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Cytogenetic Effect of Organoarsenicals on Cultured Mammalian Cells

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Arsenic is widely distributed in nature and is known to cause human skin and lung cancer (IARC, 1987). There are several reports indicating the potential of inorganic arsenic to produce clastogenic changes in higher organisms, which may be one of the reasons for its carcinogenic properties. However, such information on highly toxic organic compounds is limited or unavailable.

Inorganic arsenic when administered to humans or to experimental animals is methylated into methylarsonic acid (MMA) and dimethylarsinic acid (DMA) before being excreted in urine (Crecelius, 1977; Vahter, 1985). DMA is considered to be the ultimate metabolite in humans (Buchet et al, 1981). The trivalent form of arsenic, such as arsenite, interacts with biological materials in cells because of its strong affinity for sulfhydryl groups. Cellular levels of glutathione (GSH) and GHS-S-transferase activity are mostly responsible for cell's sensitivity to arsenite exposure (Huang et al. 1993; Lee et al. 1989). Methylation of arsenic is regarded as an important detoxification process of the metal.

If, indeed, methylation does detoxify arsenic, one would assume that the resulting compound to be less toxic compared to inorganic arsenic. However, results from many studies do not quite concur with this assumption. For instance, Oya-Ohta et al. (1996) noted that DMA caused extensive pulverization of chromosomes in most metaphases in cultured human fibroblasts. An in vitro study on V79 Chinese hamster ovary cells revealed a significant increase in frequency of both metaphase arrests and tetraploids after DMA treatment (Endo et al. 1992). The authors suggested that tetraploidy may be one of the multiple stages in carcinogenesis. In a subsequent study, DMA induced mitotic arrests to a greater extent than did arsenite, arsenate, MMA, and trimethylarsine oxide (Eguchi et al. 1997). Also, there have been other reports about DMA causing DNA damage in rodents (Yamanaka et al. 1989) and enhancing various types of cancers in mammals (Yamamoto et al. 1995 ; Wanibuchi et al. 1996).

Another notable observation is that organoarsenicals are found in increasing quantities in marine animals. Given the fact that a vast number of people consume seafood is a cause of great concern. Therefore, in addition to studies on

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methylation, investigations on biological aspects, both in vitro and in vivo are necessary.

In the present study, cytogenetic effects of organoarsenical, DMA in causing chromosome aberrations (CA) and sister-chromatid exchange (SCE) in cultured Chinese hamster ovary (CHO) cells were re-investigated. Another toxic organic arsenic compound, arsanilic acid for which the clastogenic data is lacking was also included in the study. Arsanilic acid (4-aminophenylarsonic acid), which contains arsonic moiety, is structurally similar to pentavalent arsenate. Though not investigated for its genotoxic effect, sodium arsanilate caused vestibular dysfunctions in male meadow voles when given intratympanic injections. (Ossenkopp et al.1992). Further, it induced alteration in locomotor activity, rearing, and scores of vestibular impairments in rats (Llorens and Rodriguez-Faree 1997).

MATERIALS AND METHODS

Chinese hamster ovary (CHO) cells were cultured in 75-cm^2 Corning plastic flasks in 10 ml of McCoy's 5A medium supplemented with 10% fetal bovine serum and antibiotics consisting of penicillin - G (100 U/ml) and (streptomycin 100 μg/ml). The cultures were maintained in an atmosphere of 5% CO_2 in air at 37° C. Approximately $1.5X10^6$ cells were seeded in each flask, 24 hr prior to treatment with test chemicals. The cells originally obtained from Environmental Health and Testing, Lexington, KY, were kept frozen in liquid nitrogen and regularly thawed for use. Dimethylarsenic acid and arsanilic acid were purchased from Sigma Chemical Company, St. Louis, MO.

To investigate CA, the cultures were treated with $10^{-8,}$ $10^{-7,}$ $10^{-6,}$ 10^{-5} and 10^{-4} M of test chemicals, originally dissolved in medium and suspended for 12h. The control cultures were maintained under identical conditions. The procedure for chromosome preparation and scoring is described in previous publications (Kochhar, 1982; Kochhar, 1985). Briefly, 2.5 h prior to cell harvest, the cultures were treated with colcemid (0.1 μg/ml). The medium was then discarded, cultured washed, trypsinized, spun (500 rpm, 10 min) and the supernatant aspirated. The pellet was suspended in 1 ml of warm KCL (0.075 M, 10 min); an equal quantity of fixative (distilled methanol:glacial acetic acid; 3:1) added and centrifuged for 10 min. The fixation procedure was repeated twice and concentrated cell suspension was dropped on clean wet slides and allowed to dry overnight. The slides were stained with 2% Giemsa solution. The number and the types of aberrations were scored by examining 100 well-spread metaphases for each treatment and the controls.

