

Induction of Arsenite Tolerance and Thermotolerance by Arsenite Occur by Different Mechanisms

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Both V79 and As/R28A cells (an arsenite-resistant Chinese hamster V79 cell variant) show increased resistance to toxic concentrations of arsenite after pretreatment with a nontoxic concentration. The induced tolerance can be completely inhibited by actinomycin D or cycloheximide. Pretreatment with a nontoxic heat shock (45°C, 10 min) resulted in a clear increased thermotolerance in both cell lines but failed to induce arsenite tolerance in either cell line. Pretreatment with arsenite induced a thermotolerance in V79 cells but not in As/R28A cells. These results are consistent with a model whereby the signal for induction of arsenite tolerance involves binding of arsenite to a protein effector which is amplified in the As/R28A line, thereby preventing action of arsenite in the regulation of heat shock factor which induces the heat shock response. — Environ Health Perspect 102(Suppl 3):97–100 (1994).

Key words: Chinese hamster, cell lines, arsenic, heat shock toxicity

Introduction

Arsenic is a common environmental toxicant. It is widely distributed throughout the earth and may be released as an aerosol via industrial processes such as the smelting of other metals and power generation from coal. Although arsenic is a well documented human carcinogen, most animal carcinogenesis bioassays (1) and short term gene mutagenesis assays (2–4) yield negative results. It can induce chromosome aberrations (5,6), sister chromatid exchanges (6,7), gene amplification (8,9) and cell transformation (10). The mechanism of its carcinogenesis is thus not clear.

A potentially important aspect of arsenite action is its great ability to induce a variety of cellular responses. Arsenite is able to induce expression of a variety of genes, including *c-fos* (11), multidrug resistance (MDR1) (12) and heat shock genes

(13–15). We previously reported that arsenite tolerance was inducible by low concentrations of arsenite or antimonite in V79 cells and in As/R28A cells, a Chinese hamster V79 variant established in our laboratory showing a stable arsenite resistance (16). Arsenite tolerance increased with pretreatment time, reached a plateau at 6 to 8 hr, and then remained stable up to at least 20 hr, suggesting that *de novo* mRNA and protein synthesis were required. This is further supported by our present finding that inducible arsenite tolerance can be completely inhibited by actinomycin D or cycloheximide. In this article we also compare heat shock and sodium arsenite as inducers of thermotolerance and arsenite tolerance in V79 cells and in the variant line As/R28A.

Materials and Methods

Cell Culture, Treatments, and Cytotoxicity Assay

Chinese hamster V79 cells were obtained from American Type Culture Collection. The arsenite-resistant cell line As/R28A was established in this laboratory previously (16). Both V79 cells and As/R28A cells were maintained in F12 medium containing 5% fetal calf serum, 100 units/ml of penicillin and 100 µg/ml of streptomycin, without arsenite, as a monolayer culture. Cytotoxicity was determined by colony formation. Exponentially growing cells were trypsinized, counted, and replated at 500 cells per 60 mm dish.

Arsenite was added to the medium immediately after seeding, and remained in the medium throughout the incubation. The heat treatment was carried out in a 45°C water bath immediately after seeding of the cells using 25 ml flasks instead of petri dishes. The cells were incubated for 10 days without changing medium, fixed with methanol, and stained with Giemsa. The

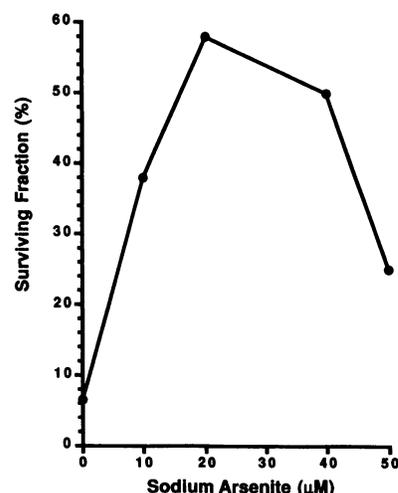


Figure 1. Inducible arsenite tolerance by various concentration of sodium arsenite. Exponentially growing mass cultures of As/R28A cells were pretreated with various concentrations of sodium arsenite for 16 hr. The cultures were then trypsinized, seeded at a density of 500 cells/dish, and challenged with 100 µM arsenite. The arsenite remained in the medium throughout 10 days' incubation without changing the medium.

