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MECHANISM OF COMUTAGENESIS OF SODIUM ARSENITE WITH N-METHYL-N-NITROSOUREA

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ABSTRACT

Arsenic compcunds are known carcinogens. Although many carcinogens are also mutagens, we have previously shown that sodium arsenite is not mutagenic at either the Na^+/K^+ ATPase or hprt locus in Chinese hamster V79 cells. It can, however, enhance UVmutagenesis. We now confirm the nonmutagenicity of sodium arsenite in line G12, a pSV2gpt-transformed V79 ($hprt^-$) cell line, which is able to detect multilocus deletions in addition to point mutations and small deletions. The lack of arsenic mutagenicity has led to studies emphasizing its comutagenicity. Sodium arsenite at relatively nontoxic concentrations (5 μM for 24 h or 10 μM for 3 h) is comutagenic with N-methyl-N-nitrosourea (MNU) at the hprt locus in V79 cells. Using a nick translation assay, which measures DNA strand breaks by incorporating radioactive deoxyribonucleoside monophosphate at their 3'OH ends in permeabilized cells, we found that much more incorporation was seen in cells treated with MNU (4 mM, 15min) followed by 3-h incubation with 10 μM sodium arsenite compared with cells exposed to the same MNU treatment followed by 3-h incubation without sodium arsenite. This result shows that in the presence of arsenite, strand breaks resulting from MNU or its repair accumulate over a 3-h period. We suggest that the repair of MNU-induced DNA lesions may be inhibited by arsenite either by affecting the incorporation of dNMPs into the MNU-damaged DNA template or by interfering with the ligation step.

Index Entries: Mutagenesis; DNA repair; arsenic; alkylating agents.

INTRODUCTION

Epidemiological studies indicate that inorganic arsenic compounds are human carcinogens. However, no animal model has been established for arsenic carcinogenicity[1-4]. Furthermore, unlike most carcinogens which are also mutagens, arsenic compounds are not mutagenic in either bacteria[5,6] or mammalian cells[5,7]. The contradiction between epidemiological evidence and experimental studies implies that inorganic arsenic compounds may act as cocarcinogens or comutagens rather than primary carcinogens or mutagens. In fact, arsenite, the probable physiologically active form[8] that is not mutagenic in E. coli or in Chinese hamster V79 cells, is comutagenic with UV in E. coli[9] and with

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UV or methyl methanesulfonate (MMS) in Chinese hamster ovary (CHO) cells[10,11]. In addition to the comutagenic effects, arsenic compounds have been shown to induce chromosomal aberrations, sister chromatid exchanges (SCE), and cell transformation[12-14].

The lack of arsenic mutagenicity and its comutagenicity with UV or MMS suggests arsenic compounds may not be direct DNA-damaging agents and they may modify DNA repair process(es) to result in an increase in mutation frequency. This theory is supported by the finding that arsenic trioxide inhibits the removal of thymine dimers from the DNA of human SF34 cells after UV irradiation[15]. However, the mechanism of arsenic comutagenesis with alkylating agents such as MMS cannot be explained in the same way, since different repair pathways are involved. Alkylating agents such as MMS and MNU react with DNA to form a variety of adducts in which MNU favors primarily the oxygens (SN_1 reaction), whereas MMS favors the nitrogens (SN_2 reaction)[16,17]. These DNA adducts (except for O^6-methylguanine, which is removed by O^6-alkylguanine-DNA alkyltransferase (O^6-AGT)) are excised by specific DNA glycosylases[18]. Since Chinese hamster cells are deficient in O^6-AGT activity and are incapable of removing O^6-methylguanine from the DNA[19-21], the comutagenicity of MMS with arsenite might, therefore, involve inhibition of the repair of the other lesions.

To determine whether arsenic compounds are also comutagenic with the other type of alkylating agents, MNU was used to study the comutagenic effect in the V79 (hprt) system. In addition, since MNU is known to cause single strand breaks (SSB)[22], a decrease of SSB with time after MNU treatment would be an indication of DNA repair. Thus, a nick translation assay[23], which measures DNA strand breaks by incorporating radioactive deoxyribonucleoside monophosphate at the free 3'OH ends by endogenous polymerase(s) or along with *E. coli* DNA polymerase I in permeabilized cells, was used to determine if the repair of MNU-induced strand breaks is inhibited by arsenite.

