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Induction of Mitotic Arrest and Aneuploidy by Organic Arsenic Compounds in Human Lymphocytes

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Inorganic arsenic is methylated in the mammalian body to methylarsonic acid (MMA), dimethylarsinic acid (DMA) and trimethylarsine oxide (TMA). To achieve a more precise understanding of arsenic carcinogenicity, we examined the genotoxic effects of organic arsenic compounds on human lymphocytes by assessing induction of mitotic arrest, sister chromatid exchange (SCE) and aneuploidy. MMA, DMA and TMA arrested mitosis, DMA induced hyperdiploid cells, and DMA and TMA induced tetraploid cells. Of the three arsenic metabolites tested, DMA had the strongest effects on cell mitosis and aneuploidy induction. DMA arrested mitosis and induced c-mitosis significantly. These results suggest that DMA arrests mitosis and induces aneuploidy through spindle disruptions similar to those observed with known spindle poisons, such as colchicine or vinblastine. Since aneuploidy has been thought to be associated with tumor induction or neoplastic transformation, induction of aneuploidy by organic metabolites of arsenic may play a major role in arsenic carcinogenesis in humans. © 1998 John Wiley & Sons, Ltd.

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INTRODUCTION

Epidemiological studies have shown that arsenic exposure is correlated with increased incidence of cancers of the skin, lung, urinary bladder, liver, kidney and other internal organs in humans. However, the ultimate carcinogen among arsenic compounds and precise mechanism of arsenic carcinogenicity are still unknown.[1]

Vahter has proposed that a substantial portion of ingested inorganic arsenic is methylated in the mammalian body to the less toxic metabolites methylarsonic acid (MMA), dimethylarsinic acid (DMA) and trimethylarsine oxide (TMA).[2] In humans, the major metabolite of inorganic arsenic excreted in the urine is DMA.[3] DMA is not only a major metabolite of arsenic but is still in widespread use as cacodylic acid in herbicides and it contaminates the meat, liver and kidney of cattle.[4] Recently, Yamamoto *et al*.[5] have shown that DMA acts as a promoter of urinary bladder, kidney, liver and thyroid gland carcinogenesis in rats, and speculated that this effect may be related to cancer induction by arsenic in humans. Chen *et al*.[6] have shown that DMA ingested by rats is mainly excreted as DMA in the urine. Endo *et al*.[7] have reported that DMA induced mitotic arrest and tetraploid cells in cultured Chinese hamster lung V79 cells.

Increase in frequency of sister chromatid exchange (SCE) in lymphocytes was correlated to the arsenic concentration in urine obtained from individuals chronically exposed to arsenic in drinking water.[8] Nordenson *et al*.[9] have reported that chromosomal aberrations in lymphocytes were significantly increased in workers exposed to arsenic. Chromosome analysis of lymphocytes from patients who displayed typical arsenic hyperkeratosis also revealed frequent

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structural and numerical aberrations.[10] This evidence suggests that arsenic in the body has genetic effects on human lymphocytes.

A few studies have succeeded in inducing cancer in rats exposed to arsenic after pretreatment with carcinogens.[5, 11, 12] However, arsenic in drinking water or in the occupational environment can induce cancer in humans.[13] Humans may be more sensitive to arsenic compounds than other animal species. It is therefore necessary to examine the genetic effect of organic arsenic compounds not only on animal cells but also on human cells. Since isoenzymes of animal cells differ from those of human cells, the uptake, distribution or metabolism of arsenic in human cells might differ from those in animal cells.

The aim of the present study is to characterize better the genotoxic effects of the organic arsenic compounds MMA, DMA and TMA on human lymphocytes, by assessing *in vitro* changes in mitotic index (which reveal cytotoxicity), SCE (which is thought to be highly correlated with chromosome damage), and aneuploidy (which is numerical chromosomal aberration).

EXPERIMENTAL

Chemicals

All arsenic compounds were purchased from Tri-Chemical, Yamanashi, Japan. Sodium arsenite (Na_2HAsO_3), sodium arsenate (Na_2HAsO_4), arsenobetaine (Asbe), MMA, DMA and TMA (all with purity > 99.99%) were dissolved in distilled water. 5-Bromo-2′-deoxyuridine (BrdU), colchicine and Hoechst 33258 were purchased from Wako Pure Chemicals, Osaka, Japan, and Giemsa's solutions were from Merck, Darmstadt, Germany. RPMI 1640 was obtained from Nissui Pharmaceutical, Tokyo, Japan, and fetal calf serum was from Gibco Laboratories, Grand Island, NY, USA. Phytohemagglutinin M (PHA-M) was purchased from Difco, Detroit, MI, USA.

