Mutagenesis vol.12 no.5 pp.353–358, 1997

Arsenite retards DNA break rejoining by inhibiting DNA ligation

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Arsenic has been shown to inhibit methyl methanesulphonate (MMS)-induced DNA repair but the exact mechanism remains controversial. The purpose of this investigation is to examine which step of DNA repair is most sensitive to arsenite (As) and how As inhibits it. The results from single-cell alkaline electrophoresis, showing post-treatment with As increased DNA strand breaks in MMS-treated cells, suggest that that the excision step seems to be less sensitive to As than later steps. To test this hypothesis, hydroxyurea (Hu) plus cytosine-β-D-arabinofuranoside (AraC) were used to block DNA polymerization, allowing the DNA strand breaks to accumulate. These experiments indicated that As had weak inhibitory effects on DNA strand break accumulation. However, As inhibited the rejoining of those DNA strand breaks which could be rejoined within 4 h after release from blockage by Hu plus AraC. To further elucidate this mechanism, a cell extract was used to compare the relative sensitivity of the various steps in DNA repair to As. The potency of the As inhibitory effect as deduced from concentration–response curves were: ligation of poly(rA)·oligo(dT) > ligation of poly(dA)·oligo(dT) ≈ DNA polymerization ≥ DNA repair synthesis > excision. As is known to inhibit the activity of pyruvate dehydrogenase by interacting with vicinal dithiol groups. Dithiothreitol could effectively remove As inhibition of both the ligation of poly(rA)·oligo(dT) and the activity of pyruvate dehydrogenase but had no obvious effect on As inhibition of poly(dA)·oligo(dT) ligation. Since DNA ligase III contains vicinal dithiol groups, we postulate that As may inhibit DNA break rejoining by interacting with the vicinal dithiols to inactivate DNA ligation in MMS-treated cells.

Introduction

Arsenic is related to the aetiology of Blackfoot disease (Chen *et al.*, 1988), and lung, skin, liver, bladder and kidney cancers in certain areas of Taiwan (Chen and Wang, 1990; Chen *et al.*, 1992). Arsenic is not a potent mutagen in short-term tests, although it can enhance the genotoxicity of many mutagens (Jha *et al.*, 1992; Lee *et al.*, 1985, 1986a,b; Lin and Tseng, 1992; Wiencke and Yager, 1992) and inhibit DNA repair (Hartwig *et al.*, 1997; Lee-Chen *et al.*, 1992, 1993, 1994; Wang *et al.*, 1994). Arsenite (As) inhibits DNA repair in cells treated with methyl nitrosourea, (Li and Rossman, 1989b), methyl methanesulphonate (MMS) (Lee-Chen *et al.*, 1993, 1994) and UV light (Lee-Chen *et al.*, 1994). As inhibition of DNA repair may be related to its cogenotoxicity, both actions could be important in As-induced human diseases.

As has been shown to increase the nick translation of MMS-treated V79 cells (Li and Rossman, 1989b) and the unscheduled DNA synthesis of methyl nitronitrosoguanidine-treated human fetal lung fibroblasts (Dong and Luo, 1994). These results are interpreted as not being due to inhibition of the incision step by As, since inhibition of this step would have resulted in fewer breaks. On the other hand, As causes the accumulation of a large amount of alkali-labile sites and only a small amount of frank strand breaks in MMS-treated cells (Lee-Chen *et al.*, 1993). This result suggests that As inhibits the action of specific DNA glycosylase, apurinic endonuclease or spontaneous hydrolysis of the cognate *N*-glycosyl bond. If As inhibits the steps after the action of apurinic endonuclease, a large number of frank breaks would have been detected. As alone does not affect nick translation in control cells, indicating that As does not inhibit DNA polymerization (Li and Rossman, 1989b). As has been shown to inhibit both constitutive and methyl nitrosourea-inducible DNA ligase activities (Li and Rossman, 1989a). In support of this finding, As has also been shown to inhibit the rejoining of DNA strand breaks in Chinese hamster ovary (CHO) cells pretreated with MMS plus hydroxyurea (Hu) and cytosine-β-D-arabinofuranoside (AraC) (Lee-Chen *et al.*, 1994). The available data are consistent in indicating that As inhibits the repair of alkylating agent-induced DNA damage; however, there is still disagreement about which step in the base-excision repair is affected by As. The purpose of this investigation was to determine which step(s) during the repair of MMS-damaged DNA are affected by As, and how As inhibits them.

Materials and methods

Cell culture and chemicals

Chemicals for cell culture were obtained from Gibco (Grand Island, New York, USA). CHO-K1 cells were grown in McCoy's 5A medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and 0.03% glutamate, at 37°C, in a water-saturated atmosphere containing 5% CO_2. $NaAsO_2$ (Merck, Darmstadt, Germany), MMS, Hu, AraC and dithiothreitol (DTT) (all from Sigma Chemical Co, St Louis, MO, USA) were dissolved in double-distilled water.