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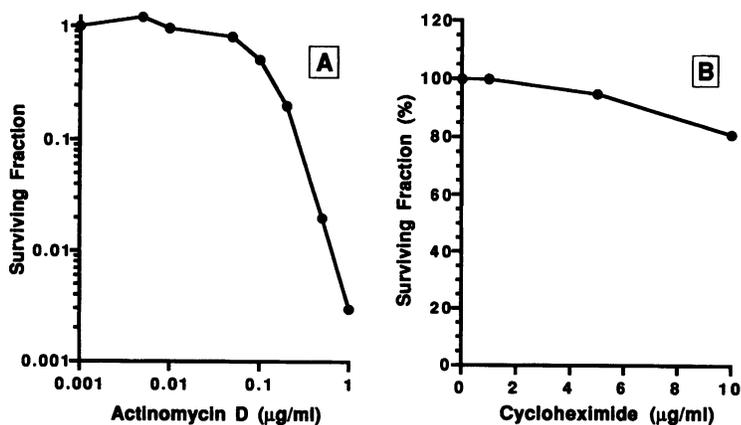


Figure 2. (A) Cytotoxicity of actinomycin D to As/R28A cells. As/R28A cells were treated with different concentrations of actinomycin D for 8 hr. The cultures were washed, trypsinized, and reseeded for clonal survival at the same dilution into 60 mm dishes. (B) Cytotoxicity of cycloheximide to As/R28A cells. The same procedure as (A) was used except that cycloheximide was used as the test compound.

number of colonies is scored and survival is defined as the fraction of the colony number in the treated group to the control group.

Inducible Responses by Heat Shock and Arsenite

Exponentially growing mass culture of V79 cells or As/R28A cells in complete F12 medium were pretreated with subtoxic concentrations of sodium arsenite (10 µM for V79 and 20 µM for As/R28A cells) for 16 hr at 37°C or with heat shock at 45°C (10 min for both cell lines) followed by a 6-hr incubation at 37°C. The cells were trypsinized, seeded for cytotoxicity as above, and challenged with either heat shock or arsenite. The cells were incubated, fixed, and stained as described above.

Inhibition of Inducible Arsenite Resistance in As/R28A Cells by Actinomycin D or Cycloheximide

To choose a nontoxic dose of actinomycin D or cycloheximide, exponentially growing As/R28A cells were trypsinized and 3×10^5 cells/60 mm dish were seeded. The cultures were incubated for 48 hr to reach subconfluence and then were treated with various concentrations of actinomycin D or cycloheximide for 8 hr. The cells were washed carefully twice with prewarmed serum-free medium, trypsinized, and seeded at the same dilution so that dishes from each dose point of actinomycin D or cycloheximide contained the same number of cells. The dishes were incubated for 10 days. Colonies were fixed, stained, and counted as described above.

To inhibit the inducible arsenite resistance in As/R28A cells, the exponentially growing mass culture was treated with actinomycin D and cycloheximide followed 15 min later with sodium arsenite (15 µM) for 8 hr. The culture was washed with prewarmed serum-free medium, trypsinized, replated, and challenged with various concentrations of sodium arsenite. The cells were incubated for 10 days. Colonies were fixed, stained, and counted as described above.

