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Study of *in vitro* cytotoxicity of arsenocholine, a trimethyl arsenic compound in seafood†

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We examined the *in vitro* cytotoxic effects of an organic arsenic compound contained in seafood, *viz.* the trimethyl (2-hydroxyethyl)-arsonium cation, or arsenocholine (AsCho), on some murine immune effector cells, such as splenocytes, thymocytes, Peyer's patch lymphocytes, peritoneal macrophages and bone marrow (BM) cells using synthesized pure material. We found that AsCho had no cytotoxicity on most immune effector cells, even at concentrations over 10 mmol dm^{-3}, and it slightly but significantly enhanced the viability of BM cells at doses over 100 μmol dm^{-3}. This biological effect of AsCho on BM cells might be direct rather than due to autocrine mechanisms mediating some factors secreted by AsCho-stimulated BM cells, because the culture supernatants of BM cells pre-stimulated with AsCho did not influence the viability of other fresh BM cells. It is interesting that this unique biological effect was found in AsCho, an organic arsenic compound contained in some marine animals that are ingested daily as seafood in many countries. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: arsenocholine; arsenic; bone marrow cell; immunotoxicity; seafood; cytotoxicity

INTRODUCTION

Arsenic has had the reputation of being a poison for centuries.[1] Epidemiological studies have shown that the inorganic arsenical, arsenite, has high toxicity; its LD_{50} in mice is 35 mg kg^{-1},[2] and it has also been shown to be carcinogenic in experimental animals and humans.[1] It has been previously reported that marine animals, such as clam, conch, crab, lobster, shrimp and fish, which are ingested daily as seafood in many countries, contain very high concentrations of arsenicals, ranging from about 4 to 80 μg g^{-1},[3] and these arsenicals are generally in the form of water-soluble organic arsenic compounds. The limit for arsenic in drinking water in Japan (10 μg dm^{-3}), is largely based on inorganic arsenicals, and if this limit were applied to seafood, as 10 ng g^{-1}, most of the seafood would be deemed unfit for consumption, having contents often 1000 times this concentration.[1] This finding has caused great concern with respect to the health of people who often ingest considerable amounts of seafood. It is necessary, therefore, to investigate the effects of organic arsenic compounds contained in marine animals on living systems; however, there have been only a few reports about them because sufficient amounts of pure compounds for biological experiments have not been obtained.

In 1985, Kaise *et al.*[2] first reported on the acute toxicity of arsenobetaine (AsBe), the trimethyl (carboxymethyl) arsonium zwitterion (which was found by Edmonds *et al.*[4] in 1977 to be a major organic arsenic compound in marine animals), using synthetic pure material, and clarified that it had no acute toxicity in murine models, even at more than 10 g kg^{-1}, when it was administered orally. Subsequently, we observed that *in vitro* cytotoxicity of AsBe was very weak compared with that of inorganic arsenicals in cultured murine splenocytes, macrophages, thymocytes, Peyer's patch (PP) lymphocytes and bone marrow (BM) cells.[5,6] These data imply that AsBe has no toxicity on mammalian living systems. Norin and Christakopoulos[7] (1982) and

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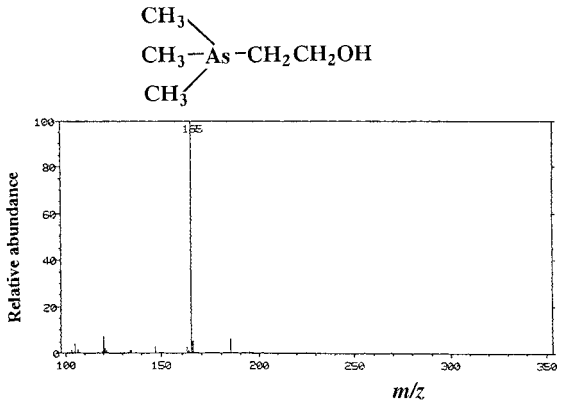

Figure 1. Primary structure of AsCho and FAB-MS of synthesized AsCho. FAB-MS was performed using a JMS DX-300 mass spectrometer (JEOL Co., Tokyo) equipped with FAB ion source and xenon atoms at 6 keV. The FAB-MS of AsCho showed *m/z* 165 $[M]^+$.

