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Evaluation of *in vivo* acute immunotoxicity of arsenocholine, a trimethyl arsenic compound in seafood

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In this study, we observed the first *in vivo* acute immunotoxicity of a trimethyl(2-hydroxyethyl)arsonium cation, namely arsenocholine (AsCho), which is present in marine animals that are ingested daily as seafood in many countries. It has been reported that AsCho has significant acute *in vivo* toxicity. A high dose of the synthetic pure AsCho was administered to CDF_1 mice intraperitoneally (0.1 g kg^{-1} mouse weight) or orally (a total of 10.0 g kg^{-1} mouse weight); its effect on the immune organs and immune effector cells was assessed. Administered AsCho, especially via the oral route, showed weak and partial, but significant, *in vivo* immunotoxicity in mice, although it did not cause any severe acute inflammatory responses.

KEYWORDS: arsenocholine; arsenic; seafood; immunotoxicity; immunology; spleen; thymus; macrophage

INTRODUCTION

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. It has been 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, about 4–80 μg g^{-1} (dry weight),[2] and that these arsenicals generally are in the form of water-soluble organic arsenic compounds.[3] The limit for arsenic in drinking water in Japan, 10 μg l^{-1}, is largely based on inorganic arsenicals; if this limit were applied to seafood as 10 ng g^{-1}, most of them would be unfit for consumption.[4] This finding has caused great concern with respect to the health of people who often ingest considerable amounts of seafood. Therefore, it is necessary to investigate the effects of organic arsenic compounds contained in marine animals on living systems; however, there have only been a few reports about them, because insufficient amounts of pure compounds for biological experiments have been obtained.

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Norin and Christakopoulos[5] and Shiomi *et al.*[6] found a minor water-soluble trimethyl arsenic compound, namely arsenocholine (AsCho), from some kinds of shrimp[5] and conch.[6] The chemical structure of this trimethyl arsenic compound was the trimethyl(2-hydroxyethyl)arsonium cation.[5] The effects of AsCho on living systems using synthesized pure material have been examined. AsCho had a weak but significant acute toxicity in murine models; the LD_{50} values of AsCho on mice were 187 mg kg^{-1} or 6.54 g kg^{-1} when administered intravenously or orally respectively.[7] In contrast, we recently observed that AsCho did not show any cytolethality in cultured mouse immune effector cells, such as macrophages, splenocytes, thymocytes, Peyer's patch lymphocytes and bone marrow cells, *in vitro*.[1,8,9]

In this study, we examined whether AsCho has any *in vivo* immunological effects using mouse models, and found that AsCho had weak immunotoxicity when administered orally.

EXPERIMENTAL

AsCho

AsCho was synthesized from trimethylarsine reacted with 2-bromo-ethanol[10] and was recrystallized twice from acetonitrile.[7] It gave white prism crystals, melting point

256–258 °C, and its structure was confirmed by ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, high-performance liquid chromatography–inductively coupled plasma mass spectrometry (MS) and fast-atom bombardment (FAB) MS. The purity of this synthesized AsCho was >99.9%, as determined by thin-layer chromatography and gas chromatography–MS. Lipopolysaccharide (LPS) contamination of AsCho was $<1.7 \times 10^{-7}$% (wt wt^{-1}) determined by the endotoxin-specific limulus test (Seikagaku Co., Tokyo, Japan). AsCho was dissolved in sterilized phosphate-buffered saline (PBS) or distilled water and filtered through a 0.2 μm filter.

Mice

Male CDF_1 (BALB/c × DBA/2) mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The mice were used at 6 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.

Administration schedule of AsCho

Synthetic AsCho was administered intraperitoneally at a dose of 0.1 g kg^{-1} on day 1 and all experiments were assessed on day 5. In the case of oral administration, AsCho was administered at a dose of 2.5 g kg^{-1} once a day on days 1, 3, 5 and 7 (four times, total 10.0 g kg^{-1}), and all experiments were assessed on day 9. As a control, mice were treated sterilized PBS or water alone via the intraperitoneal or oral routes respectively.