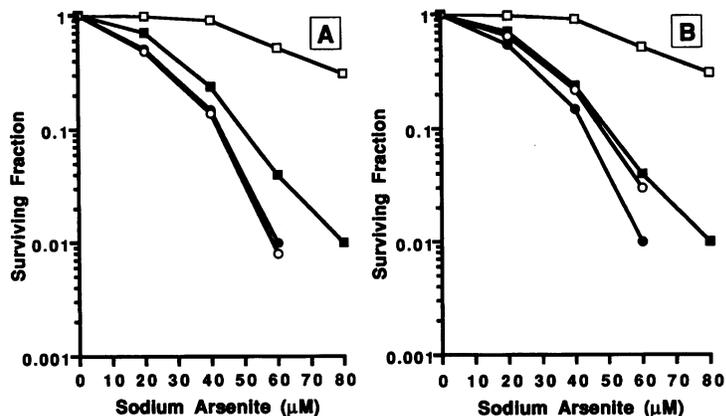


Figure 3. (A) Inhibition of inducible arsenite resistance in As/R28A cells by actinomycin D. Exponentially growing mass culture of As/R28A cells without any pretreatment (■), with 8 hr pretreatment in 15 µM sodium arsenite (□), 15 µM sodium arsenite plus 0.01 µg/ml actinomycin D (○), and 0.01 µg/ml actinomycin D (●) were trypsinized, replated, and challenged with various concentrations of arsenite. The arsenite remained in the medium throughout 10 days' incubation without change of medium. (B) Inhibition of inducible arsenite resistance in As/R28A cells by cycloheximide. Exponentially growing mass culture of As/R28A cells without any pretreatment (■), with 8 hr pretreatment of 15 µM sodium arsenite (□), 15 µM sodium arsenite plus 1 µg/ml cycloheximide (○), and 1 µg/ml cycloheximide (●) were trypsinized, replated, and challenged with various concentrations of arsenite. The arsenite remained in the medium throughout 10 days' incubation without change of medium.

Results

Inducible Arsenite Tolerance by Sodium Arsenite Pretreatment in As/R28A Cells Using *de novo* mRNA and Protein Synthesis

Cells were pretreated for 16 hr with various concentrations of arsenite and then challenged with 100 µM arsenite, which alone causes 6.5% survival (Figure 1). Arsenite tolerance increases with pretreatment up to 20 µM, and then declines, probably as a result of toxicity from the pretreatment (16). The maximal induced tolerance is about 9 times higher than that of the untreated control.

Figures 2A and 2B show the cytotoxicity of an 8 hr treatment with actinomycin D or cycloheximide, respectively. It is clear that actinomycin D is much more toxic to the cells than cycloheximide in the same concentration range. Nontoxic doses of actinomycin D (0.01 µg/ml) and cycloheximide (1 µg/ml) were chosen for the following experiments. Figure 3 demonstrates the complete inhibition of inducible arsenite tolerance by either actinomycin D (A) or cycloheximide (B). Although nontoxic doses of both compounds were chosen, pretreatment with either compound followed by arsenite challenge always results in a slightly lower survival, compared with arsenite alone.

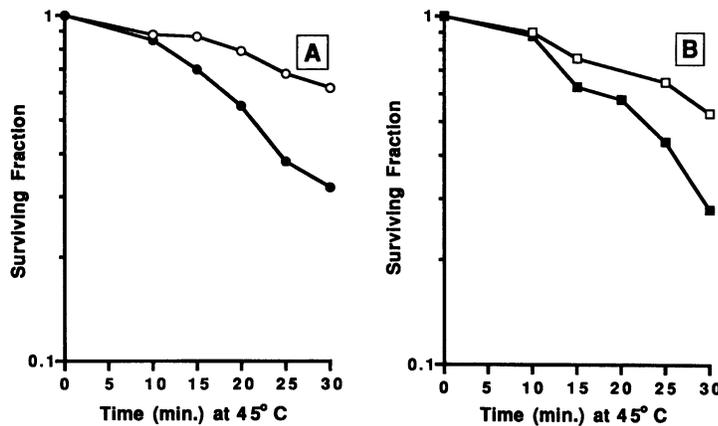


Figure 4. (A) Inducible thermotolerance in V79 cells by heat pretreatment. Exponentially growing mass cultures of V79 cells with (○) and without (●) nontoxic dose of heat pretreatment (45°C, 10 min) were incubated at 37°C for 6 hr. The cultures were then trypsinized, replated, and challenged with heat shock for various periods of time. The cells were incubated at 37°C for 10 days to form colonies. (B) The same procedure was used except that As/R28A cells with (□) and without (■) heat pretreatment were used.