Cytotoxicity

The cytotoxic effects of various treatments were determined by colony formation assay. After treatment, 200–4000 cells were plated in triplicate for the determination of colony forming efficiency as previously described (Jan *et al.*, 1990).

Single-cell alkaline electrophoresis

The method described by Gedik *et al.* (1992) was adopted with minor modifications. After electrophoresis, slides were rinsed with deionized water, and washed three times (5 min each) in 0.4 M Tris–HCl, pH 7.5. Then 10 μl of 40× Sybr Green (Molecular Probe S-7567, Eugene, OR, USA) was dropped onto the gel and a coverslip was applied. The slides were examined under an epifluorescence microscope with a 365 nm excitation filter, a 400 nm dichroic mirror and a 435 nm barrier filter. The image of 50 cells per treatment was recorded by a digital camera (Kodak DCS-420). The migration of DNA from the nucleus of each cell was measured with a computer program by using the parameter of comet moment (Kent *et al.*, 1995): comet moment = $^{TM}_{0\to n}$ {(amount of DNA at distance X)×(distance X)}/total DNA. Means and SE are shown in Figures 1 and 2.

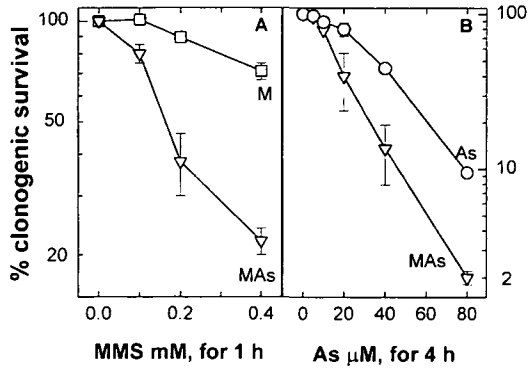

Fig. 1. Effects of $NaAsO_2$ on the clonogenic survival of MMS-treated cells. (**A**) Cells were treated with various concentrations of MMS for 1 h (□, M) and then incubated with 20 μM $NaAsO_2$ for 4 h (▽, MAs). (**B**) Cells were pretreated with 0.2 mM MMS for 1 h and then incubated with various concentrations of $NaAsO_2$ for 4 h (▽, MAs), or with $NaAsO_2$ only for 4 h (○, As). Before normalization, the plating efficiencies of untreated, treatment with 20 μM $NaAsO_2$ for 4 h, and with 0.2 mM MMS for 1 h were 102.5 ± 4.3, 81.4 ± 6.3 and 89.7 ± 3.6 respectively. Mean and SE were calculated from three independent experiments.

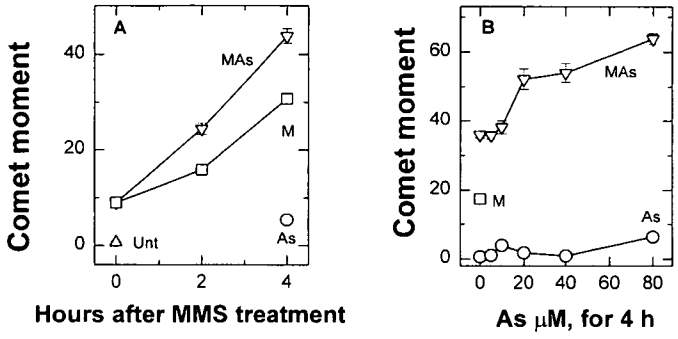

Fig. 2. Effect of $NaAsO_2$ on MMS-induced DNA repair. (**A**) Cells without treatment (Δ, Unt), or treated with 80 μM $NaAsO_2$ for 4 h (○, As), or with 0.4 mM MMS for 1 h and then further incubated in the drug-free medium (□, M), or medium containing 80 μM $NaAsO_2$ for 2 or 4 h (▽, MAs). (**B**) Cells were treated with 0.4 mM MMS for 1 h (□, M), or with 0.4 mM MMS for 1 h and then incubated with various concentrations of $NaAsO_2$ for 4 h (▽, MAs) or with various concentrations of $NaAsO_2$ for 4 h (○, As). DNA strand breaks were analysed by single-cell alkaline electrophoresis as described in the Materials and methods section.

Cell extracts

Whole-cell extracts were prepared from CHO-K1 culture containing 1×10^8 cells according to the method of Manley *et al.* (1983), except cell extracts were dialysed against buffer without DTT. The cell extracts contain proteins required for DNA repair *in vitro* (Wood *et al.*, 1988). Protein content was determined by the Bio-Rad protein assay kit (Hercules, CA, USA) using bovine serum albumin as a standard.