Since the nonmutagenicity of arsenite in bacteria or in Chinese hamster cells could not rule out the possibility of mutations caused by multilocus deletions (which are either unselectable or lethal events in these systems), we also tested the mutagenicity of arsenite in line G12, a pSV2gpt-transformed V79 (hprt) cell system that is able to detect multilocus deletions in addition to point mutations and small deletions[24].

MATERIALS AND METHODS

Cell Culture

Chinese hamster V79 cells were grown in 75 cm^2 flasks at 37°C in an atmosphere of 5% CO_2 in Ham's F12 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U penicillin and 100 μg streptomycin/mL (Gibco). A clone of V79 cells exhibiting a low spontaneous mutation frequency at the hprt locus was isolated, expanded, and stored in liquid nitrogen until needed. Ceils were thawed and utilized within 4 wk to ensure a low background mutation frequency in the hprt mutation assays.

Construction and Culture of Line G12

The line G12 was developed in this laboratory[24]. Briefly, pSV2gpt, a plasmid carrying the E. coli xanthine-guanine phosphoribosyl transferase locus (gpt), was transfected into a nonrevertible hprt$^-$ cell line of V79 induced by UV (16J/m^2). Transfectants were selected in HAT medium. Following several generations in the absence of selection, transfectants were rechecked for stable gpt integration. Southern analysis and spontaneous mutation frequencies were carried out on 23 transfectants. One (G12) had a low spontaneous mutation frequency and a single gpt insert. This cell line shows higher mutation frequency after irradiation compared with the V79 (hprt) system. This could be due to the insertion of gpt gene in an autosome, thereby allowing detection of multilocus deletions, a lethal mutation at the X-linked hprt locus.

Line G12 was stored in liquid nitrogen. The cells were thawed and maintained inHAT medium 1 wk before the mutation assay • The HAT medium was then replaced by F12 medium in the mutation assay.

Test Compounds

N-methyl-N-nitrosourea (MNU; Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO and stored at -20°C as a 1 M stock solution. Sodium arsenite was purchased from Alfa Chemical Co. (Morton Thiokol Co., Danvers, MA) and stored in a desiccator until needed. Sodium arsenite solution was made fresh by weighing and dissolving the compound in water to prepare a 1 M stock solution which was sterilize using a 0.22 μM syringe filter. The final dilutions were made in serum free media immediately prior to use.

Mutation Assays

The hprt mutation assay, using a modification of the method describe by Chang et al[25]., was performed in Chinese hamster V79 cells to study the comutagenesis of arsenite with MNU. V79 cells were seeded concurrently for mutagenesis and survival at densities of 4×10^5 and 500 cells/100 mm dish, respectively. Following a 24-h incubation, cells were treated with different doses of MNU (1,2, or 4 mM) for 15 min, washed twice with Earle's balanced salt solution (EBSS), and F12 medium was added with or without relatively nontoxic doses of sodium arsenite (5 μM for 24 h or 10 μM for 3 h). Then the cells were washed again and refed with F12 medium for a 5-d expression period by replating once to maintain exponential growth. At that time, the survival plates were ficed and stained and the mutagenesis plates trypsinized and reseeded (10 dishes, 1×10^5 cells/dish) in complete F12 medium containing 10 μg/mL 6-thioguanine (6-TG). For the reseeding survival, five hundred cells were plated concurrently in triplicate into 100 mm dishes containing F12 medium without 6-TG and stained after 7 d. Following a 10-d selection period, the mutagenesis plates were fixed and stained. The 6-TG mutation frequency per 10^6 surviving cells was calculated, using the reseeding survival values.

The gpt mutation assay was performed in a similar manner to the hprt mutation assay, except that G12 cells were used.