Shiomi *et al.*[8] (1987) detected another water-soluble trimethyl arsenic compound, namely arsenocholine (AsCho), from some kinds of shrimp[7] and conch,[8] and the chemical structure of this new trimethyl arsenic compound was found to be trimethyl (2-hydroxyethyl)-arsonium cation (see Fig. 1).[7,8] In 1992, Kaise *et al.*[9] investigated the effects of AsCho on living systems using synthetic pure material, and found that AsCho had weak but significant acute toxicity on murine models. The LD_{50} values of AsCho on mice were 187 mg kg^{-1} and 6.54 g kg^{-1} when administered intravenously and orally respectively.[9] This report means that AsCho is slightly but significantly toxic in mammals, which is different from AsBe; however, there has been no more data about the toxicity of AsCho.

In this study, we examined whether AsCho has any toxicological and/or biological effects on mammalian cells using murine immune effector cells *in vitro*. We found that AsCho was not toxic to these cells, and, interestingly, it slightly augmented the viability of BM cells.

EXPERIMENTAL

Reagents

Sodium arsenite was purchased from the Wako Pure Chemical Co. (Osaka, Japan), and was recrystallized twice from water. Trimethylarsine oxide was prepared from trimethylarsine using H_2O_2 as described elsewhere,[10] and was recrystallized twice from benzene. AsCho was synthesized from trimethylarsine reacted with 2-bromo-ethanol,[11] and was recrystallized twice from acetonitrile.[9] It gave white prism crystals, melting point 256–258 °C, and its structure was confirmed by ^1H NMR, ^{13}C NMR and fast-atom bombardment mass spectrometry (FAB-MS) (see Fig. 1). The purities of these arenicals were >99.9% as determined by thin-layer chromatography and gas chromatography (GC)–MS. Lipopolysaccharide (LPS) contamination of AsCho was $<1.7 \times 10^{-7}$% (w/w) detemined by the endotoxin-specific limulus test. Choline chloride was purchased from Wako. These reagents were dissolved in culture medium and were filtered through a 0.2 μm filter when added to the cells.

Mice

Male CDF_1 (BALB/c × DBA/2) mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The mice were used at 6–8 weeks of age and were bred under specific pathogen-free conditions. They were fed laboratory chow and given sterilized water *ad libitum* and kept in a temperature-controlled room (22 °C) in groups of ten.

Cells

Immune effector cells were obtained from mice anesthetized by ethyl ether. Single-cell suspensions of splenocytes[5] or thymocytes[12] were prepared by teasing the spleen or thymus with a sterilized steel screen in Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co., Tokyo). A single-cell suspension of PP lymphocytes was prepared by cutting the PP with a scalpel blade and then gently teasing it with two slide glasses in MEM containing 10% heat-inactivated fetal calf serum (FCS–MEM; LPS contamination of FCS was <6 pg cm^{-3} measured by the limulus test).[12] Peritoneal macrophages (PMs)[5] were collected either by washing the peritoneal cavity or by bronchial lavage, using Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.05% ethylenediaminetetraacetate. BM cells were prepared by flushing the femoral shafts using MEM.[12] These immune effector cells were washed twice with MEM and resuspended in FCS–MEM.

Assay for viability of cells

Lymphocytes (splenocytes, thymocytes and PP lymphocytes; 2.5×10^5 cells/100 μl per well), PMs (5×10^4 cells/100 μl per well) or BM cells (5×10^5 cells/100 μl per well) were incubated with AsCho (~10 mmol dm^{-3}) or arsenite (~20 μmol dm^{-3}) in flat-bottomed 96-well tissue culture plates for 48 h (PMs) or 72 h (lymphocytes and BM cells) at 37 °C in a CO_2 incubator with FCS–MEM. The viability of the cells was determined by measuring live cells using the AlamarBlue (AB) assay, which is similar to the MTT assay.[13] Briefly, 6 h before the end of the incubation, 10 μl/well of AB solution (Iwaki Glass Co., Chiba, Japan) was added directly to the 96-well plates, and the absorbance at 570 nm (reference 630 nm) was measured using a microplate reader, model 550 (Bio-Rad Laboratories, Hercules, CA). Arsenicals themselves did not affect the absorbance of the AB solution, even at a concentration over 40 mmol dm^{-3}.

