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CONTRIBUTION TO THE VALIDATION OF THE ANAPHASE-TELOPHASE TEST: ANEUGENIC AND CLASTOGENIC EFFECTS OF CADMIUM SULFATE, POTASSIUM DICHROMATE AND NICKEL CHLORIDE IN CHINESE HAMSTER OVARY CELLS

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ABSTRACT

There is increasing evidence that aneuploidy during mitosis may be a factor in the etiology of somatic malignancy. The analysis of alterations in anaphase-telophase of mitosis is a useful test for evaluating the aneuploidogenic and clastogenic ability of chemicals. Several metals have been found to be carcinogenic to humans and animals. However, the underlying mechanisms remain unclear. In the present study the aneugenic and clastogenic abilities of cadmium sulfate, potassium dichromate and nickel chloride were analyzed using the anaphase-telophase test. Chinese hamster ovary (CHO) cells cultured for two cycles were treated with the desired compound for 8 h before cell harvesting. The frequency of cells with chromatin bridges, lagging chromosomes and lagging chromosomal fragments was scored. The mitotic index was determined by counting the number of mitotic cells per 1,000 cells on each coverslip and was expressed as a percentage of the number of mitotic plates. Statistical comparisons were done using the "G" method. Correlation and regression analyses were performed to evaluate variations of the mitotic index. Chromium and cadmium were clastogenic and aneugenic and increased the frequencies of the three types of aberrations scored; nickel had only aneugenic activity because it increased the frequency of lagging chromosomes. These results indicate that the anaphase-telophase test is sufficiently sensitive to detect dose-response relationships that can distinguish clastogenic and/or aneugenic activities and that the results obtained using the anaphase-telophase test were similar to those obtained by chromosome counting.

INTRODUCTION

Although aneuploidy is a serious health problem, the experimental methodology used to investigate the condition has not been completely validated. The tests currently used to detect aneugenic compounds include chromosome counting in diploid cell lines (Danford, 1984, 1985; Dulout and Natarajan, 1987) and the fluorescence *in situ* hybridization (FISH) (van Diemen *et al*., 1995; Dulout *et al*., 1996; Natarajan *et al*., 1996). The use of diploid cell lines is necessary in order to have a low basal frequency of aneuploid cells. However, chromosome counting is time consuming even when using cells with a low chromosome number. Although accurate, FISH requires special equipment and costly reagents that are not available in most laboratories. An alternative ancillary test system is the analysis of alterations in the anaphase-telophase of cultured mitotic cells (Dulout and Olivero, 1984; Seoane and Dulout, 1994). This test is very simple and can be performed in any cytogenetic laboratory with cell culture facilities. The analysis of alterations in anaphase-telophase is restricted to the detection of lagging fragments, chromatin bridges, and lagging chromosomes. Lagging fragments arising from chromosome breaks and chromatin bridges are considered indicators of exchange-type aberrations. Lagging chromosomes are presumably produced by alterations at the level of the kinetochore (Dulout and Olivero, 1984; Seoane and Dulout, 1994).

Validation of the anaphase-telophase test has been done using heavy metal salts such as cadmium sulfate, potassium dichromate and nickel chloride. These compounds were selected based on their mutagenic and carcinogenic capacity and on previous results with cadmium chloride (Seoane and Dulout, 1994).

Compared to laboratory animals little is known of the carcinogenicity of cadmium compounds in humans (IARC, 1990). The ability of cadmium to induce genotoxicity *in vivo* and *in vitro* is well known (Lakkad *et al.*, 1986; Oberdorster, 1989; Tang *et al.*, 1990; Howard, 1991). Saplakoglu and Iscan (1998) recently reported sister chromatid exchanges (SCE) induced by cadmium chloride (10^{-7}-10^{-3} M), whereas Coogan *et al.* (1992) and Dally and Hartwig (1997) found that cadmium II induced single strand breaks at concentrations of 500 μM and 10 μM, respectively. The induction of aneuploidy during meiosis in mice has been reported (Selypes *et al.*, 1992). Cadmium chloride at concentrations of 0.5-4 μM has an aneugenic effect on Chinese hamster ovary (CHO) cells (Seoane and Dulout, 1994), and inhibits microtubule assembly at concentrations of 10-1000 μM (Wallin *et al*., 1985; Andersen and Ronne, 1990).