Cells

Mouse macrophage RAW 264.7 cells were cultured in RPMI 1640 medium (Sigma Chemical Co., St Louis, MO) containing 10% heat-inactivated fetal bovine serum (FBS-RPMII; LPS contamination of FBS was <6 pg ml^{-1} measured by the limulus test). Rat nontumorigenic liver epithelial TRL 1215 cells originally derived from the liver of 10-day-old Fisher F344 rats were graciously supplied by Dr Michael P. Waalkes, National Cancer Institute @ National Institute of Environmental Health Science, National Institute of Health, NC, USA,[11,12] and were cultured in William's medium E (Sigma) containing 10% FBS. Human normal skin fiblobrast TIG-112 cells (JCRB0533)[13] and human monocyte-like U937 cells (JCRB9021) were obtained from the Health Science Research Resources Bank (Osaka, Japan), and were cultured in Eagle's minimum essential medium (Sigma) containing 10% FBS or FBS-RPMI respectively. Mouse immune effector cells, splenocytes, thymocytes and peritoneal macrophages (PMs), were obtained from anesthetized mice by diethyl ether. Splenocytes and thymocytes were prepared by teasing the spleen or thymus with a sterilized steel screen in RPMI 1640 medium.[14] Peritoneal exude cells (PECs) were collected by washing the peritoneal cavity using Ca^{2+}- and Mg^{2+}-free PBS containing 0.05% ethylenediamine tetraacetate (EDTA).[15] PMs were prepared using their adherent abilities by 2 h incubation of PECs on tissue culture plates at 37 °C in a CO_2 incubator.[15] These mouse immune effector cells were washed twice and resuspended in fresh FBS-RPMI.[14] Cultures of all cells were maintained in a humidified atmosphere of 5% CO_2–95% air at 37 °C.

Assay for cellular viability

Raw 264.7 cells (2×10^5/100 μl/well), TRL 1215 cells (5×10^4/100 μl/well), TIG-112 cells (1×10^4/100 μl/well) or U937 cells (2×10^4/100 μl/well) were incubated with AsCho on flat-bottomed 96-well tissue culture plates for 48 h at 37 °C in a CO_2 incubator. After the incubation, the medium was removed and replaced with fresh 100 μl/well medium. The viability of these cells was determined by measuring live cells by AlamarBlue (AB) assay, similar to the methylthiazoletetrazolium assay.[16] Briefly, 10 μl/well of AB solution (Iwaki Glass Co., Chiba, Japan) was added directly to the 96-well plates and incubated for 3 h at 37 °C; then, the absorbance at 570 nm (reference 630 nm) was measured using a model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA). AsCho itself did not affect the absorbance of the AB solution, even at concentrations over 40 mmol l^{-1}.

Assay for lymphocyte blastogenesis

Mouse lymphocytes (splenocytes and thymocytes; 2.5×10^5 cells/100 μl/well) were incubated on flat-bottomed 96-well tissue culture plates for 72 h at 37 °C in a CO_2 incubator in the presence or absence of a submitogenic concentration of T-lymphocyte mitogen, concanavalin A (Con A; Sigma; 2.5 μg ml^{-1}) or B-lymphocyte mitogen, LPS (O111:B4; Sigma; 50 μg ml^{-1}). The viability of the cells was determined by AB assay.[8]

Assay for cellular lysosomal enzyme activity of PMs

Cellular lysosomal enzyme (acid phosphatase) activity in PM lysates (cells were dissolved by 0.1% Triton X-100) was assayed by a method described previously using *p*-nitrophenyl phosphate (Sigma) as a substrate.[14] Enzyme activity was expressed as amount of *p*-nitrophenol (PNP) per microgram of cellular protein determined by BCA protein assay reagent (Pierce Co., Rockford, IL) with bovine serum albumin as a standard.

Measurement of cellular reduced glutathione (GSH) levels of PMs

Cellular GSH levels were measured by a method described elsewhere using *o*-phthaldialdehyde as a substrate.[17] Briefly, PMs were lysed in 150 μl of ice-cold 0.8 mol l^{-1} perchloric acid including 8 mmol dm^{-3} EDTA. Cellular debris was removed by centrifugation, and 100 μl of the supernatant of each cell was then added to 2 ml of 0.1 mol l^{-1} sodium phosphate buffer (pH 8.0) including 5 mmol l^{-1} EDTA and 1 mg ml^{-1} *o*-phthaldialdehyde, and was kept for 15 min at

room temperature in the dark. The fluorescence intensity of sample solutions was measured at excitation and emission wavelengths of 350 nm and 425 nm respectively. Aliquots of GSH were used to construct a standard curve, and the results were expressed as GSH per milligram of cellular protein determined by BCA protein assay reagent with bovine serum albumin as a standard.