Culture of human lymphocytes

Peripheral blood was obtained from two healthy non-smoking donors who had not taken any drugs for at least one week before sampling. A 0.3-ml portion of whole blood was seeded in 5 ml of RPMI culture medium supplemented with fetal calf serum (15%), penicillin (100 units/ml), streptomycin (100 μg/ml), and 0.15 ml of PHA-M. Cultures were preincubated for 48 h at 37 °C under 5% CO_2 before the addition of arsenic compounds and 10 μg/ml of BrdU. Since Endo *et al.*[7] have shown that DMA decreases the hemagglutinating activity of PHA-M, PHA-M was removed from the culture medium after preincubation. It has been reported that exposure to PHA-M for 24 h is sufficient to stimulate lymphocytes.[14] For analysis of mitotic index (MI), sister chromatid exchange (SCE), and aneuploidy, proliferating lymphocytes were exposed to arsenic compounds for 24- or 48-h periods following preincubation. Two hours before fixation, colchicine was added to the culture medium. To examine the effect of DMA alone on the mitotic index and induction of c-mitosis, cells were exposed to DMA alone for 5 h without the addition of colchicine to the culture medium for the last 2 h. For each chemical, two or more experiments have been repeated to determine the effective dose. The data reported are the final values obtained with a more complete dose range.

Chromosome preparations

The cells were collected by centrifugation, exposed to 0.075 M KCl hypotonic solution for 20 min at 37 °C to spread chromosomes and hemolyze red blood cells, and fixed three times in methanol : acetate (3 : 1). Drops of a concentrated suspension of cells were placed on slides in a humidified atmosphere, which were allowed to air-dry. The slides were then stained by a modification of the fluorescent-plus-Giemsa technique to obtain harlequin chromosomes.[15] The slides were stained for 20 min in a solution of 1 μg Hoechst 33258 ml^{-1} in Sorensen phosphate buffer, pH 6.8. The slides were then washed, mounted in buffer under a coverslip, and irradiated by a mercury lamp for 19 min. They were then stained for 20 min in a 2% Giemsa solution in Sorensen phosphate buffer.

Scoring

The mitotic index (MI) was determined as the proportion of cells with mitotic figures in 500 mononuclear cells. For determination of the cell-

cycle kinetics, the proportions of first (M_1), second (M_2) and third or more (M_3) cell-division metaphases in 100 mitoses were determined. SCEs per lymphocyte were scored by analyzing second-division metaphases for each point. The average generation time (AGT) was calculated using the method of Ivett and Tice.[16] To evaluate c-mitotic cells, 100 metaphases per dose were classified by increasing degree of chromatid spreading and contraction. Both complete and partial c-mitosis were scored as c-mitosis. Hyperdiploid cells with between 47 and 80 chromosomes and tetraploid cells with more than 80 chromosomes were scored. Statistical evaluation was performed by analysis of variance (ANOVA), the chi-square test, or the Fisher's exact test.

RESULTS

We examined the effects on mitotic index of three organic arsenic metabolites, as shown in Fig. 1. Usually, a decrease in the mitotic index is evidence of cytotoxicity induced by various chemicals. A 50% decrease in mitotic index compared with the control was taken to be evidence of cytotoxicity. MMA, DMA and TMA significantly increased the mitotic index in human lymphocytes by the end of 24 or 48 h of treatment. MMA at 20 $\mu g\ ml^{-1}$ increased the mitotic index to twice the control level by the end of 24 or 48 h of treatment. MMA at concentrations above 80 $\mu g\ ml^{-1}$ decreased the mitotic index significantly by the end of 48 h of treatment due to its cytotoxic effect. No cytotoxicity was observed with 24 h of treatment. The dose range of 5–40 $\mu g\ ml^{-1}$ DMA significantly increased the mitotic index by the end of 24 h of treatment. A significant correlation was observed between DMA concentrations of 0–20 $\mu g\ ml^{-1}$ for 24 h and the MI ($\gamma = 0.97$, $n = 6$, $P < 0.01$). By the end of 48 h of treatment, the dose range of 2.5–10 $\mu g\ ml^{-1}$ DMA had increased the mitotic index significantly. A significant correlation was also observed between DMA concentrations of 0–10 $\mu g\ ml^{-1}$ for 48 h and the MI ($\gamma = 0.987$, $n = 5$, $P < 0.01$). DMA at 20 $\mu g\ ml^{-1}$ for 24 h or DMA at 10 $\mu g\ ml^{-1}$ for 48 h significantly increased the mitotic index to approximately four times the control level. Treatment for 48 h with DMA was more effective than 24 h of treatment with DMA in increasing mitotic index; however, in the case of MMA, 24 h of treatment was more effective than 48 h of treatment in increasing the mitotic index. DMA at 20 $\mu g\ ml^{-1}$ decreased the mitotic index significantly by the end of 48 h of treatment, due to its cytotoxic effect. TMA at 640 $\mu g\ ml^{-1}$ and 1280 $\mu g\ ml^{-1}$ for 24 h, and