For the sister-chromatid exchange (SCE) assay, the cells were treated with the same levels of arsenicals expect that the highest concentration (10^{-4} M) was omitted. At the time of the initiation of treatments, the culture flasks, including the controls were wrapped in foil and injected with 10 μM of 5-bromo-2-deoxyuridine (BrdU) under OC Kodak safe light and incubated for 24hr. The cell

harvest was done by mitotic shake-off procedure. About 2.5 h before the harvest 0.1 μg/ml of colcemid was added to each culture. The culture flasks were gently shaken and the contents poured into labeled centrifuge tubes and centrifuged at 550 rmp for 10 min. The pellet was treated with the osmoticum and incubated for 10 min. After spinning at 550 rpm for 5 min, the supernatant was discarded again and the cells resuspended in 1 ml of freshly made fixative followed by centrifugation for 10 min. The fixation process was repeated twice The harvest of SCE cultures was carried out under safe light up to the second fixation to avoid photoactivation of BrdU. The final fixation of cells was done in 0.5 ml of fixative and concentrated suspension dropped onto clean, wet slides at 45 degree angle and dried over night.

The SCE slides were stained with Hoechst 33258 dye, according to the procedure of Perry and Wolff (1974) with a black light modification of Goto et al. (1978). SCE were scored at 1000X magnification. For each of 2 slides/treatment, 25 well-spread metaphase plates were scored and an average SCE was calculated. The mean SCE /cell was determined by dividing total SCE by 25. Finally, standard error of mean of observed SCE and controls was calculated.

RESULTS AND DISCUSSION

Table. 1 summarizes the observations on the frequencies of various types of chromosome aberrations caused by DMA and arsanilic acid. It was noticed that both of the organoarsenicals tested caused substantial increase in CA rates compared to the controls. The percentage of total increase in aberrations was dose related. The increase was more than 4 folds at higher concentrations (10^{-5} M) for both compounds compared with controls. 10^{-4} M of DMA proved too toxic to the cells. The aberrations rates were even higher (5 to 6 folds) in 10^{-5} and 10^{-4} M arsanilic acid. Both arsenicals were particularly effective in producing chromosome gaps and multiple centromeres (more than one). Other anomalies observed were the ring chromosomes and the chromatid type of abnormalities, such as breaks and the exchanges.

The test compounds also produced significant increase in SCE rates compared to the controls (Table 2). Like aberrations, higher concentrations (10^{-5}M) of DMA and arsanilic acid produced more than 4-folds increase in SCE. The elevated SCE frequencies seemed to be dose dependent.

Once simply regarded as detoxification product of inorganic arsenic, evidence has accumulated that DMA has unique properties of its own. It has been shown that DMA induced DNA damage in lung cells of mice or rat within 24 hr of oral administration (Yamanaka et al. 1989). It was suggested that this damage is mainly due to the peroxyl radical of DMA and the production of active oxygen species by pulmonary tissue. More recently, multi-organ studies further demonstrated that DMA treatment enhanced cancer induction in urinary bladder, kidney, and thyroid glands of and promoted lung tumors in mice (Yamamoto et

Table 1. Frequencies of chromosome aberrations produced at metaphase by various concentrations of organoarsenicals.

Treatment & Concentration (M)	Cells with Aberrations (%)	ABERRATIONS/100 Metaphases					
		Multiple Centromeres	Gaps	Rings	Chromatid breaks	Chromatid exchanges	Others[a]
Control	5.0±1.41	1.0 ±1.41	3.5 ± 0.71	1.5 ± 0.71	2.8 ±0.71	0	1.5 ±0.71
DMA							
10^{-8}	11.0 ±1.41*	4.0 ±1.41*	7.0 ±4.24	3.0 ±1.41	2.0 ±0.00	0	4.0±0.00*
10^{-7}	17.5 ±0.71*	5.0 ±0.00*	7.5 ±2.12*	2.0 ±2.12	4.5 ±0.71	2.0 ±0.00*	3.5±0.71*
10^{-6}	17.0 ±0.00*	4.5 ±2.12*	12.0 ±1.41*	2.5 ± 0.71	2.5 ±0.71	3.5 ±0.71*	3.0 ±0.00*
10^{-5}	21.5 ±0.71*	4.5± 0.71*	11.5±0.71*	4.5 ±0.71*	5.0 ±1.41*	3.0±0.00*	5.0 ±1.40*
10^{-4}	-	-	-	-	-	-	-
Arsanilic acid							
10^{-8}	11.5 ±2.12*	2.5±3.53	5.5 ±0.71	2.0 ±1.41	1.0 ±1.41	0	3.0±0.00
10^{-7}	10.5 ±0.71*	4.0 ±1.41*	5.5 ±2.12	1.0 ±1.41	2.0 ±1.41	0	2.5 ± 0.71
10^{-6}	15.5 ±0.71*	2.5 ±0.71	6.5 ±0.71	2.0 ±1.41	2.5± 0.71	1.5 ±0.71*	4.5 ±0.71*
10^{-5}	25.5 ±2.12*	3.5 ±0.71*	8.0 ±4.24*	1.5 ± 0.71	5.0 ±1.41*	1.5 ±0.71*	5.5 ±0.71*
10^{-4}	31.0 ±1.41*	5.0±0.00*	10.5 ±3.54*	3.0 ±1.41	6.0 ±0.00*	2.0±0.00*	7.0 ±4.24*

All values represent means ± S.D. of 2 independent experiments.