Thermotolerance and Inducible Thermotolerance by Nontoxic Heat Pretreatment

Figure 4 shows the survival curves for treatment at 45°C in V79 and As/R28A cells. It is clear that As/R28A cells are not more thermotolerant than V79 cells. After induction by a nontoxic heat treatment (10 min), both V79 and As/R28A cells show a clear increase in thermotolerance with approximately equal enhancement in survival.

Arsenite Induces Thermotolerance in V79 Cells but Not in As/R28A Cells

In order to determine whether arsenite can induce thermotolerance in both cell lines, cells were pretreated with subtoxic concentrations of arsenite and then challenged with heat (45°C). Figure 5A shows that increased thermotolerance can be induced by arsenite pretreatment in V79 cells. In contrast, As/R28A cells become more sensitive to heat if pretreated with sodium arsenite (Figure 5B), using the same dose

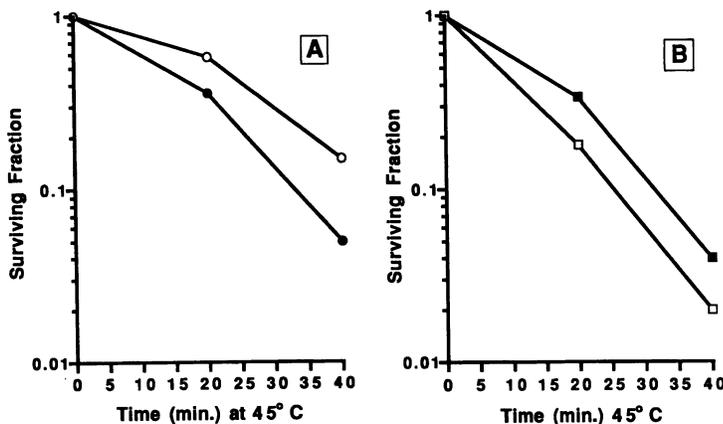


Figure 5. The effects of arsenite pretreatment on thermotolerance in V79 and As/R28A cells. (A) Inducible thermotolerance by arsenite in V79 cells. Exponentially growing mass cultures of V79 cells with (○) or without (●) arsenite pretreatment (10 μM, 16 hr) were trypsinized, replated, and challenged with heat shock for various periods of time. The cells were then incubated at 37°C for 10 days to form colonies. (B) Inhibition of heat tolerance by arsenite pretreatment in As/R28A cells. The same procedure was used except that As/R28A cells with (□) and without (■) sodium arsenite pretreatment (20 μM, 16 hr) were used.

which induced arsenite tolerance as described above (Figure 3).

Heat Shock Pretreatment Does Not Induce Arsenite Tolerance in V79 and As/R28A Cells

It was also of interest to determine whether arsenite tolerance was induced by heat. Using the same heat shock pretreatment (10 min at 45°C) shown to induce thermotolerance (Figure 4), V79 and As/R28A cells were then challenged with arsenite. No significant enhancement in survival was observed in either V79 or As/R28A cells (Figure 6).