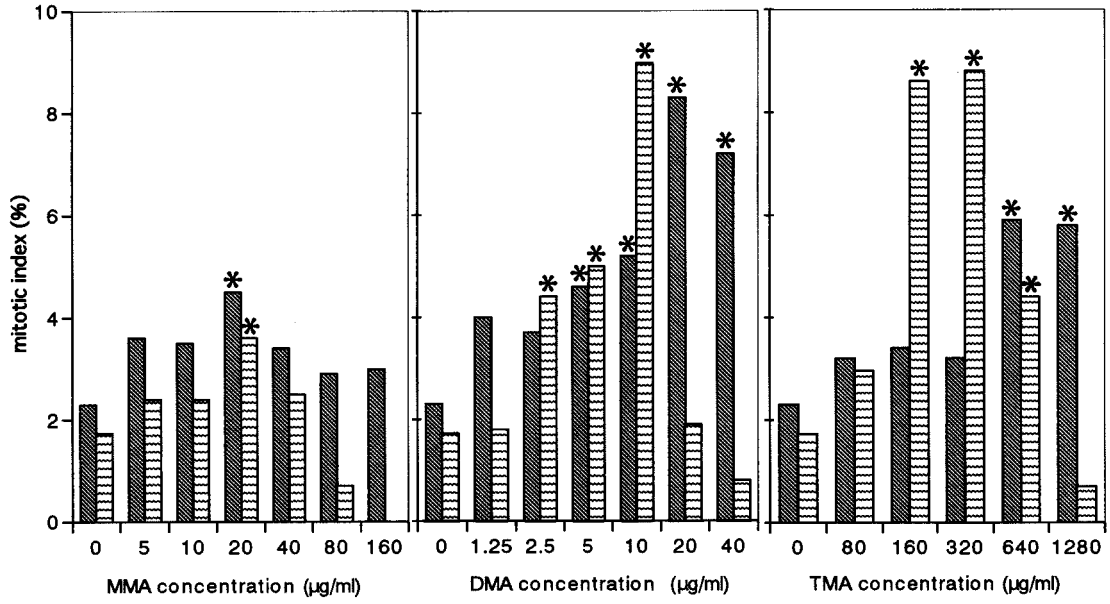

Figure 1 Mitotic index of human lymphocytes exposed to MMA, DMA, or TMA for 24 h (left columns) or 48 h (right columns). * $P < 0.05$ by the chi-square test.

TMA concentrations of 160–640 μg ml^{-1} for 48 h increased the mitotic index of lymphocytes markedly. However, dose–response effects were not observed between concentrations and MI for either the MMA- or TMA-exposed group. The concentration of TMA required to arrest mitosis (160 μg ml^{-1} for 48 h of treatment) was extremely high, i.e. 64-fold higher than that of DMA (2.5 μg ml^{-1} for 48 h of treatment). TMA at 640 μg ml^{-1} for 24 h and 320 μg ml^{-1} for 48 h resulted in maximal mitotic indices, from three- to four-fold the control value. TMA at 1280 μg ml^{-1} for 48 h exhibited cytotoxicity. These results indicate that DMA arrested the mitoses of lymphocytes at the lowest concentration among the three organic arsenic compounds tested.