Cell-free system to measure the base excision activity

This assay was based on monitoring the excision of MMS-damaged closed-circular plasmid to open-circular form. Plasmid pRSVcat DNA was prepared from chloramphenicol-amplified *Escherichia coli* HB101 cells by alkaline sodium dodecyl sulphate (SDS) lysis and CsCl gradient ultracentrifugation and further purified by sucrose gradient. The plasmids were then treated with 9 mM MMS at 30°C for 30 min. The excision reaction mixture (50 μl) containing the 400 ng MMS-damaged plasmids, 1 μg cell extracts, 50 mM HEPES–KOH pH 7.8, 70 mM KCl, 5 mM $MgCl_2$, 0.4 mM EDTA, 2 mM ATP, 40 mM phosphocreatine and 2.5 μg creatine phosphokinase were incubated at 37°C for the times indicated and the reaction terminated by the addition of SDS (to 0.6%), EDTA (to 20 mM) and proteinase K (240 μg/ml). Following digestion for 1 h at 37°C and phenol/chloroform extraction, the DNA was precipitated with ethanol (2-fold volume) and 3 M sodium acetate (1/10-fold volume). The precipitated DNA was dissolved in 20 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), treated with 2 μg/ml of RNase A at 37°C for 30 min, and then the open-circular and close-circular form of plasmids were separated by 0.8% agarose gel electrophoresis. DNA bands were photographed with UV light and photographic negatives were analysed by a densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

Cell-free assay of DNA polymerization activity

Monitoring DNA polymerase activity was carried out by the procedure of Chiu and Baril (1975) with a slight modification. A final volume of 20 μl of reaction mixture containing 50 mM Tris–HCl (pH 6.8), 8 mM $MgCl_2$, 50 μM dATP, 50 μM dGTP, 50 μM dCTP, 5 μCi/ml dTTP (21 Ci/mmol, Amersham, Bucks, UK), 1 mg/ml nuclease-free bovine serum albumin, 1 μg DNase I-activated calf thymus DNA (Baril *et al.*, 1977) and 1 μg cell extract was allowed to react for 45 min at 37°C. The reaction was terminated by addition of 10% trichloroacetic acid (TCA). The incorporation of radioactivity into the acid insoluble fraction was determined by precipitating the reaction mixture with 10% TCA and filtration of the mixture onto GF/C filters (Whatman, Maidstone, UK). The filters were washed twice with 10% TCA and twice with 90% ethanol, dried on a heat plate and counted in scintillation fluid (Beckman, Fullerton, CA, USA). The repair synthesis in this assay presumably involves excision and polymerization.

Cell-free system to measure the DNA-repair synthetic activity

This assay was based on monitoring the repair synthesis of closed-circular plasmid DNA damaged by MMS as described previously (Lynn *et al.*, 1994). Briefly, 400 ng of 9 mM MMS 30 min-treated plasmid DNA was incubated with 10 μg cell extract in a reaction mixture containing the four deoxynucleoside triphosphates and [α-^{32}P]dATP without DTT at 37°C for 3 h. The plasmid DNA was then extracted, linearized by restriction enzyme *Pvu*II, electrophoresed in a 0.8% agarose gel, and analysed by PhosphorImager (Molecular Dynamics). The activity of DNA repair was estimated by the amount of repair incorporation divided by the amount of DNA.

Cell-free assay of DNA ligation activity

The activities of DNA ligases in cell extracts were measured by the activities that join oligo(dT) molecules hydrogen-bonded to poly(dA) or poly(rA). The joining of poly(dA)·oligo(dT) was taken to measure the activities of ligases I + II + III + IV (Wei *et al.*, 1995) and the joining of poly(rA)·oligo(dT) was taken to measure the activities of ligases II + III as described by Yang *et al.* (1992). Briefly, oligo(dT)$_{16}$ (Pharmacia, Uppsala, Sweden) was end-labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase and then annealed to poly(dA) or poly(rA) (Pharmacia). A final volume of 20 μl of reaction mixture containing 70 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$, 2 mM ATP, 50 μg/ml nuclease-free bovine serum albumin, the hybrid substrate (1.4 ng) and 1 μg cell extract was allowed to react for 30 min at 16°C. The reaction was terminated by addition of a stop solution (95% formamide, 20 mM EDTA) and denatured by heating at 90°C for 5 min. The reaction mixture was electrophoresed in a 15% polyacrylamide/urea gel. The radioactivity of oligo(dT) was estimated by a PhosphorImager. The DNA ligation activity = [radioactivity of ligated oligo(dT)/total radioactivity of oligo(dT)]×100%

Pyruvate dehydrogenase activity assay

Pyruvate dehydrogenase activity was analysed based on Brown and Perham (1976) with modifications. Pyruvate dehydrogenase (1 μg/40 μl) was incubated with reaction mixture (50 mM potassium phosphate buffer, pH 8.0, 2.5 mM NAD^+, 0.2 mM thiamine pyrophosphate, 1 mM $MgCl_2$, 0.13 mM coenzyme A, 2.6 mM GSH and 2.0 mM pyruvate) for 40 min. NADH formation was detected by fluorescence (excitation 363 nm/emission 447 nm) measured with a Hitachi fluorescence spectrophotometer model F-4010, with a 30 s detection time.