Discussion

The As/R28A cell line is a subline of V79 cells showing a stable arsenite-resistant phenotype. Both V79 and As/R28A cells also show an inducible arsenite tolerance. The extent of the increased tolerance is greater in As/R28A cells than in V79 cells (16). The As/R28A line was established by selecting arsenite-resistant colonies in progressively increasing concentrations of arsenite. After maintenance for more than one year in the absence of arsenite, the cell line retains its arsenite-resistant phenotype. Because arsenite is known to induce heat shock proteins, it was of interest to determine whether the arsenite resistant phenotype of As/R28A was due to alterations in the heat shock response. When its thermotolerance was compared with that of the parental V79 cells, no significant difference was found (Figure 4). The arsenite-resistant phenotype of As/R28A, therefore, is not due to changes in heat shock proteins which confer thermotolerance. It was further found that As/R28A lost the ability to induce thermotolerance by arsenite (Figure 5B), which was observed in its parental V79 cell line (Figure 5A) and in other cell lines (17–19). This is not due to an inability to induce the heat shock response, since As/R28A cells retain the ability to induce thermotolerance by heat to the same extent that V79 cells do (Figure 4).

Although pretreatment of As/R28A cells with arsenite was unable to induce thermotolerance, it did induce a clear arsenite tolerance (Figure 1). The inducible arsenite tolerance increases with the pretreatment concentration of arsenite (Figure 1) and the pretreatment time (16). Since it required about 8 hr for As/R28A cells to reach maximal inducible arsenite tolerance, *de novo* mRNA and protein synthesis appeared to be required for the inducible arsenite tolerance. This hypothesis was further supported by the finding that

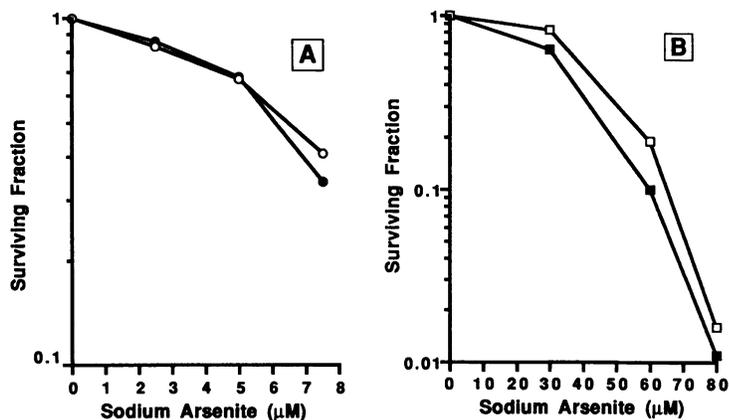


Figure 6. The effects of heat pretreatment on arsenite tolerance in V79 (A) and As/R28A (B) cells. The same procedure was used as described in Figure 5 except that the cells with (●) or without (○) heat-pretreatment were challenged by sodium arsenite instead of heat.

inducible arsenite tolerance was completely inhibited by either actinomycin D (Figure 3A) or cycloheximide (Figure 3B). It has been reported that cycloheximide markedly suppressed arsenite-inducible thermotolerance but not heat-inducible thermotoler-

ance (17–19), suggesting that different mechanisms might be involved for the induction of thermotolerance by heat shock and arsenite.

The mechanism for the constitutive and inducible arsenite tolerance in As/R28A

cells is not known. We have previously reported cross-tolerance and cross-inducibility between arsenite and antimonite resistance (16). The plasmid mediated arsenite-resistance of *E. coli*, which is mediated by an arsenite efflux pump, also shows cross-resistance and cross-inducibility to antimonite (20). These similarities between observations in *E. coli* and our results in Chinese hamster cells lead us to speculate that mammalian cells might also have an arsenic efflux pump and suggest the following working hypothesis:

As a result of up-regulation or gene amplification, As/R28A cells contain increased levels of an arsenite-binding protein, which may be part of an efflux pump. When this protein is bound to arsenite, it activates its own transcription. The affinity of the hypothetical arsenite-binding protein for arsenite is probably very strong. In As/R28A cells this would prevent arsenite's access to other cellular targets, (such as the heat shock factor) thereby preventing the signals needed to regulate other genes which are normally inducible by arsenite.

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