On the other hand, as shown in Fig. 2, arsenite, arsenate and Asbe did not increase the mitotic index. Arsenite at 2 μg ml^{-1} for 24 h or arsenate at 20 μg ml^{-1} for 24 h completely inhibited the mitosis of lymphocytes. Asbe exhibited no cytotoxicity, even at 2000 μg ml^{-1}.

Findings for cell-cycle progression and numerical chromosome changes in human lymphocytes after 48 h of incubation with organic arsenic compounds are shown in Table 1. The concentrations of each chemical yielding the maximal mitotic index in Fig. 1 were chosen for the present analyses. Mitotic cells were classified into three categories, M_1, M_2 and M_3. An increase in the proportion of M_1 and a decrease in that of M_3 by treatment with organic arsenic compounds was followed by elongation of the AGT.

The aneuploidogenic effects of organic arsenic compounds in human lymphocytes were examined. To avoid recording of false aneuploids due to cell breakage during slide preparation, cells with 45 or fewer chromosomes were rejected and only those metaphases with 47 or more chromosomes were scored. Significant induction of hyperdiploid cells was observed with DMA at 10 μg ml^{-1} for 48 h. Although TMA at 320 μg ml^{-1} also increased hyperdiploid cells, this increase was not statistically significant. The frequency of aneuploid cells with an additional one or two chromosomes was higher than that of such cells with 6–16 additional chromosomes. Significant induction of tetraploid cells with more than 80 chromosomes was observed for DMA at 10 μg ml^{-1} and TMA at 320 μg ml^{-1}.

Since DMA increased the mitotic index by 24 or 48 h of treatment, as shown in Fig. 1, the mitotic index for human lymphocytes exposed to DMA alone for 5 h after 72 h of preincubation was examined, as shown in Fig. 3(A). Colchicine was added to the culture medium 2 h before fixation to make the analysis of chromosomal figures easy in the experiment shown in Fig. 1. However, in this experiment, colchicine was not added in order to avoid its arresting effect.

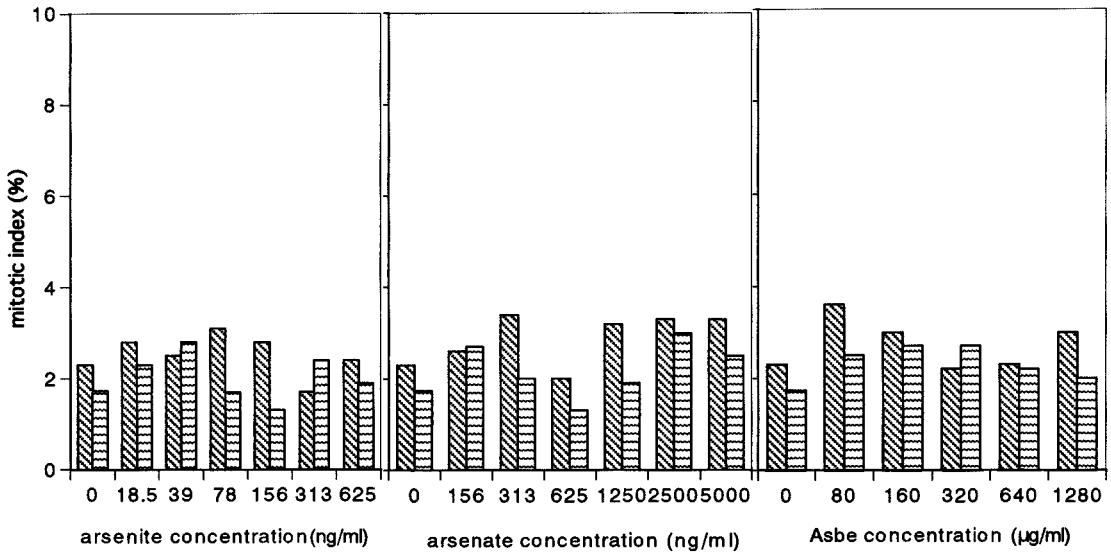

Figure 2 Mitotic index of human lymphocytes exposed to arsenite, arsenate or Asbe for 24 h (left columns) or 48 h (right columns).

