucleus assay is fast, convenient and inexpensive (Lasne et al. 1984). In the current study, two-hexavalent compounds --CrO_3 and $K_2Cr_2O_7$-- were tested for their influence on the frequency of micronucleus production in cultured CHO cells as an indication of carcinogenic potential.

MATERIALS AND METHODS

Exponentially growing CHO cells were cultured in 75-cm^2 Corning plastic flasks in 10ml of McCoy's 5A modified culture medium supplemented with 10% fetal calf serum and the antibiotics penicillin-G (100 U/ml) and streptomycin (100µg/ml). The cells were incubated in an atmosphere of 5% CO_2 in air at 37°C. The cell

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cultures were set up 24 hr prior to exposure to the test compounds. Approximately 1.5 x 10^6 cells were seeded per flask. The CHO cells were obtained from Enviromental Health Research and Testing, Lexington, KY and were regularly thawed from liquid nitrogen storage and maintained by subculturing twice a week. Chromium salts used in this investigation were purchased from Sigma Chemical, St. Louis, MO.

It should be noted that micronuclei can be conveniently scored from the same preparations used to score SCE (Raj and Heddle 1980). This is because SCE is assessed at the second metaphase after treatment and micronuclei that have arisen from acentric fragments at first and second divisions should be present on the same slide.

The cell treatments were made by administering the test compound in concentrations of 10^{-8}, 10^{-7}, and 10^{-6} M, dissolved in deionized water and suspended in the culture medium. At the time treatments were made, all cultures, including the controls, were wrapped in foil, injected with 10μM 5-bromo-2′-deoxyuridine (BrdU), under red light, and reincubated for 24 hr.

The cell harvest was conducted under red light to prevent premature BrdU activation. Two to four hours prior to the harvest, colcemid (0.1μg/ml) was added to each flask. The cells were loosened by the mitotic shake off procedure (Kochhar 1988), and the contents were then poured into labeled centrifuge tubes and spun for 10 min at 550 rpm. The supernatant was discarded and the cell pellet was resuspended in 5ml of 0.075 M KCl for 5 min. The cells were again centrifuged at 550 rpm for 5 min. The supernatant was discarded and the cells were resuspended in 1 ml of freshly prepared fixative (3:1; methanol : glacial acetic acid) followed by centrifugation at 550 rpm for 10 min. The fixation procedure was repeated twice. The cells were then suspended in 0.5ml of fixative and dropped onto clean, wet slides (which had been chilled to around 2-8°C) at a 45° angle and air dried overnight.

The slides were stained with Hoechst dye, essentially according to the method of Perry and Wolff (1974) except for the exposure to the black light modification introduced by Goto et al. (1978) and then counter stained with giemsa. 250 interphase cells per treatment were scored for the detection of micronuclei and the results recorded as number of micronuclei/250 cells. Micronuclei were scored essentially by the criteria according to Raj and Heddle (1980): a micronucleus should (1) have the same staining intensity as the nucleus; (2) have a diameter less than 1/3 that of the nucleus; and (3) be within 4 nuclear diameters of the nucleus without being in contact with the nucleus.

RESULTS AND DISCUSSION

The effect of hexavalent chromium compounds on the frequency of micronucleus production are summarized in Table 1. It was observed that CrO_3 produced a steady increase in the frequency of micronuclei with the increasing concentrations of this compound. At the lowest concentration (10^{-8}M), CrO_3 caused only a marginal increase in micronuclei while 10^{-7} M of this compound caused a 2-fold increase of micronuclei compared to the controls. The highest concentration of CrO_3 (10^{-6} M) was the most effective, as increase in micronuclei was nearly 3-fold compared to the controls. $K_2Cr_2O_7$ also caused enhancement in the induction of micronuclei and was comparatively more effective than CrO_3. Micronuclei increased in frequency with increasing concentrations of the compound. The lower concentrations (10^{-8} and 10^{-7}M) showed a 2-fold to a 2.5 fold increase in micronuclei compared to the controls. 10^{-6} M produced the greatest number of micronuclei which was more than 3-fold compared to the controls. An additional higher concentration of chromium compounds (10^{-5} M) was also used but proved to be somewhat toxic and did not reveal the dividing cells clearly.

Table 1. Induction of micronuclei by hexavalent chromium compounds in cultured CHO cells.

Treatment	Concentration (M)	Micronuclei/250 cells
CrO_3	control	5
	10^{-8}	6
	10^{-7}	10
	10^{-6}	14
$K_2Cr_2O_7$	control	6
	10^{-8}	12
	10^{-7}	15
	10^{-6}	19

The results obtained in this study demonstrate that hexavalent chromium compounds such as CrO_3 and $K_2Cr_2O_7$ have a positive effect in causing the production of micronuclei in cultured CHO cells. The data gathered in this study concurs with results from previous cytogenetic investigations (Tsuda and Kato 1977; Umeda and Nishimura 1979; Howard et al. 1991) in which CrO_3 and $K_2Cr_2O_7$ were found to be effective in producing various types of chromosome aber-

rations and SCE in cultured mammalian cells. Moreover, these investigators noted little or no effect on chromosomes by exposing cells to chromium with oxidation states of 2 and 3, which is in agreement with our recent observations (unpublished data). In another study dealing with hexavalent chromium compounds, Nishioka (1975) noted that these agents were active in rec assay with Bacillus subtilis which demonstrate these agents capacity to cause DNA damage. Also, Sirover and Loeb (1976) observed that CrO_3 could cause infidelity of DNA synthesis in vitro.