Arsenic analysis

BM cells (1×10^7/well) on flat-bottomed 12-well tissue culture plates were incubated with 10 mmol dm^{-3} AsCho

Table 1. *In vitro* cytotoxicity of AsCho on immune effector cells[a]

	AsCho (10 mmol dm^{-3})	Reagents[b]	Metabolite integrity (% control)[c]
Splenocytes	−	−	100.2 ± 1.5
	+	−	90.7 ± 4.6[d]
	−	+ Con A	100.1 ± 4.1
	+	+ Con A	92.5 ± 0.4
	−	+ LPS	100.0 ± 3.8
	+	+ LPS	75.3 ± 1.9[e]
Thymocytes	−	−	100.2 ± 1.6
	+	−	98.3 ± 4.8
	−	+ Con A	100.2 ± 1.5
	+	+ Con A	96.6 ± 3.4
PP lymphocytes	−	−	102.6 ± 14.5
	+	−	78.1 ± 23.6
PMs	−	−	99.7 ± 7.7
	+	+	114.5 ± 5.8
	−	+ BSO	100.0 ± 6.1
	+	+ BSO	116.2 ± 5.1
BM cells	−	−	99.8 ± 0.0
	−	+	122.4 ± 0.4[f]

[a] Immune effector cells isolated from CDF_1 mice were incubated with AsCho (10 mmol dm^{-3}) or medium alone for 72 h (splenocytes, thymocytes, PP lymphocytes and BM cells) or 48 h (PMs), and the viability of cells was measured by AB assay. One representative experiment out of three similarly performed is given. Results are expressed as arithmetic mean plus/minus the standard error of the mean (SEM) ($n = 5$).

[b] Splenocytes, thymocytes and PP lymphocytes were incubated with AsCho or medium alone in the presence or absence of submitogenic concentrations of T-lymphocyte mitogen, concanavalin A (Con A; 2.5 μg cm^{-3}) or B-lymphocyte mitogen, LPS (50 μg cm^{-3}). PMs were pre-treated with or without BSO (1 mmol dm^{-3}) for 24 h, and continuously incubated with AsCho or medium alone.

[c] Percentage absorbance with respect to control cells treated with same reagents and incubated with medium alone.

[d] $p < 0.05$, comparison with control cells treated with the same reagent and incubated with medium alone.

[e] $p < 0.01$.

[f] $p < 0.001$.

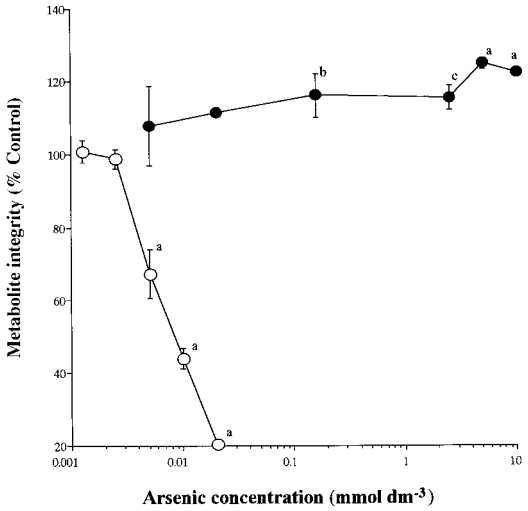

Figure 2. Effect of arsenicals on the viability of BM cells. BM cells isolated from CDF_1 mice were incubated with various doses of arsenite (○), AsCho (●) or medium alone for 72 h at 37 °C, and the viability of cells was determined by AB assay. One representative experiment out of five similarly performed is given. Results are expressed as the arithmetic mean plus/minus SEM of triplicate dishes. [a] $p < 0.001$ comparison with control BM cells incubated with medium alone. [b] $p < 0.05$. [c] $p < 0.01$.