Table 1 Percentages of M_1, M_2 and M_3 cells, MI, AGT, and percentages of hyperdiploid cells and tetraploid cells in human lymphocytes after 48 h incubation with organic arsenic compounds

	Percentage of cells					No. of cells					
Chemical	M_1	M_2	M_3	MI (%)	AGT[a]	With 47 chrom.	With 48 chrom.	With 51–64 chrom.	Total no. of cells	Hyperdiploid cells (%)[b]	Tetraploid cells (%)[c]
Control	3.5	5.5	92	1.73	16.52	2	0	0	91	2.2	0
MMA (20 μg ml^{-1})	5	7	88	3.6	16.96	2	1	1	101	3.96	0.5
DMA (10 μg ml^{-1})	18	23	59	8.97	19.92	5	1	2	95	8.42*	3*
TMA (320 μg ml^{-1})	8	18	74	8.8	18.05	4	2	0	114	6.14	5*

[a] Average generation time (AGT): $48/(M_1+2M_2+3M_3)$.
[b] Number of cells with 47–80 chromosomes/number of mitotic cells) × 100%.
[c] Number of cells with more than 80 chromosomes/number of mitotic cells) × 100%. 200 cells were examined for tetraploidy.
* Significantly different from control ($P<0.05$) by the Fisher's exact test.

Treatment with 120–200 μg ml^{-1} DMA resulted in a significant and dose-dependent increase in mitotic index. DMA at 160 and 200 μg ml^{-1} yielded maximal mitotic index values of 5.9%. In contrast, colchicine at 0.15 μg ml^{-1} for 5 h induced a 12.9% mitotic index. To examine whether DMA affects spindles, the presence of c-mitosis, which is thought to be a sign of spindle disruption, was also investigated, as shown in Fig. 3(B). Treatment with colchicine or DMA resulted in significant induction of c-mitosis with chromatid separation and contraction. DMA at 200 μg ml^{-1}, which yielded a maximal mitotic index, induced 61% c-mitosis. Colchicine at 0.15 μg ml^{-1} induced a 68% c-mitosis.

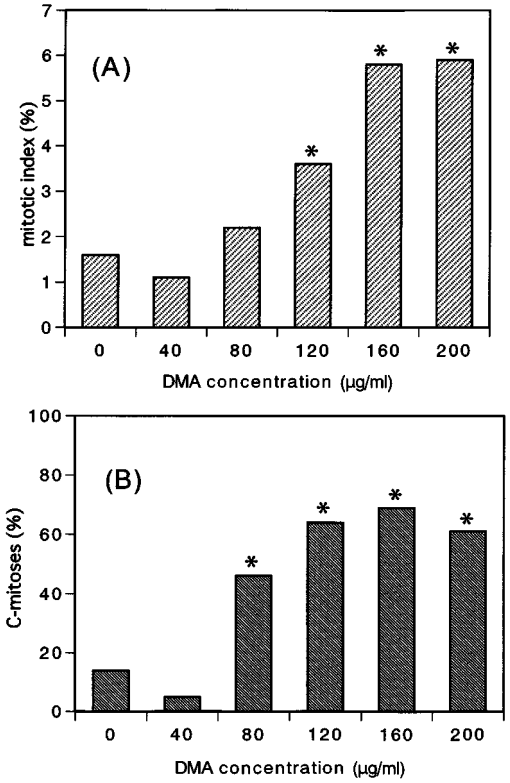

Figure 3 Mitotic index (A) and c-mitosis (B) of human lymphocytes after 5 h incubation with DMA alone. * $P<0.05$ by the chi-square test.

As shown in Table 2, the SCEs/lymphocyte ratio was significantly increased after 24 h of incubation with 640 μg ml^{-1} of TMA. DMA at 20 μg ml^{-1} did not significantly increase the SCEs/lymphocyte ratio.

Table 2 SCEs/cell frequencies in human lymphocytes after 24 h incubation with organic arsenic compounds

Chemical	No. of cells analyzed	No. of SCEs	SCEs/cell
Control	63	364	5.78 ± 2.478[a]
MMA (20 μg ml^{-1})	84	470	5.60 ± 2.17
DMA (20 μg ml^{-1})	54	347	6.43 ± 2.76
TMA (640 μg ml^{-1})	28	267	9.54 ± 3.02*

[a] Mean ± S.E.M.
* Significantly different from control ($P<0.0001$) by ANOVA.

DISCUSSION

In the present study, we examined the mitotic index, SCE and aneuploidy induction in human lymphocytes exposed to three metabolites of arsenic, MMA, DMA and TMA.