Statistics

All results are expressed as the arithmetic mean plus/minus the standard error of the mean SEM, and one-way analysis of variance (ANOVA) followed by a Student's *t*-test were used for the statistical analysis. Probabilities less than 5% ($P < 0.05$) were considered significant.

RESULTS

In vitro cytolethality of AsCho

Mouse macrophage RAW 264.7 cells, rat liver TRL 1215 cells, human skin TIG-112 cells and human monocyte-like U937 cells were incubated with various doses of AsCho in the presence or absence of 25 μmol l^{-1} specific GSH synthase inhibitor, L-buthionine-[*S*,*R*]-sulfoximine (BSO; Sigma), for 48 h; the cellular viability was observed. As a result, AsCho had no cytolethality in any of these cells, even at concentrations over 25 mmol l^{-1} with or without the BSO treatment.

In vivo immunotoxicity of AsCho administered intraperitoneally

The synthetic AsCho was intraperitoneally administered to mice at a high dose of 0.1 g kg^{-1} mouse weight on day 1; immune organs and immune effector cells were obtained from these mice on day 5. All mice survived without a significant decrease of body weight by AsCho at this intraperitoneal dose; the body weight of control mice on day 5 was 26.1 ± 0.4 g and that of AsCho-treated mice was 25.9 ± 0.2 g ($n = 5$). As shown in Table 1, intraperitoneally administered AsCho did not affect immune organs and immune effector cells. No significant changes were found in the organ weights of either spleen or thymus, or in the number of immune effector cells, such as splenocytes, thymocytes, PMs and peripheral blood leukocytes. Only the number of peripheral blood erythrocytes

Table 1. *In vivo* immunotoxicity of AsCho administered intraperitoneally[a]

		Control	AsCho
Spleen	Organ weight		
	Absolute (mg)[b]	74.8 ± 3.1	83.8 ± 3.8
	Relative (mg%)[b]	0.29 ± 0.01	0.32 ± 0.01
	Number of splenocytes ($\times 10^7$/spleen)	5.20 ± 0.39	4.80 ± 0.93
	Splenocyte blastogenesis (metabolic integrity, % control)[c]		
	Medium	100.5 ± 26.6	99.5 ± 7.5
	+ Con A	99.7 ± 6.7	100.0 ± 2.4
	+ LPS	100.2 ± 9.4	99.6 ± 9.6
Thymus	Organ weight		
	Absolute (mg)	39.3 ± 2.3	34.8 ± 1.3
	Relative (mg%)	0.16 ± 0.01	0.14 ± 0.01
	Number of thymocytes ($\times 10^7$/thymus)	7.50 ± 1.68	8.00 ± 1.10
	Thymocyte blastogenesis (metabolic integrity, % control)[c]		
	Medium	100.0 ± 18.5	59.4 ± 21.3
	+ Con A	101.0 ± 24.8	121.3 ± 14.6
PMs	Number of PMs ($\times 10^6$/mouse)	1.60 ± 0.48	2.10 ± 0.33
	Lysosomal enzyme activity (PNP nmol/100 μg cellular protein)	3.25 ± 0.03	3.64 ± 0.01
	GSH level (nmol/mg cellular protein)	28.8 ± 7.9	18.6 ± 1.2
Peripheral blood cells	Leukocytes ($\times 10^6$/ml blood)	9.15 ± 0.31	8.14 ± 0.47
	Erythrocytes ($\times 10^{10}$/ml blood)	2.75 ± 0.10	2.31 ± 0.18[d]

[a] AsCho was administered intraperitoneally to CDF_1 mice at a dose of 0.1 g kg^{-1} on day 1, and immune organs and immune effector cells were obtained from these mice on day 5. One representative experiment out of three similar performed is given. Results are expressed as the arithmetic mean plus/minus SEM ($n = 5$).

[b] Data are expressed as absolute organ weights and relative organ weights. Relative organ weight (mg%) = absolute organ weight (mg)/mouse body weight (mg) ×100.