Nick Translation Assay

The nick translation assay was performed according to the method of Snyder and Matheson[23] with modifications. Appropriately treated V79 cells (10^6) were harvested

with trypsin, collected in an Eppendorf tube by centrifugation, and suspended with gentle pipeting in 1 mL solution containing 0.25 M sucrose, 0.1 M Tris-HCI (pH7.4), 10mM $MgCI_2$, and 0.5 mM dithiothreitol. Lysolecithin(60 μg/mL) was added just prior to use. The cell suspension was kept on ice for 2 min and then centrifuged. The cell pellet was resuspended in nick translation assay mix containing 50 mM Tris-HCI (pH7.4), 5 mM $MgCI_2$, 10 mM 2-mercaptoethanol, 50 μg/mL bovine serum albumin, 0.01 mM each dATP, dGTP, dTTP and 100 μCi/mL [^{32}P] dCTP. The reaction was allowed to proceed with or without E coli DNA polymerase I (200 U/mL) for 30 min at room temperature. Ten percent trichloroacetic acid (TCA) (1 mL) was added to stop the reaction. The entire assay mix was applied onto Whatman 3 mM filter discs prewetted with 2% pyrophosphate, rinsed with 59% ethanol and air dried following a brief ether wash. Acid insoluble radioactivity was measured by scintillation counting.

RESULTS

Mutagenesis at the gpt Locus in G12 Cells

As shown in Table 1, sodium arsenite at the doses tested does not cause significant ($p<0.05$) increases in mutation frequency at the gpt locus in G12 cells. Although the mutation frequencies increase slightly as the doses of arsenite are increased, the effect is not significant at doses giving up to 62% killing (38% survival). It is possible that extremely toxic doses might result in significant mutagenesis at the locus.

Comutagenesis Assay of Arsenite with MNU

In V79 cells, the presence of relatively nontoxic (96-98% survival), nonmutagenic doses of arsenite (5 μM for 24 h or 10μM for 3 h) significantly ($p<0.001$) enhance MNU-induced mutagenesis at the hprt locus (Table 2). This comutagenic effect is more evident at low MNU concentrations. The MNU-induced cytotoxic effect is also enhanced by the relatively nontoxic doses of arsenite.

Nick Translation Assay in Permeabilized V79 Cells

The nick translation assay was

Table 1 Mutagenicity of Sodium Arsenite at the gpt Locus in Line G12

Sodium arsenite, μM	Time of exposure, h	Survival (% control)	Numbers of mutants	Mutation frequency (mutants/10^6 surv)	p value[a]
0	24	(100)	22	40.2	
5	24	88.5	34	52.8	$0.9>p>0.8$
10	24	69.2	33	60.2	$0.2>p>0.1$
15	24	38.1	30	66.4	$0.1>p>0.05$
0	3	(100)	23	49.1	
10	3	98.0	22	60.8	$0.9>p>0.8$
25	3	92.3	23	70.1	$0.3>p>0.2$
50	3	60.1	26	72.7	$0.3>p>0.2$

[a] Obtained by X^2 analysis.

Table 2 Comutagenicity of Sodiwn Arsenite with MNU at th hprt Locus in V79 Cells

MNU, mM	Sodium arsenite post-treatment	Survival (% control)	Numbers of mutants	Mutation frequency (mutants/ 10^6 surv)	Comutagenic ratio[a]
Experiment 1					
0	none	(100)	0	< 1.17	
0	5 μM, 24 h	98.0	0	< 1.39	
1	none	95.6	140	247	
1	5 μM, 24 h	86.8	213	284[b]	1.57
2	none	89.8	285	542	
2	5 μM, 24 h	79.3	444	827[b]	1.53
4	none	62.1	537	1589	
4	5 μM, 24 h	44.6	675	2103[b]	1.32
Experiment 2					
0	none	(100)	1	1.29	
0	10 μM, 3 h	96.1	0	< 1.37	
1	none	85.7	178	331	
1	10 μM, 3 h	77.4	382	759[b]	2.29
2	none	76.1	660	1020	
2	10 μM, 3 h	60.4	884	1796[b]	1.76
4	none	62.2	918	1639	
4	10 μM, 3 h	53.5	967	2213[b]	1.35

[a] Comutagenic ratio = $\frac{\text{Mutation Frequency of MNU with Sodium Arsenite Posttreatment}}{\text{Mutation Frequency of MNU Alone.}}$

[b] Statistically significant, $p < 0.001$, according to X^2 analysis, compared with MNU alone.