MMA at 20 $\mu g\ ml^{-1}$ for 24 or 48 h of treatment, 5–40 $\mu g\ ml^{-1}$ DMA for 24 h of treatment or 2.5–10 $\mu g\ ml^{-1}$ DMA for 48 h of treatment, and TMA at 640 and 1280 $\mu g\ ml^{-1}$ for 24 h or 160–640 $\mu g\ ml^{-1}$ TMA for 48 h significantly arrested human lymphocyte mitosis (Fig. 1). The increase in mitotic index was not due to induction of proliferation of lymphocytes but rather to induction of arrest by these organic arsenic compounds, since the percentage of M_3 was decreased and the percentage of M_1 was increased compared with the control (Table 1). Groups treated with organic arsenic compounds differ in their AGT from the control group, but the difference was only under 3 h. Since all mitotic cells which had divided three or more times were classified in the third-division metaphase in AGT calculations, the control AGT was considered longer than the real generation time (Table 1). Endo *et al*.[7] have reported that DMA at 250 $\mu g\ ml^{-1}$ increased the mitotic index markedly in Chinese hamster V79 cells. In contrast, in the present study, mitosis of human lymphocytes was arrested significantly by 24 h of exposure to DMA at 5 $\mu g\ ml^{-1}$. This result indicates that human primary cells may be more sensitive to arsenic compounds than other cell lines originating from rodents.

On the other hand, arsenite, arsenate and Asbe did not induce mitotic arrest (Fig. 2). Vega *et al*.[17] have reported that sodium arsenite had an aneuploidogenic and a mitotic-arrestant effect. However, we observed neither induction of mitotic arrest nor aneuploidogenic effects even with a 1000-fold higher concentration of arsenic and a longer period of exposure to arsenic than those in their experiment.

We found that DMA increased the mitotic index and induced c-mitosis (Fig. 3). Mitotic poisons interfere with the progression of mitosis, inducing mitotic arrest, and c-mitosis is thought to be a sign of inhibition or disturbance of spindle function.[18] A significant increase in c-mitosis by DMA indicates that DMA affects cell mitosis through spindle disruptions similar to those observed with known spindle poisons, such as colchicine or vinblastine.

In our experiment, a 530-fold lower concentration of colchicine (0.15 $\mu\ ml^{-1}$) than DMA (80 $\mu g\ ml^{-1}$) increased the mitotic index and c-mitosis significantly (Fig. 3). There are several mechanisms by which mitotic poisons induce c-mitosis. Colchicine arrests metaphases by inhibiting polymerization of tubulin,[19] and vinblastine by crystallizing tubulin.[20] The colchicine–tubulin subunit complex might add to the growing end of tubules to inhibit polymerization.[21] Therefore, a small amount of colchicine has the ability to inhibit the polymerization, compared with the amount of tubulin required for this.[19] On the other hand, Vogel *et al*.[22] reported that methylmercury appeared to be a potent microtubule assembly inhibitor by virtue of binding to free sulfhydryl groups both on the ends and on the surface of microtubules. Rozynkowa and Raczkiewicz[23] have shown that the c-mitotic efficiency of methylmercury is weaker than that of colcemid by a factor of 100. Since arsenic is also able to bind to sulfhydryl groups,[24] the mechanism of impairment of spindle function may resemble that of methylmercury. Inorganic arsenic did not increase the MI In the present study. The discrepancy in results for organic arsenic compounds and inorganic arsenic is now being investigated.

In the present study, numerical chromosomal changes were observed in DMA- and TMA-treated lymphocytes (Table 1). Some authors have suggested that induction of mitotic arrest and c-mitosis may be mechanisms of induction of aneuploidy.[25, 26] Aneuploidy is the result of mis-segregation of chromosomes during cell division. There are a number of mechanisms by which chemicals might increase the incidence of chromosome segregational errors.[27] It is clear that the mechanism in the present study by which organic arsenic compounds induce aneuploidy is spindle disturbance. Recent study indicates that some chemicals, including carcinogens, despite causing no detectable DNA damage, may cause mitotic abnormalities resulting in aneuploidy through interactions with the cell-division apparatus. A well-known carcinogen, diethylstilbestrol (DES), induces numerical chromosome change but not gene mutations, and a possible role of aneuploidy in carcinogenesis has been suggested.[28] Since it has been reported that DMA does not have mutagenic effects on *E. coli* B tester strains,[29] DMA may belong to the same class as DES. DMA-mediated induction of aneuploidy may play a role in DMA carcinogenesis.

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