The genotoxicity of Cr (VI) has been extensively studied with some authors reporting an increase in the frequency of single strand breaks (Gao *et al.*, 1992; Manning *et al.*, 1994) and higher frequency of micronucleus (Howard *et al.*, 1992; Godet *et al.*, 1996). Costa (1991) demostrated the formation of DNA-protein complexes by chromate. Little is known about other cadmium-induced genotoxic endpoints such as aneuploidy. Sora *et al.* (1986)

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reported the aneugenic effects of potassium dichromate (0.07-0.85 mM) in *Saccharomyces cerevisiae*. Some reports have shown that chromate can induce cellular transformation up to 200 μM (Lanfranchi *et al.*, 1988; Biedermann and Landolph, 1990). Li *et al.* (1992) found that microtubules bind 3.13 μM potassium dichromate for 16 h and that microtubule depolymerization and disruption occurred in 3T3 cells at concentrations of 25 μM.

There is substantial evidence for nickel carcinogenicity in humans (IARC, 1990). Nickel can induce DNA-breakage (Stinson *et al.*, 1992; Saplakoglu *et al.*, 1997) and DNA-protein crosslinking (Patierno and Costa, 1985; Zhuang *et al.*, 1994; Lei *et al.*, 1995). Hartwig and Beyersmann (1989) showed that nickel chloride was cytotoxic and mutagenic in V79 cells treated with concentrations of 0.5-2 mM for 5 h. Littlefield *et al.* (1994) reported that this salt (100 μM/l for 16 h) induced DNA strand breaks in a human B-lymphoblastoid cell line and in rat primary splenocytes. Au *et al.* (1994) found no increase in SCE and chromosomal aberrations in lymphocytes exposed to 0.1-100 μM for 1 h. Patierno *et al.* (1993) reported that nickel sulfate was cytotoxic to rat epithelial cells at doses of 50-200 μg/ml and induced transformation in these cells.

The objective of this study is to analyze the aneugenic and clastogenic abilities of cadmium sulfate, potassium dichromate and nickel chloride using the anaphase-telophase test, and to validate, if possible, the usefulness of such analysis to distinguish between these two mentioned kinds of genetic damage.

MATERIAL AND METHODS

CHO cells obtained from the American Type Culture Collection were cultured in Ham's F10 medium (Gibco) supplemented with 10% fetal bovine serum and antibiotics (50 IU penicillin and 50 μg streptomycin/ml).

Metal salts were obtained from Sigma Chemical Company (St. Louis, MO, USA.) and were dissolved in bidistilled water. One hundred microliters of stock solution was added to each culture containing 10 ml of culture medium to give final concentrations of 0.033, 0.067 and 0.134 μM cadmium sulfate; 1, 2, 3 and 4 μM potassium dichromate and 0.003, 0.006, 0.009 and 0.012 μM nickel chloride. These concentrations were selected based on pilot experiments, and represented the highest levels that could be studied. Untreated cultures were used as controls.

The cells were cultured as monolayers on 24 x 36-mm glass coverslips attached to a small drop of siliconized grease (Merck) to the bottom of 90-mm diameter Petri dishes. Three coverslips were placed in each Petri dish. Each coverslip was seeded with 1.5 ml of culture medium containing about 50,000 cells. After one hour, 8.5 ml of culture medium was added to each Petri dish followed by incubation at 37°C in a humidified atmosphere of 5% CO_2. Treatments were performed during the logarithmic growth phase. The cells were treated simultaneously 8 h before cell harvesting. To avoid detachment of the cells from coverslips, the cells were harvested by adding an equal volume of fixative (methanol:acetic acid 3:1 v/v) to the culture medium. The fixative was changed twice at 10-min intervals. The coverslips were stained with carbol fuchsin and attached with DPX mounting medium to coded slides. Each treatment was performed in quadruplicate.