The above studies collectively indicate that only hexavalent chromium is capable of producing substantial biological effects. It is suggested that this specificity of oxidation states of chromium is due to difficulty in absorption of di and trivalent salts into the cell, while the chromates can be taken in with relative ease (Miller and Miller 1979). There is evidence that chromates are reduced to Cr (III) in cells (Costa et al. 1984; Jennette 1979) and this formation of Cr (III) and/or other oxidation states of Cr (as V and IV) might be needed to cause DNA damage. In this regard, De Flora and coworkers (De Flora et al. 1990; De flora and Wetterhahn 1989) have noted that Cr (VI) was the only species that was able to cause damage in investigations which used intact cells, while Cr (III) caused damage in subcellular and acellular targets. These investigators suggest that Cr (VI) enters the cell via the general anion channel protein and a variety of cytosolic, microsomal and mitochondrial enzymes reduce Cr (VI) to Cr (III) that might cause the damage. A recent study conducted by Rodney et al. (1989) also showed the ability of Cr (V) intermediates to produce DNA breaks in vitro and to induce mutation in bacterial cell systems - which might suggest Cr (V) to be the critical form for genotoxic and mutagenic effect of chromate compounds. Furthermore, Cr (VI) has been noted to metabolize in Cr (V) with simultaneous formation of active oxygens (Kawanishi et al. 1986; Shi and Dalal 1989) and glutathionyl radicals (Shi and Dalal 1988). It would therefore be unwise to exclude the possibility of a role played by these radical species in chromium-induced chromosomal damage in mammalian cells.

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REFERENCES

Costa M, Kraker AJ, Patierno SR (1984) Toxicity and carcinogenecity of essential and non-essential metals. Prog Clin Biochem 1: 1-49

De Flora, S., M. Bagnasco, D. Serra, and P. Zanacchi (1990) Genotoxicity of chromium compounds. A review. Mut Res 238: 99-172

De Flora, S., Wetterhahn KE (1989) Mechanisms of chromium metabolism and genotoxicity. Life Chem Rep 7: 169-244
Goto K, Maeda S, Kano Y, Sugiyama T (1978) Factors involved in differential Giemsa staining of sister chromatids. Chromosoma 66: 351-359
Howard W, Leonard B, Moody W, Kochhar TS (1991) Induction of chromosome changes by metal compounds in CHO cells. Toxicol Lett 56: 179-186
Jennette KW (1979) Chromate metabolism in liver microsomes. Biol Trace Elem Res 1: 55-62
Kawanishi S, Inoue S, Sano S (1986) Mechanism of DNA clevage induced by sodium chromate (VI) in presence of hydrogen peroxide. J Biol Chem 261: 5952-5958
Kochhar TS (1988) Steroid hormones enhanced sister-chromatid exchange in cultured CHO cells. Experientia 44: 62-63
Lasne C, Gu ZW, Venegas W, Chouroulinkov (1984) The in vitro micronucleus assay for detection of cytogenetic effects induced by mutagen - carcinogen: comparison with the in vitro sister-chromatid exchange assay. Mut Res 130: 273-282
Miller EC, Miller JA (1979) Overview on the relevance of naturally occuring carcinogens, promoters, and modulators of carcinogenesis in human cancer In: EC Miller et al. (eds) Naturally occuring carcinogens-mutagens and modualtors of carcinogenesis. Japan Sci Soc Press, Tokyo/University Park Press, Balimore, pp 1-17
Nishioka H (1975) Mutagenic activities of metal compounds in bacteria. Mut Res 31: 185-189
Perry P, Wolff S (1974) New Giemsa method for differential staining of sister chromatids. Nature 251: 156-158
Raj AS, Heddle JA (1980) Simultaneous detection of chromosomal aberrations and sister-chromatid exchanges. Mut Res 78:253-260
Rodney PF, Robert JJ, Lay PA, Dixon NE, Raker RSU, Bonin AM (1989) Chromium (V)-induced clevage of DNA: are chromium (V) complexes the active carcinogens in chromium (VI)- induced cancer? Chem Res Toxicol 2: 227-229
Shi X, Dalal NS (1988) The mechanism of chromate reduction by glutathione: ESR evidence for the glutathionyl radical and isoable Cr(V) intermediate. Biochem Biophys Res Commun 163: 627-634
Shi X, Dalal NS (1989) Chromium (V) and hydroxyl radical formation during glutathione reductase-catalyzed reduction of chromium (VI). Biochem Biophys Res Commun 163: 627-634
Sirover MA, Loeb LA (1976) Infidelity of DNA synthesis in vitro: screening for potential metal mutagens or carcinogens. Science 194: 1434-1436
Tsuada J, Kato (1977) Chromasomal aberrations and morphological

transformations in hamster embryonic cells treated with potassium dichromate in vitro. Mut Res 46: 87-94
Umeda M, Nishimura M (1979) Inducibility of chromosomal aberrations by metal compounds in cultured mammalian cells. Mut Res 67:221-229

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