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Dimethylarsenic Acid Induces Tetraploids in Chinese Hamster Cells

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Arsenic has been documented as a human carcinogen of the skin and lungs (IARC 1980). However, attempts to induce tumors in experimental animals with inorganoarsenic compounds have mostly failed (IARC 1980) except in a few studies in which animals were given arsenic trioxide by intratracheal instillation (Ishinishi et al. 1980, Pershagen et al. 1984). Moreover, inorganoarsenics are either inactive or too weak to induce gene mutations in vitro (Jacobson-Kram and Montalbalo 1985). The mechanism of arsenic carcinogenicity has not yet been discovered.

Most mammals including human are able to methylate inorganoarsenic compounds to methylarsonic acid and dimethylarsenic acid (cacodylic acid; DMA) (Vahter and Marafante 1985). However, the genotoxicity of organoarsenic compounds has hardly been examined.

We therefore decided to study this genotoxicity, including the frequency of sister chromatid exchange (SCE) of nine organic and three inorganic arsenic compounds. Observation of the metaphases in the SCE test revealed that only DMA of the organo- and inorgano-arsenic compounds induces tetraploids and mitotic arrest. This indicates that the role of DMA may be important in arsenic genotoxicity and may give a clue to the carcinogenic mechanism of arsenic.

MATERIALS AND METHODS

Arsenic compounds examined in the experiments and their sources and purities are listed in Table 1. These compounds were dissolved in distilled water or dimethylsulfoxide and were used for the experiments. 5-bromodeoxyuridine (BrdU), colchicine, colcemid, and Hoechst 33258 were purchased from Wako Pure Chemicals, Osaka, Japan, and Giemsa's solution from Merck, Darmstadt, Germany. V79 cells which originated from Chinese hamster lung and diploid cells, were purchased from ATCC, Rockville, Maryland. Human peripheral blood was obtained from a healthy male volunteer by venipuncture using a heparinized syringe. RPMI 1640, Eagle's MEM

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Table 1. The purity and source of examined arsenic compounds

Arsenic compounds	CAS Number	Purity	Source
Sodium arsenate, dibasic	55957-14-7	99%<	Wako Pure Chemical, Osaka
Arsenic trichloride	7784-34-1	96%	Nacalai Tesque, Kyoto
Arsenic trioxide	1327-53-3	99.999%	Nacalai Tesque, Kyoto
4-Aminophenylarsonic acid (p-Arsanilic acid)	98-50-0	98%	Nacalai Tesque, Kyoto
Tetraphenylarsonium chloride, hydrochloride	507-28-8	98%	Tokyo Kasei, Tokyo
4-((2-Arsonophenyl)azo)-3-hydroxy-2,7-naphthalendisulfonic acid, disodium salt (Thorin)	3688-92-4	80%	Tokyo Kasei, Tokyo
p-Arsenosobenzoic acid, sodium salt	1197-16-6	80%	Tokyo Kasei, Tokyo
Methylthioarsine (Asozin)	2533-82-6		Nihon Noyaku, Tokyo
Dimethylarsenic acid (Cacodylic acid; DMA)	75-60-5	98%	Nacalai Tesque, Kyoto
2-Amonobenzenearsonic acid	2045-00-3	98%	Aldrich Chemicals, Milwaukee
Oxophenylarsine	637-03-6		Aldrich Chemicals, Milwaukee
(2-Diphenylarsinoethyl)diphenylphosphine (Arphos)	23582-06-1		Alfa Products, Danvers

and fetal calf serum were obtained from ICN Biomedicals, Costa Mesa, California. Kanamycin sulfate was purchased from Meiji, Tokyo. Trypsin and phytohemagglutinin M (PHA-M) were purchased from Difco, Detroit, Michigan.