The coded slides were analyzed by one investigator. A total of 1,000 cells per treatment were scored. To avoid the erroneous scoring of chromatin bridges and lagging chromosomes or fragments, the cells were analyzed in late anaphase-early telophase. In early or middle anaphase, chromosome arms cannot be distinguished from chromatin bridges and lagging chromosomes or fragments may be masked by chromosome arms. The frequency of cells with chromatin bridges, lagging chromosomes and lagging chromosomal fragments was scored (Figures 1 A-D). The

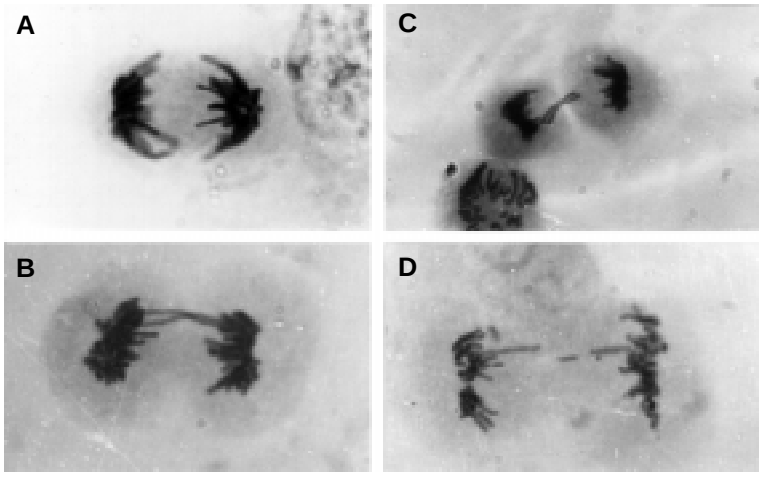

Figure 1 - A, Normal anaphase. B, Anaphase with a chromatin bridge. C, Anaphase with a lagging chromosome. D, Anaphase with a lagging fragment.

mitotic index was determined by counting the number of mitotic cells per 1,000 cells on each coverslip and was expressed as the percentage of mitotic plates.

Statistical comparisons were done using the "G" method of Sokal and Rohlf (1979), which is a frequency comparison test for non-parametric variables. Correlation and regression analyses were used to evaluate the variations in the mitotic index.

RESULTS

Cadmium sulfate increased the frequencies of chromatid bridges, lagging fragments and lagging chromosomes (Table I), although this was significantly different from the untreated controls only at the highest dose ($G = 9.71$, $P < 0.01$ for lagging chromosomes; $G = 6.88$, $P < 0.01$ for lagging fragments; $G = 8.41$, $P < 0.01$ for chromatin bridges). Variations of mitotic index were inversely correlated with dose ($r = 0.868$, $P = 0.068$).

Potassium dichromate increased the frequencies of lagging chromosomes at the four doses ($G = 10.12$, $P < 0.01$; $G = 17.02$, $P < 0.001$; $G = 14.32$, $P < 0.001$, and $G = 22.74$, $P < 0.001$, respectively) (Table II). The frequency of lagging fragments increased significantly at the three highest doses ($G = 11.03$, $P < 0.001$; $G = 7.39$, $P < 0.01$, and $G = 9.80$, $P < 0.01$, respectively) whereas the frequency of chromatin bridges increased significantly ($G = 8.76$, $P < 0.01$; $G = 27.90$, $P < 0.001$, respectively) only at the two highest concentrations. Variations of mitotic index were inversely correlated with dose ($r = 0.899$, $P = 0.013$).

Nickel chloride increased only the frequency of lagging chromosomes (Table III). However, significant differences from the controls were found only between cells treated with the second ($G = 9.73$, $P < 0.01$) and fourth ($G = 7.78$, $P < 0.01$) doses. No effect on the mitotic index was observed, except at the highest dose; the regression analysis was not significant ($r = 0.50$, $P = 0.181$).