Results

Effects of As on DNA repair in cells

The cytotoxicity of MMS was enhanced by a 4 h post-treatment with As (Figure 1A and B). With a 4 h treatment, the concentration of As required to decrease the colony forming efficiency by 50% was estimated as 40 μM. As 10 μM could enhance the cytotoxicity of MMS (Figure 1B); however, the enhancing effect could be more clearly detected with As concentrations of >20 μM (Figure 1A).

To investigate the effects of As on DNA repair in MMS-treated cells, DNA strand breaks were monitored by the comet assay. This technique measures DNA strand breaks which migrate from the nucleus upon denaturation and electrophoresis in alkaline buffer. DNA strand breaks were detected immediately after a 1 h 0.4 mM MMS treatment (Figure 2). DNA strand breaks in MMS-treated cells were increased by the

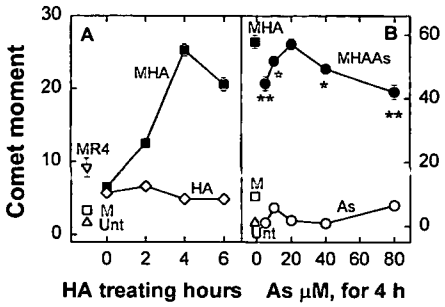

Fig. 3. Effect of $NaAsO_2$ on DNA strand breaks generation. **(A)** Cells without treatment (Δ, Unt), or treated with 0.4 mM MMS for 1 h (□, M), or with 0.4 mM MMS for 1 h and then incubated in drug-free medium for 4 h (▽, MR4), or with 10 mM Hu plus 100 μM AraC for various times (◇, HA), or with 0.4 mM MMS for 1 h and then with Hu plus AraC for various lengths of time (■, MHA). **(B)** Cells without treatment (Δ, Unt), or treated with 0.4 mM MMS for 1 h (□, M), or with various concentrations of $NaAsO_2$ for 4 h (○, As), or with 0.4 mM MMS plus 10 mM Hu plus 100 μM AraC for 1 h and incubated in medium containing Hu plus AraC, both without $NaAsO_2$ (■, MHA) or with $NaAsO_2$ for 4 h (●, MHAAs). *P < 0.05 and **P < 0.01 according to Student's *t*-test comparing MHAAs with MHA.

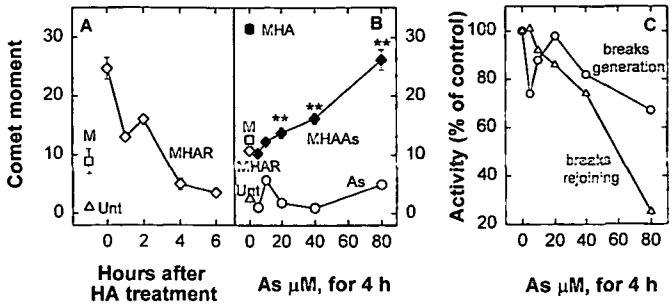

Fig. 4. Effect of $NaAsO_2$ on DNA strand breaks rejoining. **(A)** Cells without treatment (Δ, Unt), or treated with 0.4 mM MMS for 1 h (□, M), or with 0.4 mM MMS plus 10 mM Hu plus 100 μM AraC for 1 h, then with Hu plus AraC for 4 h, and then incubated in drug-free medium for various lengths of time (◇, MHAR). **(B)** Cells without treatment (Δ, Unt), or treated with various concentrations of $NaAsO_2$ for 4 h (○, As), or with 0.4 mM MMS for 1 h (□, M), or with 0.4 mM MMS plus 10 mM Hu plus 100 μM AraC for 1 h and then with Hu plus AraC for 4 h (■, MHA), or with 0.4 mM MMS plus 10 mM Hu plus 100 μM AraC for 1 h, then with Hu plus AraC for 4 h, and then without (◇, MHAR) or with various concentrations of $NaAsO_2$ for 4 h (◆, MHAAs). *$P < 0.05$ and **$P < 0.01$ according to Student's *t*-test when comparing MHAAs and MHAR. **(C)** A comparison of the effects of $NaAsO_2$ on DNA strand breaks generation and rejoining. The activity of break's generation (○) was from Figure 3B by using the equation: {(MHAAs − M)/(MHA − M)}×100%. The activity of breaks rejoining (Δ) was from Figure 4B by using the equation: {(MHA − MHAAs)/(MHA − MHAR)}×100%.