Chromosomes exposed to the test compounds were stained in the same way as in the SCE test in order to observe the chromosome numbers in a metaphase twice divided. The procedure for V79 cells was done according to the fluorescent plus Giemsa (FPG) method (Perry and Wolff 1974), and is described briefly as follows. 4×10^4 V79 cells were plated in each 6 cm diameter petri dish with 5 mL of Eagle's MEM medium supplemented with 7% fetal calf serum, and 100 μg/mL kanamycin sulfate, and cultured for 24 hours at 37°C in a 5% CO_2 atmosphere. The doubling time of the cells was about 18 hours. The cells were incubated with 50 or 100 μL of various concentrations of the tested compounds and 1 μg/mL final concentration of BrdU for 28 hours in the dark. Colcemid of 0.1 μg/mL final concentration was added to the cultures before the last 2 hours. The cells were harvested by trypsin solution, and were spread on microscopic slide glasses after being treated with a hypotonic solution of 0.075 M KCl, and fixed by methanol-acetic acid (3:1). The metaphases were stained the next day by 0.1 μg/mL of Hoechst 33258, irradiated by a mercury lamp, and stained by 2% Giemsa's solution.

The mitotic indices of the V79 cells were determined as follows. V79 cells were cultured in a petri dish for 24 hours in the same way as in the chromosome preparation. They were then incubated with 50 or 100 μL of various concentrations of the test compounds for 8 hours at 37°C in a 5% CO_2 atmosphere. The cells were spread on a microscopic slide glass after beeing treated by hypotonic solution and fixed by methanol-acetic acid. The cells were stained with 3% Giemsa's solution. Mitotic indices were determined by counting metaphases in 500 cells.

Chromosomes of human lymphocytes were prepared as follows. Human whole blood of 0.3 mL was incubated with 10 mL RPMI 1640 supplemented with 14% fetal calf serum, 100 μg/mL of kanamycin sulfate, 0.1 mL PHA-M, 5 μg/mL BrdU, and various concentrations of DMA for 72 hours in the dark at 37° C in a 5% CO_2 atmosphere. 0.1 μ g/mL of colcemid was added to the cultures before the last 3 hours. The metaphases of lymphocytes were stained by the FPG in the same way as the V79 cells.

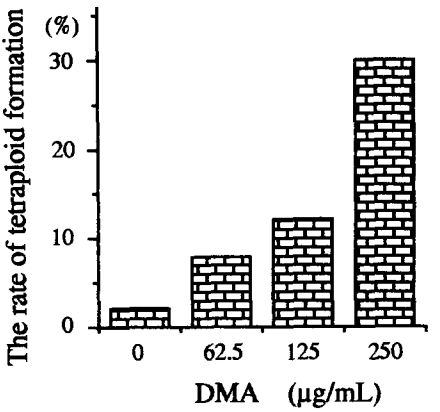

Figure 1. Effect of DMA on tetraploid formation in V79 cells. The cells had been exposed to DMA for 28 hrs.

Titration of hemmagglutining activity of PHA-M were carried out by the methods described by Rigas and Osgood (1955), using human blood cells.

RESULTS AND DISCUSSION

The number of chromosomes in the V79 cells ranged from 20 to 25, and 90% of the cells had 22 chromosomes. We concluded that the metaphases which had 40 to 50 chromosomes were tetraploids cells. The rate of spontaneous tetraploid formation was about 2%. Excess tetraploids were observed in the V79 cells exposed only to the DMA of nine organo- and three inorgano-arsenic compounds. Completely differentially stained metaphases were observed in 80 - 90% or more of all metaphases in DMA concentrations of 0 - 250 μg/mL. Few metaphases were observed at 500 μg/mL of DMA. In a twice divided metaphase, newly synthesized sister chromatids and old chromatids are completely differentially stained. In once divided metaphases they are not differentially stained, and in three or more times divided metaphases, they are partially differentially stained.

The cell division ability in the V79 cells was not inhibited by DMA concentrations of 0 - 250 μg/mL. All of the tetraploids were observed in the twice divided metaphases. None of the tetraploids were observed in once or three or more times divided metaphases. As shown in Figure 1, the rate of tetraploid formation obviously increased with increasing concentrations of DMA.