Table I - Anaphase-telophase alterations induced by cadmium sulfate (CS) in CHO cells.

Treatment	Anaphase-telophase alterations per 100 cells			Mitotic index (%)
	CB	LC	LF	
Control	0.60 ± 0.07*	1.00 ± 0.09	0.80 ± 0.08	2.32
CS 0.033 μM	1.84 ± 0.13	1.07 ± 0.10	1.15 ± 0.03	2.40
CS 0.067 μM	2.00 ± 0.14	1.60 ± 0.12	0.60 ± 0.07	2.00
CS 0.134 μM	4.28 ± 0.20	5.71 ± 0.23	4.28 ± 0.20	0.45

* Mean ± standard error of the mean. CB: Chromatin bridges. LC: Lagging chromosomes. LF: Lagging fragments.

Table II - Anaphase-telophase alterations induced by potassium dichromate (PD) in CHO cells.

Treatment	Anaphase-telophase alterations per 100 cells			Mitotic Index (%)
	CB	LC	LF	
Control	0.99 ± 0.09*	1.10 ± 0.10	0.10 ± 0.03	2.18
PD 1 μM	1.40 ± 0.11	3.10 ± 0.17	0.80 ± 0.08	2.08
PD 2 μM	1.60 ± 0.12	3.90 ± 0.19	1.20 ± 0.10	1.27
PD 3 μM	2.60 ± 0.15	3.60 ± 0.18	0.90 ± 0.09	1.18
PD 4 μM	4.60 ± 0.20	4.50 ± 0.20	1.10 ± 0.10	0.96

*Mean ± standard error of the mean. For abbreviations see legend to Table I.

Table III - Anaphase-telophase alterations induced by nickel chloride (NC) in CHO cells.

Treatment	Anaphase-telophase alterations per 100 cells			Mitotic index (%)
	CB	LC	LF	
Control	0.40 ± 0.06*	0.40 ± 0.06	0.00 ± 0.00	2.35
NC 0.013 mM	0.30 ± 0.05	1.00 ± 0.09	0.10 ± 0.03	2.35
NC 0.027 mM	0.20 ± 0.04	1.80 ± 0.13	0.20 ± 0.40	2.35
NC 0.040 mM	0.40 ± 0.06	1.30 ± 0.11	0.30 ± 0.05	2.35
NC 0.054 mM	0.50 ± 0.07	1.60 ± 0.12	0.30 ± 0.05	1.55

* Mean ± standard error of the mean. For abbreviations see legend to Table I.

DISCUSSION

The induction of lagging chromosomes in anaphase-telophase is an indication of aneugenic ability (Seoane and Dulout, 1994) whereas the induction of lagging fragments and chromatin bridges signals clastogenic activity. Consequently, the anaphase-telophase test can detect both aneugenic and/or clastogenic compounds.

The carcinogenicity and genotoxicity of cadmium, chromium and nickel strongly depends on their chemical ligands which modulate their bioavailability and reactivity with biochemical targets. With the exception of hexavalent chromium, carcinogenic metal compounds are only weakly genotoxic (Beyersmann, 1994). Nevertheless our results showed that the three cations were genotoxic (chromium and cadmium were clastogenic and aneugenic, and nickel was aneugenic).

Cadmium salt produced significant damage only at the highest concentration (0.134 μM = 0.1 x 10^{-2} μg Cd/ml). This salt has higher toxicity than cadmium chloride, which is active only at doses up to 4 μM (Seoane and Dulout, 1994).

Potassium dichromate produced lagging chromosomes in a dose-dependent manner. The concentration range used (1-4 μM) was lower than that which induced aneuploidy in *Saccharomyces cerevisiae* (Sora *et al.*, 1986) and similar to or lower than doses able to induce cellular transformation in Syrian hamster cells and BHK fibroblasts (Lanfranchi *et al.*, 1988).