or medium alone for 72 h at 37 °C. After incubation, the cells were rinsed three times with PBS, lysed with 0.5 ml of 2 mol dm^{-3} NaOH and transferred into polypropylene tubes. Cell lysates in tubes were heated at 80 °C for 2 h in a water bath, cooled and neutralized with 1 mol dm^{-3} HCl. The aqueous solution was made up to a volume of 3.5 ml and filtered through a 0.2 μm filter. The sample solutions were introduced to the fully automated continuous arsine generation system as described in a previous paper.[14] Briefly, 0.6 mol dm^{-3} HCl and 2% $NaBH_4$ in 0.2 mol dm^{-3} NaOH were continuously pumped through the mixing coil of the arsine generator at 6 ml min^{-1}. The arsines generated were collected in a U-shaped tube by cooling in liquid nitrogen and flashed into the gas chromatograph–Mass spectrometer equipped with a cryofocus system using selected ion monitoring. Methyl, dimethyl or trimethyl arsenic compounds were identified as methylarsine, dimethylarsine or trimethylarsine respectively.

Statistics

Statistical evaluations in some experiments were performed using Student's *t*-test. A value of $p < 0.05$ was considered significant.

RESULTS

Effect of AsCho on the viability of splenocytes, thymocytes, PP lymphocytes and PMs *in vitro*

Table 1 shows the effect of AsCho on the viability of splenocytes, thymocytes, PP lymphocytes and PMs *in vitro*. As to the results, AsCho was less toxic to most cells even at concentrations over 10 mmol dm^{-3}. Exceptionally, 10 mmol dm^{-3} AsCho showed weak but significant cytotoxicity on splenocytes when they were incubated with a B-lymphocyte mitogen, LPS, although AsCho was not cytotoxic at concentrations of 0.3–5 mmol dm^{-3} (data not shown). In the case of PMs, AsCho also had no cytotoxicity, even at a concentration of 10 mmol dm^{-3}, when PMs were incubated with or without a specific gluthatione synthase inhibitor, L-buthionine-[*S,R*]-sulfoximine (BSO; Sigma Chemical Co., St Louis, MO), which can deplete the cellular reduced glutathione (GSH) levels.

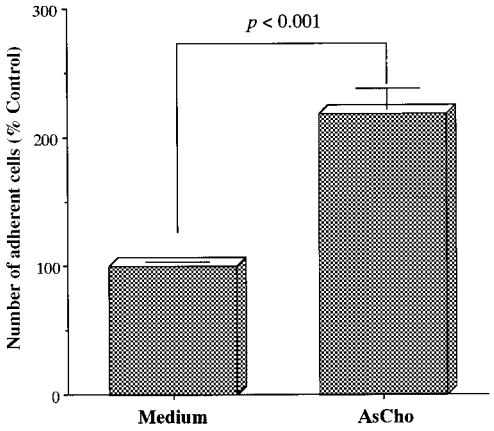

Figure 3. Effect of AsCho on the adherent ability of BM cells. BM cells isolated from CDF_1 mice were incubated with 10 mmol dm^{-3} AsCho or medium alone (control) on 96-well tissue culture plates for 72 h at 37 °C. After the incubation, the wells were washed twice by warmed PBS to remove any nonadherent cells, and the remaining adherent cells on the culture plates were stained using the Diff-Quik stain kit (Kokusai Shiyaku Co., Hyogo, Japan) and counted under the microscope. Results are expressed as arithmetic mean plus/minus SEM of three separate experiments performed in triplicate ($n = 9$).