designed for detection of DNA single-strand breaks (SSB) with free 3'OH ends [23]. As shown in Fig. 1, arsenite (10 μM, 3 h) alone does not cause any significant increase in SSB compared with the control. After MNU treatment (4 mM, 15min), on the other hand, increased incorporation of [^{32}P] -dCMP by either endogenous polymerase(s) or with *E. coli* DNA polymerase I is observed, denoting increased SSB. If cells are incubated for 3 h after MNU treatment, the incorporation of [^{32}P] -dCMPdecreases, indicating that DNA repair has occurred. However, if cells are incubated with arsenite for 3 h after MNU treatment, the d-CMP incorporation by *E. coli* DNA polymerase I remains at a level about 3 times higher than

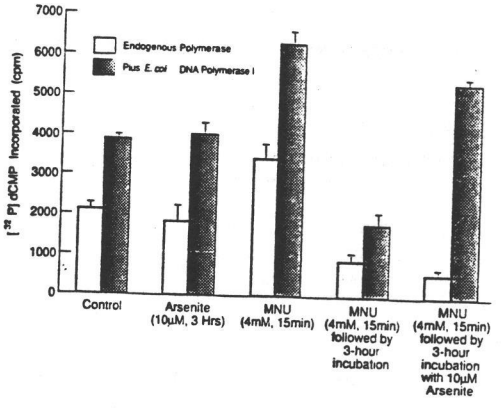

Fig. 1. The availability of 3'-OH sites for nick translation by endogenous polymerases and by *E. coli* DNA polymerases I in permeabilized Chinese hamster V79 cells.

that of MNU treated cells incubated 3 h without arsenite.

DISCUSSION

Sodium arsenite is not mutagenic in G12 cells (Table 1). This result confirms our previous observations that arsenite per se is not a mutagen[5] and extends the negative results from point mutations or intragenic deletions to multilocus deletions.

Sodium arsenite at relatively nontoxic, nonmutagenic doses is comutagenic with MNU at the hprt locus in V79 cells (Table 2). It has been reported that arsenite enhances the mutagenicity of MMS in CHO cells, although a toxic concentration of arsenite (50% survival) was used[11]. Thus, arsenite is comutagenic with both SN_1 and SN_2 alkvlating agents. Since V79 cells lack O^6-AGT[19-21], the comutagenic effect can not be the result of inhibition of O^6-AGT activity by arsenite.

In the nick translation assay (Fig.1), arsenite per se does not cause SSB (additional sites for nick translation). MNU alone increases sites available for nick translation by both endogenous polymerase(s) and *E. coli* DNA polymerase I. After a 3-h incubation, the sites decrease, probable owing to the completion of excision repair. Increased nick translation by *E. coli* DNA polymerase I is seen in V79 cells treated with MNU followed by a 3-h arsenite treatment compared with cells exposed to MNU followed by 3-h incubation without arsenite. The increase indicates that sites resulting from MNU or its repair accumulate over a 3-h period in the presence of arsenite. The sites persisting over this period could be due to inhibition of a step in excision repair by arsenite. There is no inhibition of the incision step, because such an inhibition would have resulted in fewer sites available for nick translation. It is possible that arsenite could inhibit the polymerase enzyme(s) involved in the gap-filling step during the repair of MNU-induced DNA damage. However, arsenite alone did not significantly affect nick translation in control cells (Fig.1). It is not known at this point if DNA polymerase alpha, the major replicative enzyme in animal cells[26], which is also believed to participate in DNA repair synthesis induced by MNU[27,28], is inhibited by arsenite. However, if DNA polymerase beta were involved in the repair of MNU-induced DNA damage, the paradox could be reasonably explained. Since DNA polymerase beta is a repair enzyme, it is activated or produced predominantly after DNA damage[26]. If DNA polymerase beta were the target for arsenite, we would expect that nick translation in MNU-damaged cells would be more sensitive than in intact cells.

Alternatively, arsenite could inhibit DNA ligase activity. The inhibition could be exerted either by inhibiting the ligase enzyme directly or by inhibiting MNU-induced poly (ADP-ribose) synthesis, thereby suppressing the induction of DNA ligase II, the presumed repair enzyme[29]. Additional studies are now in progress to purify ligases and polymerases from V79 cells to further investigate the inhibitory effects of arsenite.

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