Nickel chloride increased the frequency of lagging chromosomes. The frequencies of lagging fragments and chromatin bridges were not significantly different from the controls. Nevertheless, others have reported increased frequencies with higher concentrations (0.5-2 mM for 5 h) (Hartwig and Beyersmann, 1989). Littlefield *et al.* (1994) found that Ni (II) was less genotoxic than Cd (II) at the same dose. Our results showed that Ni (II) had little or no clastogenic activity under the experimental conditions employed. According to Au *et al.* (1994), nickel compounds are weakly mutagenic and may therefore be carcinogenic because of non-conventional genotoxic mechanisms including aneuploidy. On the other hand, the concentrations employed in these experiments may have been too low to induce DNA breakage.

The main cause of mitotic aneuploidy is nondisjunction, although chromosomal delay, chromatid missegregation, and monopolar division (Ford and Correl, 1991) must also be considered. Like another aneuploidy inducers, cadmium chloride modifies the microtubular organization and reduces the fidelity of the splindle apparatus in *Hordeum vulgare* (Voutsinas *et al.*, 1997). Chromium (VI) readily enters cell through non-specific anion channels and reactive intermediates formed during intracellular Cr (VI) reduction may be responsible for some of this compound's genotoxicity (Biedermann and Landolph, 1990; De Flora *et al.*, 1990; Katz and Salem, 1993). There is more than one explanation for chromate-induced aneuploidy. Li *et al.* (1992) suggested that this effect could be related to cytoskeletal damage which may result from the direct inhibition of cytoskeletal protein synthesis. Bridgewater *et al.* (1994) suggested that the interference with DNA replication by chromium-mediated DNA-DNA cross-linking at the level of the centromere may give rise to lagging chromosomes. Nevertheless, the mechanisms by which these metal salts induced aneuploidy remain uncertain.

All of the compounds tested are cytotoxic, although in the case of Ni (II) this effect was weak since it was seen only at the highest concentration.

These results may indicate that the anaphase-telophase test for detecting aneugenic/clastogenic compounds is sensitive enough to detect dose-response relationships, that it can identify clastogenic and/or aneugenic compounds, and that the results obtained are similar to those obtained by chromosome counting (Seoane and Dulout, 1994). Nevertheless, further validation of this test is still required.

RESUMO

As evidências de que a aneuploidia durante a mitose pode ser um fator na etiologia de malignidades somáticas estão cada vez mais fortes. A análise de alterações em anáfase-telófase da mitose é um teste útil para a avaliação da capacidade aneuploidogênica e clastogênica de substâncias químicas. Vários metais têm sido identificados como carcinogênicos para o homem e para animais. Contudo, os mecanismos de ação permanecem obscuros. No presente estudo, a capacidade aneugênica e clastogênica do sulfato de cádmio, do dicromato de potássio e do cloreto de níquel foi analisada usando o teste anáfase-telófase. Células do ovário do hamster chinês cultivadas por dois ciclos foram tratadas com o composto desejado por 8 horas antes da colheita das células. Foram quantificadas as freqüências de células com pontes de cromatina, lagging cromossomos e lagging fragmentos cromossômicos. O índice mitótico foi determinado pela contagem do número de células em mitose por 1000 células em cada lamínula e foi expresso como uma porcentagem do número de placas mitóticas. A análise estatística foi feita usando o método "G". Análises de correlação e de regressão foram realizadas para avaliar as variações do índice mitótico. O crômio e o cádmio foram clastogênicos e aneugênicos e aumentaram as freqüências dos três tipos de aberrações avaliadas; o níquel teve apenas atividade aneugênica porque ele aumentou a freqüência de lagging cromossomos. Estes resultados indicam que o teste anáfase-telófase é sensível o suficiente para detectar as relações dependentes da dose que podem distinguir as atividades clastogênicas e/ou aneugênicas e que os resultados obtidos usando o teste anáfase-telófase foram semelhantes aos obtidos pela contagem cromossômica.

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