[a] Includes elongated chromosomes, fragmentation and stickiness of chromosomes.

*$P < 0.05$. Significant difference from controls by planned contrast following randomized complete block ANOVA.

al. 1995). Further, a prolonged exposure to DMA in diet or drinking water caused a dose-dependent increase in cancer of urinary bladder in rats (Wei et al. 1999).

Table 2. Frequencies of SCE produced by organoarsenicals in cultured CHO cells.

Treatment	Concentration (M)	SCE/Cell ± SE[a]
Control	0	1.72 ± 0.23
DMA	10^{-8}	4.60 ± 0.22*
	10^{-7}	5.12 ± 0.31*
	10^{-6}	6.24 ± 0.31*
	10^{-5}	8.72 ± 0.37*
Arsanilic Acid	10^{-8}	4.48 ± 0.28*
	10^{-7}	5.80 ± 0.32*
	10^{-6}	6.68 ± 0.32*
	10^{-5}	7.0 ± 0.24*

[a] Mean S.E. from two independent experiments
Significant at $p<0.05$ by Student's t-test

Since chromosome aberrations, in general, and the cell transformation in vitro have been linked to neoplastic development, it is useful to employ such assays on animal cells in vitro as well as in vivo. As for instance, it has been observed that the cytogenetic assay such as aneuploidy and a numerical change in chromosome number is important to carcinogenesis in somatic cells (Oshimura and Barrett 1989). In this regard, Kashiwada et al. (1998) noted a significant induction of aneuploidy in mouse bone marrow cells treated with DMA. These studies further revealed that DMA treatment produced hyperploids with 1 or 2 extra chromosomes in 80% of all aneuploids. It was suggest that aneuploidy caused by DMA might be associated with carcinogenecity of arsenic. Another study, which seems unique to DMA effect is the excess tetraploids and mitotic arrest induction in Chinese hamster V 79 lung cells exposed to concentrations in excess of 0.7 M, but not in cells exposed to arsenite and arsenate (Endo et al. 1992). It seems that higher mitotic toxicity reported for organoarsenicals in some studies may be due to a greater disruptive effect on microtubular organization of the cell (Bernstam and Nriagu 2000). In present study on CHO cells, aneuploidy was not observed, however, cumulative chromosome aberrations (multiple centromeres, chromosome gaps, and chromatid gaps) and SCE increased significantly. This may be due to source of cells from a different species/or organ. Also, the aforementioned studies did not report any SCE data.

Moore et al (1997) compared the relative mutagenic and genotoxic potential of inorganic (arsenate and arsenite) and organic arsenicals (MMA and DMA) in L5 178Y TK+/- mouse lymphoma cells. All four chemicals were found to be mutagenic with relative potency as: arsenite>arsenate>MMA>DMA. Clastogenic determinations revealed arsenite, arsenate, and MMA as clastogens, while DMA induced only a slight increase in aberrations, to be declared as clastogen. Another interesting observation is the sensitivity of DMA effect is dependent on cellular glutathione (GSH) level. In this regard Oya-Ohta et al (1996) saw suppression of clastogenic effect of DMA by depletion of GSH in human fibroblasts. Additionally, there are reports of DMA induced apoptosis in human renal carcinoma cells, whereas MMA and trimethylarsine oxide (TMAO) did not produce this change (Ochi et al., 1996). This apoptotic effect was greater than that seen with sodium arsenite, but higher levels of DMA were needed. The study again indicated that GSH might play a unique role in the induction of apoptotic response by DMA as depletion of GSH by buthione sulfoximine (BSO) diminished this response.

Collective cytogenetic data on the effect of DMA might suggest that this compound is associated with carcinogenesis induced by inorganic arsenic compounds. However, the data are still controversial as DMA induced excessive tetraploids and mitotic arrests compared to inorganic As species (stated above). Therefore, further studies are warranted to elucidate the mechanism of the induction of aneuploidy, clastogenecity, and gross chromosome alterations caused by DMA.

The present study has demonstrated that though DMA is considered to be a detoxification product of inorganic arsenic, yet it was responsible for a significant increase in chromosome aberrations and SCE in CHO cells; the frequencies, however, were lower compared to our previous studies conducted with arsenite (Kochhar et al. 1996). Furthermore, it should be noted that the other organoarsenicals such as arsanilic acid for which the cytogenetic data is lacking, significantly enhanced the frequencies of CA and SCE in this study. As matter of fact, the increase was even higher (at higher levels) compared to DMA which suggests that other organoarsenicals should not be overlooked and be thoroughly investigated for their effects.

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