Effect of AsCho on BM cells *in vitro*

As shown in Table 1, 10 mmol dm^{-3} AsCho interestingly enhanced the viability of BM cells. Figure 2 shows the dose response effect of AsCho on the viability of BM cells compared with that of sodium arsenite. The results show that sodium arsenite had strong cytotoxicity on BM cells; its LC_{50} (the concentration that reduced the number of surviving cells to 50% of that in untreated controls) was 5 μmol dm^{-3}; conversely, AsCho slightly but significantly increased the viability of BM cells in a dose-dependent manner. In approximate 1.3-fold increase in the viability of BM cells compared with that of control cells cultured with medium alone was observed when cells were incubated with over 100 μmol dm^{-3} AsCho for 72 h. This increasing effect was not observed when cells were incubated with trimethylarsine oxide [viability of cells (metabolite integrity; percent control) was 84.1 ± 1.3% ($n = 3$) at 10 mmol dm^{-3}] or choline chloride [103.8 ± 13.1% ($n = 3$) at 10 mmol dm^{-3}], which were added as constituents of AsCho, and the simultaneous addition of trimethylarsine oxide and choline chloride also did not have an influence on the viability of BM cells (data not shown).

AsCho significantly increased the number of adherent cells from immature BM cells. After the 72 h incubation with AsCho, many spreading cells appeared from BM cells; over twofold, compared with control cells cultured with medium alone (Fig. 3). From morphological investigations, the form of the cytoplasm and nuclei of these spreading adherent cells showed the characteristics of granulocytes and/or macrophages.

Table 2. Effect of culture supernatants of BM cells incubated with AsCho on the viability of fresh BM cells[a]

Culture supernatants		Metabolite integrity (% control)	
First 24 h	Medium	106.5 ± 3.3	$p < 0.001$
	AsCho	129.4 ± 5.6[c]	
Second 48 h	Medium	115.3 ± 3.3[d]	not significant
	AsCho	116.5 ± 3.9[d]	
Total 72 h[b]	Medium	112.6 ± 5.3[e]	$p < 0.001$
	AsCho	134.8 ± 4.6[c]	

[a] BM cells isolated from CDF_1 mice were incubated with AsCho (10 mmol dm^{-3}) or medium alone for 24 h at 37 °C, and the culture supernatants were collected (Culture supernatant–first 24 h). Cells were washed three times by warmed PBS, further incubated with medium alone for 48 h at 37 °C, and the culture supernatants were collected again (culture supernatant–second 48 h). These culture supernatants were added to fresh other BM cells and incubated for 72 h at 37 °C, and the viability of the cells was determined by measuring by AB assay. One representative experiment out of four similarly performed is given. Results are expressed as arithmetic mean plus/minus SEM ($n = 3$).

[b] BM cells isolated from CDF_1 mice were incubated with AsCho (10 mmol dm^{-3}) or medium alone for 72 h at 37 °C. After the incubation period, culture supernatants were collected (culture supernatant–total 72 h), added to fresh other BM cells and further incubated for 72 h at 37 °C.

[c] $p < 0.001$, comparison with control BM cells cultured in the absence of any culture supernatants.

[d] $p < 0.01$.

[e] $p < 0.05$.

Effect of culture supernatants of BM cells incubated with AsCho on the viability of other fresh BM cells

BM cells were incubated with 10 mmol dm^{-3} AsCho or medium alone for 24 h, and the culture supernatants containing AsCho were collected (culture supernatant–first 24 h). The cells were then washed three times and further incubated with medium alone for 48 h. After the second 48 h incubation, the culture supernatants, which did not contain AsCho but were thought to contain some factors that were secreted from the BM cells stimulated by AsCho during the first 24 h, were collected (culture supernatants–second 48 h). These culture supernatants were added to other fresh BM cells, incubated for 72 h, and the viability of the fresh cells was determined by AB assay. As shown in Table 2, the addition of the culture supernatants collected after the first 24 h incubation with AsCho significantly enhanced the viability of fresh BM cells; however, the culture supernatants collected after the second 48 h incubation did not have an influence on the viability of fresh BM cells. Other culture supernatants that were collected after 72 h continuous

Q3 incubation with AsCho also showed a potent increasing effect on the viability of fresh BM cells.