incubation of cells in drug-free medium; however, the increase was greater in As-containing medium. As increased the amount of DNA strand breaks in MMS-pretreated cells in a time- and concentration-dependent manner (Figure 2A and B). Similar observations have been reported in human blood and SV40-transformed fibroblasts (Hartmann and Speit, 1996). These results suggest that the excision of MMS-induced DNA adducts seems to be not or less severely inhibited by As than are DNA polymerization and ligation. If excision was more severely inhibited than the downstream steps, then fewer DNA breaks would have resulted. To test this hypothesis, the DNA polymerase inhibitors Hu and AraC were used to accumulate the DNA breaks resulting from the excision of MMS–DNA adducts. Hu at 10 mM plus 100 μM AraC did not induce a large number of DNA strand breaks, but they effectively accumulated the DNA strand breaks of MMS-treated cells (Figure 3A). The presence of As during the period of Hu–AraC treatment slightly reduced the level of DNA breaks (Figure 3B). The effect of As on the rejoining of DNA breaks resulting from treatment of MMS and Hu plus AraC was also studied. A large proportion of the DNA breaks resulting from the treatment with MMS and Hu plus AraC rejoined during a 4 h incubation in medium without Hu–AraC (Figure 4A). The presence of As during this period significantly retarded strand break rejoining (Figure 4B). The effects of As on DNA strand break generation and rejoining were compared in Figure 4C. The results showed that the rejoining of DNA strand breaks was more sensitive to As than the generation of DNA strand breaks.

Effects of As on DNA repair in cell extract

The effects of As on the various steps in DNA repair were also investigated using cell extracts. By incubating untreated or MMS-damaged plasmids in the excision reaction mixture, the plasmids could be time-dependently excised into open-circular form (Figure 5A). In both cases, the excision activity in cell extract was lost by heating cell extract at 90°C for 5 min. These results suggest that both the non-specific excision and MMS–DNA adduct-specific excision are enzymatic processes. After a 16 min incubation, the percentages of open-circular forms in untreated and MMS-treated plasmids were ~15 and 80% respectively. This results suggest that the MMS–

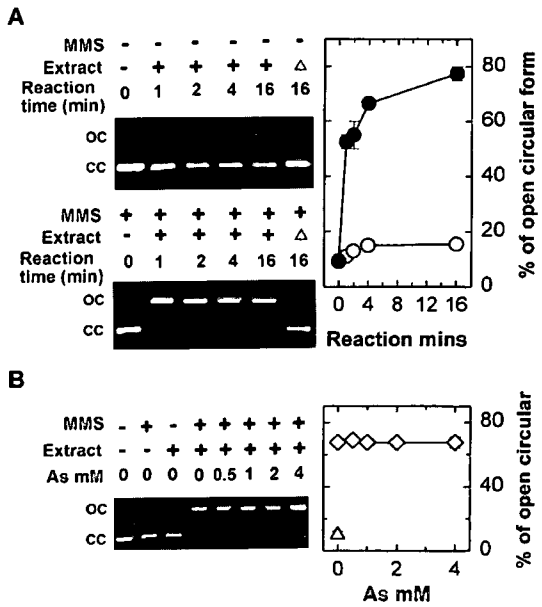

Fig. 5. (A) The excision of MMS-damaged plasmids *in vitro*. MMS-treated plasmids were incubated in an excision reaction mixture containing cell extracts as described in Materials and methods, for the time indicated. After incubation, the plasmids were extracted and separated by electrophoresis. Δ, the cell extract was heated at 90°C for 5 min. The quantitative results of excision of untreated (○) and MMS-treated plasmids (●) were presented in the plot chat. **(B)** Effects of $NaAsO_2$ on excision of MMS-damaged plasmids *in vitro*. Cell extracts in reaction buffer were incubated with various concentrations of $NaAsO_2$ for 30 min on ice and then the MMS-damaged plasmids were added and incubated for 2 min. The percentage of open-circular form in the reaction mixture without cell extract (Δ) and with $NaAsO_2$ treated cell extracts (◇) are shown in the plot chart. Mean and SE were calculated from three experiments.