In order to understand the mechanisms of tetraploid formation by DMA, we surveyed the arresting metaphases of V79 cells of arsenic compounds. Excess mitotic arrest was observed in cells exposed only to the DMA of the nine organo- and three inorgano-arsenic compounds. As shown in Figure 2, the rate of mitotic arrest was increased with increasing concentrations of DMA. The mitotic arrest activity of DMA was much

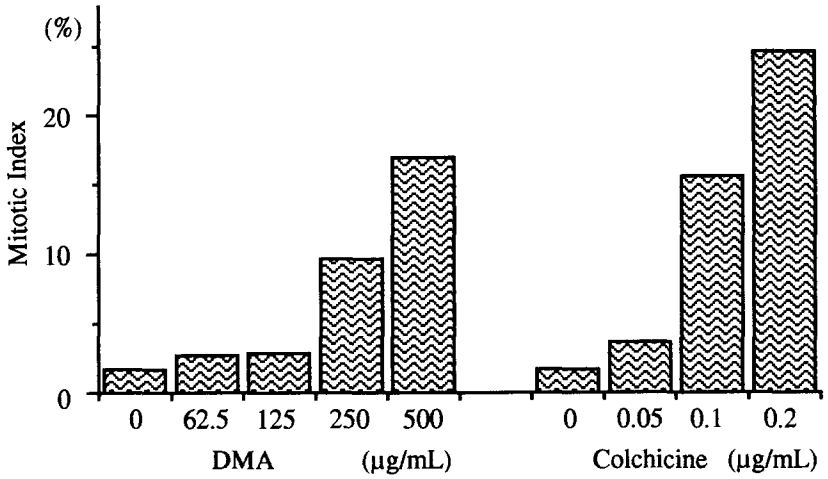

Figure 2. The rate of mitotic arrest in V79 cell exposed to DMA or colchicine for 8 hours

lower (4000 times) than colchicine which is a well known inhibitor of mitosis (Taylor 1965) and a good tetraploid-inducer.

Dustin and Piton (1929) showed the profound disturbance of cell division which followed the injection of arsenic trioxide or DMA into mice, and King and Ludford (1950) found that DMA had a mitotic arresting effect on mammalian cells. Many of the mitotic arrest inducers such as colchcine or colcemid, vinblastin and steroids, which are not structurely related to each other, cause aneuploid and cell transformation (Tsutui et al. 1984, Satya-Prakash et al.1984). Our tests demonstrated that DMA causes mitotic arrest and tetraproid formation. Therefore, it might also cause cell transformation.

In order to investigate the cell division ability of the tetraploid cells, the cells were treated with 250 μg DMA/mL for 28 hours, washed to remove the DMA, and passaged after being diluted by one fourth with fresh medium. As shown in Figure 3, the rate of

Table 2. Effect of DMA on human lymphocytes

DMA dose (μg/mL)	Metaphase	Metaphase division (%)		
		Once	Twice	Three or more times
31.3	Not observed	ND	ND	ND
15.6	Not observed	ND	ND	ND
7.8	Small amounts observed	100	0	0
3.9	Observed	90	10	0
2.0	Observed	35	30	30
control	Observed	10	45	45

ND: not detected because mitosis was absent

tetraploid formation in the first passaged cells (2 days after removal of DMA and about twice divided) were almost the same as that in the no passaged cells. But, the rate of tetraploid formation in the second passaged cells (4 days after removal of DMA and about four times divided) were greatly decreased to 5% of the metaphases. This result suggests that the tetraploid cells have low cell division ability or a slower cell cycle than diploid cells.

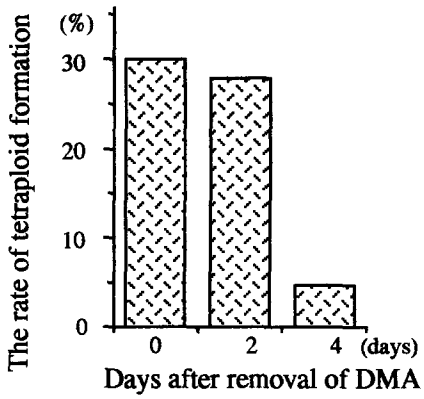

Figure 3. The cells were exposed to DMA of 250μg/mL for 28 hrs, and were passaged in fresh medium after removal of DMA by centrifugation.