[c] Splenocytes or thymocytes were incubated in the presence or absence of submitogenic concentrations of Con A (2.5 μg ml^{-1}) or LPS (50 μg ml^{-1}) for 72 h. After the incubation, the viability of cells was measured by AB assay, and data are expressed as percentage control cells which were obtained from control mice and incubated with the same mitogen.

[d] $P < 0.05$ comparison with that of control mice which were treated with sterilized PBS alone.

was slightly reduced by the AsCho administration. The cell populations of PECs and peripheral blood leukocytes were not changed by the AsCho treatment; about 60% of the PECs were macrophages and 30% were granulocytes, and most of the peripheral blood leukocytes were granulocytes. Also, intraperitoneally administered AsCho did not affect the immune functions of cells, such as the blatogenesis of splenocytes and thymocytes, the cellular lysosomal enzyme activity of PMs and the GSH levels of PMs.

In vivo immunotoxicity of AsCho administered orally

The synthetic AsCho was administered orally to mice at a high dose of 2.5 g kg^{-1} mouse weight once a day on days 1, 3, 5 and 7 (four times, total 10.0 g kg^{-1} mouse weight), and immune organs and immune effector cells were obtained from these mice on day 9. All mice survived without a significant decrease of body weight by AsCho at this oral dose; body weight of control mice on day 8 was 25.2 ± 0.7 g and AsCho-treated mice was 23.6 ± 0.7 g ($n = 5$). As shown in Table 2, orally administered AsCho slightly, but significantly, suppressed the survival of splenocytes, although the responses to mitogens of splenocytes were not affected. The spleen weights and the number of splenocytes were not changed by orally administered AsCho. Significant toxicity was also observed in the thymus by AsCho treatment. AsCho administered orally slightly reduced the weight of the thymus and the survival of thymocytes, although the number and the responses of thymocytes to mitogens were not changed.

DISCUSSION

The inorganic arsenicals, arsenite and arsenate, are known to have highly toxic effects, and are known to be metabolically methylated in animals. In the marine ecosystem, inorganic arsenicals in sea water are probably first taken up into seaweed and are metabolically methylated to dimethylarsinoyl ribosides, namely arsenosugars.[4,18] They are further methylated and converted to trimethyl arsenic compounds, including AsCho, in marine animals.[5,6] It was reported that AsCho had a weak, but significant, *in vivo* acute toxicity in mice,[7] but we recently demonstrated that AsCho had no *in vitro* cytolethality on cultured mouse splenocytes, thymocytes, Peyer's patch lymphocytes, macrophages and bone marrow cells.[1,8,9] In this study, we examined whether AsCho has any *in vivo* immunotoxicity, and found that it was slightly, but significantly, immunotoxic *in vivo*.

We first confirmed whether or not AsCho had *in vitro* cytolethality, using those mammalian cultured cell lines that are often used for the evaluation of the *in vitro* cytolethality of chemicals, such as mouse macrophage RAW 264.7 cells, rat liver TRL 1215 cells, human skin TIG-112 cells and human

Table 2. *In vivo* immunotoxicity of AsCho administered orally[a]

		Control	AsCho
Spleen	Organ weight		
	Absolute (mg)[b]	74.5 ± 1.3	68.7 ± 3.0
	Relative (mg%)[b]	0.30 ± 0.00	0.29 ± 0.01
	Number of splenocytes ($\times 10^7$/spleen)	6.00 ± 0.87	6.59 ± 0.32
	Splenocyte blastogenesis (metabolic integrity, % control)[c]		
	Medium	100.5 ± 27.0	58.9 ± 15.0[d]
	+ Con A	100.1 ± 26.5	73.5 ± 11.7
	+ LPS	100.0 ± 24.0	102.1 ± 24.3
Thymus	Organ weight		
	Absolute (mg)	39.7 ± 1.2	30.9 ± 1.7[e]
	Relative (mg%)	0.16 ± 0.00	0.13 ± 0.01[d]
	Number of thymocytes ($\times 10^7$/thymus)	8.30 ± 0.73	7.39 ± 0.72
	Thymocyte blastogenesis (metabolic integrity, % control)[c]		
	Medium	100.0 ± 14.3	51.8 ± 12.5[e]
	+ Con A	100.0 ± 26.2	79.5 ± 14.4

[a] AsCho was administered orally to CDF_1 mice at a dose of 2.5 g kg^{-1} once a day on days 1, 3, 5 and 7 (four times, total 10.0 g kg^{-1}), and immune organs and immune effector cells were obtained from these mice on day 9. One representative experiment out of three similar performed is given. Results are expressed as arithmetic mean plus/minus SEM ($n = 5$).