DNA adduct-specific excision activity in cell extracts was far greater than the non-specific endonuclease activity. Prior to incubation in the excision reaction mixture, ~10% of the untreated plasmids were in open circular form. Incubation in

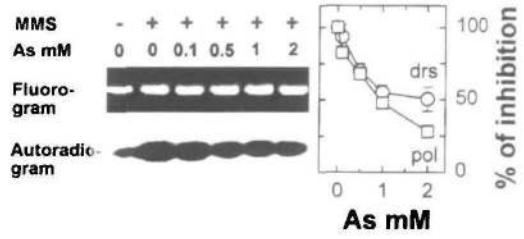

Fig. 6. Effects of $NaAsO_2$ on DNA repair synthesis and polymerization *in vitro*. Cell extracts in reaction buffer were incubated with various concentrations of $NaAsO_2$ for 30 min on ice and then the activities of DNA repair synthesis (O, drs) and DNA polymerization (□, pol) were measured as described in Materials and methods. The activity without $NaAsO_2$ treatment was taken as the control value. In the experiment of DNA polymerization the control activity was 2053 ± 154 c.p.m. Mean and SE were calculated from three experiments. A picture of DNA repair synthesis was presented in left panel.

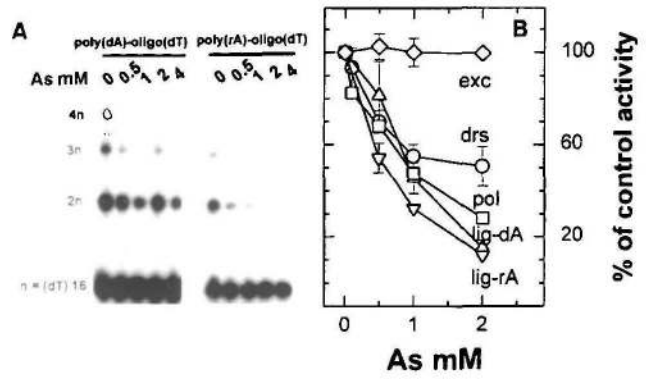

Fig. 7. **(A)** Effects of $NaAsO_2$ on DNA ligation *in vitro*. Cell extracts in reaction buffer were incubated with various concentrations of $NaAsO_2$ for 30 min on ice, and then the ligation activities of poly(dA)·oligo(dT) or poly(rA)·oligo(dT) were analysed as described in the Materials and methods section. **(B)** A comparison of the effects of $NaAsO_2$ on the excision of MMS-damaged DNA (◇, exc), DNA repair synthesis (O, drs), DNA polymerization (□, pol), the ligation of poly(dA)·oligo(dT) (Δ, lig-dA) and the ligation of poly(rA)·oligo(dT) (▽,lig-rA). The data are from Figures 5, 6 and 7. Mean and SE were calculated from three experiments.

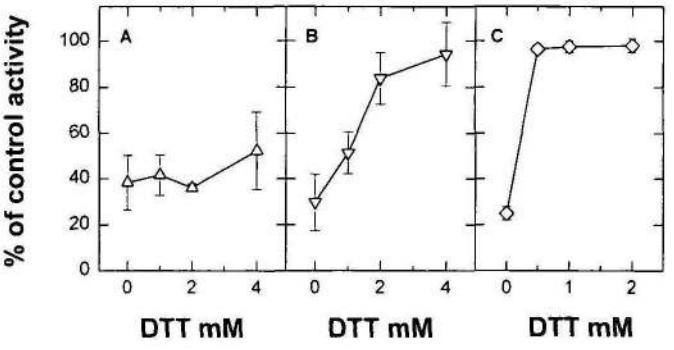

Fig. 8. Effects of DTT on As inhibition of **(A)** poly(dA)·oligo(dT) ligation, **(B)** poly(rA)·oligo(dT) ligation or **(C)** pyruvate dehydrogenase activity. For experiments (A) and (B), cell extracts were incubated with 1 mM $NaAsO_2$ for 30 min on ice, then DTT was added and incubation were continued for another 30 min. The DNA hybrids were added and the ligation assay was carried out. For experiment C, pyruvate dehydrogenase in potassium phosphate buffer was incubated with 0.2 mM $NaAsO_2$ for 30 min on ice, then DTT was added and incubation was continued for another 30 min. The activity of pyruvate dehydrogenase was then measured as described in Materials and methods. The activity of treatment without As was taken as the control. Mean and SE were calculated from two or three experiments.

9 mM MMS for 30 min at 30°C did not significantly increase the percentage of open circular forms. Preincubation of cell extract with As up to 4 mM did not significantly alter its efficiency in excising the MMS-damaged plasmid into the open-circular form (Figure 5B). This suggests that As has a very weak inhibitory effect on the MMS–DNA adduct-specific excision activity in cell extract.

The ability of cell extract to carry out DNA polymerization was measured by using DNase I-activated calf thymus DNA as a template for the incorporation of radiolabelled TTP into acid-insoluble materials. Preincubation of cell extract with As reduced the activity of DNA polymerization. The concentration of As required to inhibit 50% (IC_{50}) of DNA polymerization activity was ~1 mM (Figure 6).