In order to determine if DMA induces tetraploid formation and mitotic arrest in human lymphocytes stimulated by PHA-M in vitro, similar to that observed in V79 cells, whole human peripheral blood was cultured with PHA-M and various concentrations of DMA.

As shown in Table 2, the mitogenesis of lymphocytes stimulated by PHA-M was completely inhibited by the DMA in the final concentration of 15.6 μg/mL. The rate of completely stained (twice divided) or partially stained (three times or more divided) metaphases were decreased with increased DMA concentrations of 2 to 7.8 μg/mL. This result indicates that the cell division time of lymphocytes was prolonged in very low concentrations of DMA. No tetraploid or mitotic arrest of lymphocytes were observed in DMA concentrations of 3.9 -15.6 μg/mL.

The concentrations were about one tenth the concentrations in which excess tetraploids and mitotic arrest were observed in V79 cells. These results suggest that mitogenesis of human lymphocytes stimulated by PHA-M were inhibited in DMA, similar to the results reported on sodium arsenite and sodium arsenate (McCabe et al. 1983). Also, we could not observe excess tetraploid or mitotic arrest of lymphocytes because mitogenesis of stimulated lymphocytes was inhibited in such a low DMA concentration.

Table 3. DMA Effect in PHA-M hemagglutination

DMA dose	Dilution of PHA-M					
(μg/mL)	x50	x75	x100	x150	x200	x300
0	○	○	○	○	×	×
125	○	○	○	×	×	×
250	○	○	×	×	×	×
500	○	○	×	×	×	×

○ ; hemagglutinated ×; not hemagglutinated

In order to investigate whether the ability of PHA-M was inhibited by DMA, the hemagglutinating activity of PHA-M (which is another activity of PHA-M) was examined using human blood cells. It was found that a DMA concentration of 125 -250 μg/mL decreased the hemagglutinating activity of PHA-M (Table 3). But the concentrations were far higher than the concentrations in which mitogenesis of lymphocytes were inhibited.

In order to examine the binding ability of DMA and PHA-M, we tried to test for arsenic in PHA-M which had been incubated with DMA of 500 μg/mL for 2 hours. The PHA-M was then precipitated with ethanol-ether (9:1) and resuspended in 0.9% NaCl solution to remove free DMA. Arsenic, however, was not detected in the PHA-M. These results suggest that DMA directly inhibits the cell division of lymphocytes.
As Wheeler et al. (1986) surmised in synthetic estrogens, tetraploid formation is the potential cause of carcinogenicity. None of the nine organo-arsenic compounds induced gene mutations, caused great DNA damage, or induced high frequency of SCEs (Endo et al. 1988). Only DMA, of the nine organoarsenics examined, is a metabolite of arsenic in mammals.

The concentration of urinary metabolites of inorganic arsenic in subjects inhaling air containing about 10 $\mu g/m^3$ arsenic varied from about 40-200 μg As/ g creatinine (Vahter et al. 1986). In subjects exposed to inorganoarsenics, urinary excretion consists of 60-80% DMA (Vahter 1986). The ACGIH TWA value of arsenic and soluble compounds is 0.2 mg/m^3 (ACGIH 1990). It has been calculated that arsenic inhalation of 0.2 mg/m^3 could result in a total urinary arsenic concentration of 800-4000 μ g/g creatinine, which is equal to 884 - 5895 μg DMA/g creatinine. DMA of 62.5 μg/mL is an acceptable level in urine of arsenic exposed workers. Also there are many differences between in-vitro examinations and examinations of workers. Tetraploid induced by DMA may be one of the multiple stages which cause carcinogenicity, and give a clue to the arsenic carcinogenic mechanism, and DMA might be the ultimate carcinogen of arsenic.

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