[b] Data are expressed as absolute organ weights and relative organ weights. Relative organ weight (mg%) = absolute organ weight (mg)/mouse body weight (mg) $\times 100$.

[c] Splenocytes or thymocytes were incubated in the presence or absence of submitogenic concentrations of Con A (2.5 μg ml^{-1}) or LPS (50 μg ml^{-1}) for 72 h. After the incubation, the viability of cells was measured by AB assay, and data are expressed as percentage control cells which were obtained from control mice and incubated with the same mitogen.

[d] $P < 0.05$ comparison with that of control mice which were treated with sterilized water alone.

[e] $P < 0.01$.

monocyte-like U937 cells. The results indicate that AsCho shows no significant cytolethality in these cells even at concentrations over 25 mmol l^{-1} if the cellular GSH was depleted by the BSO treatment. Cellular GSH generally protects the *in vitro* cytolethality of arsenic compounds, and some organic arsenic compounds showed significant cytolethality when cellular GSH was depleted by BSO if itself was not cytotoxic in the normal cells.[19] We subsequently examined the *in vivo* immunotoxicity of AsCho using mouse models. The synthetic AsCho was intraperitoneally or orally administered to mice at a high dose of 0.1 g kg^{-1} mouse weight or total 10.0 g kg^{-1} respectively. We determined this intraperitoneal or oral dose by reference to the LD_{50} value of AsCho.[7] As shown in Tables 1 and 2, administered AsCho, especially via the oral route, showed weak and partial, but significant, *in vivo* immunotoxicity in mice, although it did not cause any severe acute inflammatory responses *in vivo*. Oral AsCho was mainly toxic in T-lymphocytes. It has sometimes been reported that inorganic arsenicals are also selectively toxic in T-lymphocytes,[20–22] thus, T-lymphocytes may be one of the arsenic-sensitive immune effector cells.

The reason why the study of acute *in vivo* toxicity of AsCho does not reflect its *in vitro* cytolethality has not yet been clarified. We previously observed similar differences between *in vitro* and *in vivo* toxic assays in another minor organic arsenic compound in marine animals, i.e. tetramethylarsonium hydroxide.[1,8,23] It is believed that orally administered AsCho is absorbed from the gastrointestinal tract, rapidly oxidized and converted to the trimethyl (carboxymethyl) arsnoium zwitterions, namely arsenobetaine, and then excreted into the urine.[7] It is well known that arsenobetaine is not toxic *in vitro* or *in vivo*.[1,7,8] We recently reported that a human final methylated metabolite of inorganic arsenite, dimethylarsinic acid, made a conjugate with cellular GSH that induced apoptosis during subsequent enzymatic metabolic reactions.[15,19,23] This finding suggests that the enzymatic oxidation process of AsCho to arsenobetaine may be important for exhibiting significant toxicity *in vivo*. Further experiments will be needed to clarify the role of the enzymatic metabolic process of AsCho in inducing significant *in vivo* acute toxicity.

The average daily consumption of arsenic by the Japanese from seafood is about 3.9 mg kg^{-1} body weight per day, with a high level of 8.6 mg kg^{-1} per day estimated from a previous report.[24] And it is reported that the main soluble organic arsenic compound in marine animals is arsenobetaine and that the amount of AsCho is very low, i.e. about 0.25% the amount of arsenobetaine.[1] In this study, we used a high oral dose of AsCho, a total of 10.0 g kg^{-1} mouse weight (4.5 g kg^{-1} as arsenic) during the 7 days (642.9 mg kg^{-1} per day as arsenic) for the investigation of its *in vivo* acute immunotoxicity. At the very least, a high dose of oral AsCho did not cause any fatal *in vivo* immunotoxicity. Considering these facts, this implies that AsCho contained in marine animals may be not very immunotoxic to people who often consume seafood. This information could be useful in the risk assessment of AsCho; however, further examinations will be necessary to clarify its chronic effects.

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