The level of DNA repair synthesis in cell extract was measured by the incorporation of radiolabelled ATP into MMS-damaged plasmids. Specific incorporation of [α-^{32}P]dATP into plasmids treated with MMS, and a small amount of incorporation into the untreated plasmids, were observed (Figure 6). The incorporation into the untreated plasmids presumably came from contamination of nicked plasmids and/or nicks produced by the non-specific endonuclease in the cell extract as shown in Figure 5A. However, there was no incorporation into undamaged plasmids without cell extract, MMS-treated plasmids without extract, and MMS-treated plasmids with extract heated for 10 min at 90°C (Lynn *et al.*, 1994). Preincubation of cell extract with As reduced the activity of DNA repair synthesis. The IC_{50} of As was estimated to be ~2 mM (Figure 6).

The activity of DNA ligases in cell extracts was measured by the joining of oligo(dT) molecules hydrogen-bonded to poly(dA) or poly(rA). Preincubation of cell extract with As reduced the joining of poly(dA)·oligo(dT) and poly(rA)·oligo(dT) (Figure 7A). The IC_{50} of As of the ligation of poly(dA)·oligo(dT) and poly(rA)·oligo(dT) were estimated to be ~1 and 0.5 mM respectively (Figure 7B). A comparison of the relative sensitivity of the various steps in base-excision repair to As (Figure 7B) indicates that As inhibited these activities in cell extract in the following order: ligation of poly(rA)·oligo(dT) > ligation of poly(dA)·oligo(dT) ≈ DNA polymerization ≥ DNA repair synthesis > excision.

Effect of DTT on As-inhibited enzymatic activities

The activities of DNA ligases II and III can be measured using poly(rA)·oligo(dT) as the substrate (Lindahl and Barnes, 1992). As is known to inhibit the activity of pyruvate dehydrogenase by interacting with vicinal dithiol groups (Alkonyi *et al.*, 1976). Since there are vicinal dithiol groups in DNA ligase III (Wei *et al.*, 1995), the possibility that As interacts with protein thiols to inhibit DNA ligation was investigated. By incubating As with pyruvate dehydrogenase in potassium phosphate buffer, an As concentration-dependent inhibition curve was obtained, and the IC_{50} was estimated to be 0.2 mM (data not shown). Inhibition by As of poly(dA)·oligo(dT) ligation could not be restored by post-As incubation with DTT (Figure 8A). However, post-As incubation with DTT allowed the levels of ligation of poly(rA)·oligo(dT) and the activity of pyruvate dehydrogenase to recover to those without As treatment (Figure 8B and C).

Discussion

The inhibitory effects of As on the various base-excision repair steps in cell extract were compared in Figure 7B. However, the amount of cell extract was not the same in measuring the

activity of each repair step, and a higher concentration of cell extract protein may need a higher concentration of As to interact with. Thus, the amount of cell extract used in different assays may affect the IC_{50} values. To this end, we have calculated the final cell extract concentration in each reaction. The final concentrations of proteins in the reaction mixture of DNA repair synthesis, excision, polymerization and ligation of poly(dA)·oligo(dT) and poly(rA)·oligo(dT) of were 0.25, 0.065, 0.1, 0.1 and 0.1 μg protein/μl respectively. Taking ligation of poly(rA)·oligo(dT) as the standard, the protein concentrations in the reaction mixture of DNA repair, polymerization and ligation of poly(dA)·oligo(dT) were 2.5-, 1- and 1-fold, and the IC_{50} of As were 0.8, 1 and 1 mM respectively. Therefore, the ligation activity of poly(rA)·oligo(dT) was still relatively sensitive to As inhibition. Since As up to 2 mM had no apparent inhibitory effect on base excision, the IC_{50} could not be estimated.

The present results suggest that DNA polymerization and ligation are more sensitive than MMS–DNA adduct excision to As. This conclusion is derived from the following observations: (i) post-treatment with As enhanced the accumulation of DNA breaks in MMS-damaged cells (Figure 2). There would be fewer DNA breaks if the excision step was the most sensitive step to As inhibition; (ii) as had a stronger inhibitory effect on the rejoining of DNA breaks accumulated by Hu and AraC than on the generation of DNA breaks accumulated by Hu plus AraC in MMS-damaged cells (Figure 4C). The rejoining includes DNA polymerization and DNA ligation; (iii) the curve of As inhibition of DNA repair synthesis was similar to that of DNA polymerization at concentrations of As <1 mM (Figure 6); (iv) excision of MMS-damaged plasmids into open-circular form (Figure 5B) was relatively insensitive to As in comparison with DNA polymerization and ligation (Figures 6 and 7).