Metabolism of AsCho in BM cells

It was previously reported that orally administered AsCho was oxidized and biotransformed into AsBe in the body using murine models.[9] We subsequently examined if AsCho converted to other chemicals, such as AsBe, in BM cells. BM cells (1×10^7/well) on flat-bottomed 12-well tissue culture plates were incubated with 10 mmol dm^{-3} AsCho for 72 h at 37 °C, and the chemical forms of arsenicals in the BM cell lysates were analyzed by the fully automated continuous arsine generation system as described in the Experimental section. If AsCho was converted into AsBe in BM cells it would be identified as trimethylarsine, since AsBe is quantitatively changed to trimethylarsine oxide under hot alkaline conditions and would be continuously reduced to trimethylarsine by HCl and $NaBH_4$.[14] However, no trimethylarsine gas was detected from BM cell lysates that had been pre-incubated with AsCho. This finding means that AsCho taken up into BM cells was not metabolized, at least not to AsBe, because AsCho cannot form trimethylarsine oxide on treatment with hot base, a situation different from AsBe.[14] Other kinds of arsine gas were also not detected.

DISCUSSION

Although certain arsenic compounds are toxic to animals and humans, others are not.[1] The toxicity of arsenic varies a great deal with the chemical species. In the marine ecosystem, it has been demonstrated that inorganic arsenicals in sea water are probably taken up into seaweed and are metabolically methylated to dimethylarsinoyl ribosides, i.e. arsenosugars.[15] We previously reported that arsenosugar enhanced the viability of murine PMs *in vitro*, but, conversely, it is cytotoxic to other immune effector cells.[16] Arsenosugars are further methylated and finally converted to the trimethyl arsenic compound AsBe in many species of marine animals.[3,4] We and Kaise *et al*. previously demonstrated that AsBe was not toxic at all to mammals *in vivo*[2] and *in vitro*[5,6] using murine models. AsCho was found at low levels from some species of marine animals[7,8] and thought to be a precursor of AsBe.[17,18] We have also examined the toxic effects of AsCho on living systems,[5] and Kaise *et al*. previously reported that AsCho had weak but significant acute toxicity in mice *in vivo*.[9] In this study, we examined whether AsCho had any biological effects on mammalian cells using murine immune effector cells *in vitro*, and found that it slightly but significantly augmented the viability of immature BM cells, although it had no cytotoxic and/or immunomodulating effect on other cells, such as splenocytes, thymocytes, PP lymphocytes and PMs.

The reasons why AsCho enhances the viability of murine BM cells are not yet precisely clarified. Because the culture supernatants of BM cells pre-stimulated with AsCho did not influence the viability of other fresh BM cells (Table 2), we postulated that this biological effect of AsCho might be direct rather than due to autocrine mechanisms mediating some factors secreted by AsCho-stimulated BM cells. It has been reported that the biological effects of arsenic compounds depend on their chemical structures.[1,19] In this paper, a significant enhancement effect on the viability of BM cells was observed by AsCho, but not by sodium arsenite or trimethylarsine oxide. Choline chloride, the nitrogenous analogue of AsCho, did not show a potent effect on BM cells, and the simultaneous addition of trimethylarsine oxide and choline chloride also did not influence them. Furthermore, we previously reported that no potent biological effects were observed on BM cells with any other methyl arsenic compounds, such as monomethylarsonic acid, dimethylarsinic acid and tetramethylarsonium hydroxide.[20] We examined in this paper if AsCho was converted to other arsenic chemicals in BM cells by the fully automated continuous arsine generation system using GC–MS. The results show that AsCho is not methylated and/or demethylated in BM cells, because no species of arsine gas was detected from BM cell lysates that had been pre-treated with AsCho. Taken together, these findings suggest that the chemical structure of AsCho is a very important factor, at least in part, in the expression of significant enhancing effects on the viability of BM cells. In our most recent report, a strong effect on the survival of BM cells was also observed with AsBe, which has a chemical structure similar to that of AsCho.[6]

It is interesting that this unique biological effect was found in AsCho, an arsenic compound contained in some marine animals, such as shrimp[7] and conch,[8] which are ingested daily as seafood in many countries; however, it has been reported that the concentration of AsCho contained in seafood is very low;[7,8,17,18] thus, it is suggested that ingestion of AsCho contained in seafood does not influence the health of people who often consume seafood.

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