The insensitivity of base excision steps to As in rodent cells is in contrast to the apparent inhibitory effect of As in the excision of UV-irradiated DNA in both rodent (Wang *et al.*, 1994) and human cells (Hartwig *et al.*, 1997). This could be due to different proteins and endonucleases being involved in base excision (Seeberg *et al.*, 1995) and nucleotide excision (Aboussekhra *et al.*, 1995).

Figure 7 indicates that the ligation of poly(rA)·oligo(dT) was more sensitive to As inhibition than the ligation of poly(dA)·oligo(dT). Since all four types of DNA ligases can use poly(dA)·oligo(dT) as a substrate (Wei *et al.*, 1995), only DNA ligases II and III can use poly(rA)·oligo(dT) as a substrate (Lindahl and Barnes, 1992): this suggests that DNA ligases II and/or III could be highly sensitive to As. As is known to exert its toxic effects by reacting primarily with thiols, especially vicinal dithiols (Aposhian, 1989; Aposhian and Aposhian, 1989). As inhibition of pyruvate dehyrogenase is known to stem from its binding to the vicinal dithiol groups of pyruvate dehydrogenase (Alkonyi *et al.*, 1976). The result showing that As inhibition of pyruvate dehydrogenase activity, and poly(rA)·oligo(dT) ligation could be removed by DTT, suggests that As may interact with vicinal dithiol groups of proteins participating in poly(rA)·oligo(dT) ligation. Zinc finger, which contains vicinal dithiols, has been predicted to exist in DNA ligase III (Wei *et al.*, 1995). It is tempting to surmise that As interacting with vicinal dithiols of DNA ligase III may be the reason why the ligation of poly(rA)·oligo(dT) is particularly sensitive to As. Poly(dA)·oligo(dT) ligation was less sensitive to As and the fact that As inhibition could not be removed by DTT seems to support this hypothesis because 85% DNA ligation activities come from DNA ligase I (Lindahl and Barnes, 1992), which has no putative zinc finger or vicinal dithiol groups in its amino acid sequence (Barnes *et al.*, 1990). Using atomic absorption, we have previously determined that, when treating CHO-K1 cells with 100 μM arsenite for 4 h, ~35.9 ng of total arsenic was accumulated/10^6 cells (Huang *et al.*, 1993). We estimated the volume of a CHO-K1 cell under a microscope and came to a value of ~560 μm^3, which is similar to the value reported by Korte and Yasui (1993). By this calculation, the intracellular concentration of total arsenic is ~857 μM after treating CHO-K1 cells with 100 μM As for 4 h. This intracellular total arsenic concentration is more than the IC_{50} of As inhibition of poly(rA)·oligo(dT) ligation in cell extract (which is 500 μM). Therefore, As inactivation of DNA ligase II and/or III in cell extract could also occur within cells. Since DNA ligase III has been proposed to play a role in DNA ligation in base excision repair (Caldecott, 1994), inhibition of DNA ligase III may be a pathway in the retardation of the rejoining of MMS-induced DNA strand breaks.

The present results suggest that DNA strand break rejoining is more sensitive to As than base excision, and the interaction of As with the vicinal dithiols of DNA ligase III may play a role in As inhibition of DNA repair in MMS-treated cells. However, the possibility that As may inhibit DNA repair by other mechanisms is not excluded. Several recent studies suggest that As may generate reactive oxygen species (ROS) during its metabolism in cells (Lee and Ho, 1995; Wang *et al.*, 1996; Yamanaka *et al.*, 1991). ROS have been shown to induce oxidative DNA damage (Wagner *et al.*, 1992) and to inhibit DNA repair (Hu *et al.*, 1995). Thus, arsenic may affect cellular DNA repair by creating more DNA damage or inactivating repair enzymes through the generation of ROS. As has also been shown to inhibit ATP generation (Yih *et al.*, 1991), and to induce DNA–protein cross-linking (Dong and Luo, 1993) that may also affect DNA repair. Cellular DNA repair may require gene activation that could be modulated by As through increasing AP-1 binding activity (Bergelson *et al.*, 1994), increasing protein phosphorylation (Trigon and Morange, 1995) or decreasing protein phosphatase activity (Huang *et al.*, 1995). The observations that protein synthesis inhibitors can suppress the coclastogenicity of As (Huang *et al.*, 1986), and that the activity of poly(rA)·oligo(dT) ligation induced by *N*-methyl-*N*-nitrosourea can be suppressed by As (Li and Rossman, 1989b) seem to support this hypothesis.

Acknowledgements

We thank Drs T.C.Lee, R.Wu and J.L.Yang for their valuable suggestions and Mr Dan Chamberlin for English editorial service. This work was supported by grants from the National Science Council, ROC (NSC 86-2311-B-001-074).

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Received on January 14, 1997; accepted on May 28, 1997