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Evaluation of *in vitro* Cytotoxicity of Tetramethylarsonium Hydroxide in Marine Animals

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We have studied the cytotoxicity *in vitro* of tetramethylarsonium hydroxide (TetMA-OH), which is found in some marine animals, in various murine immune effector cells, including splenocytes, thymocytes, Peyer's patch (PP) lymphocytes, peritoneal macrophages (PMs) alveolar macrophages (AMs) and bone-marrow (BM) cells, using synthetic material which was compared with an inorganic arsenical, sodium arsenite. Arsenite showed strong cytotoxicity in these cells, with an IC_{50} (the concentration that reduced the number of surviving cells to 50% of that in untreated controls) of about 2–9 μmol dm^{-3}. In contrast, TetMA-OH was less toxic, even at a concentration above 10 mmol dm^{-3}, in these immune effector cells, and no enhancement effect on the viability of the cells was observed. These data suggested that TetMA-OH had no biological effect, either toxic or modulating on any immune effector cells *in vitro*.

Keywords: tetramethylarsonium hydroxide; cytotoxicity; immunotoxicity; lymphocyte; macrophage; bone-marrow cell

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INTRODUCTION

Arsenic has had the reputation of being a poison for centuries.[1] Epidemiological studies have suggested that an 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 human.[1] We previously reported that marine animals, such as clam, 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}.[3] These arsenicals are generally in the form of water-soluble organic arsenic compounds, and the trimethyl(carboxymethyl)arsonium zwitterion, namely arsenobetaine, is a major organic arsenic compound in marine animals.[4] We examined the toxic effect of arsenobetaine on living systems using synthesized material, and found that it had no acute toxicity in murine models even above 10 g kg^{-1}, when it was orally administered.[2] Subsequently we observed that the cytotoxicity *in vitro* of arsenobetaine is very weak compared with those of inorganic arsenicals in cultured murine macrophages and splenocytes.[5] These data imply that arsenobetaine has no toxicity in mammalian living systems.

In 1987, Shiomi *et al.* detected a new minor water-soluble organic arsenic compound from the branchia of a clam, *Meretrix lusoria*,[6] and some lower marine animals, such as the sea hare, *Aplysia kurodai*, and the sea anemone, *Parasicyonis actinostoloides*.[7] The chemical structure of this organic arsenic compound was that of a tetramethylarsonium salt, and this caused great concern with respect to human health because the tetramethylammonium ion, i.e. tetramine, the nitrogenous analogue of the tetramethylarsonium ion, has been known to be a causative agent of numerous intoxications in Japan due to the ingestion of sea snails, such as *Neptunea arthritica*.[8,9] Therefore, we examined the lethal toxicity of the tetramethylarsonium ion in mice using synthetic tetramethylarsonium iodide or chloride, and found that these tetramethylarsonium halide salts showed significant

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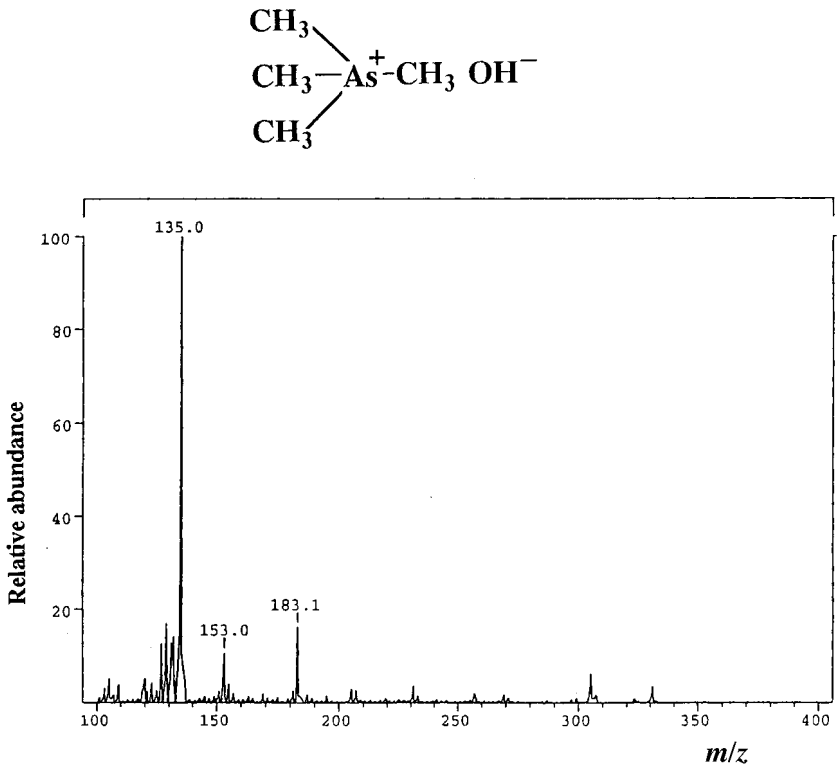

Figure 1 Primary structure and mass spectrum of synthetic TetMA-OH. The FAB MS of the TetMA-OH was performed using a Finnigan MAT TSQ-700 mass spectrometer (Finnigan Co., San Jose, CA, USA) equipped with a FAB ion source and xenon atoms at 8 keV. There are TetMA-OH signals at *m/z* 135 $[M-OH]^+$ and *m/z* 153 $[M+H_2O]^+$.

acute toxicity; their LD_{50} was 890 mg kg^{-1} or 580 mg kg^{-1}, respectively.[9,10] Additionally, we recently reported that tetramethylarsonium iodide also exhibited a weak cytotoxicity *in vitro* in cultured murine splenocytes; the concentration that reduced the number of surviving cells to 50% of that in untreated controls (IC_{50}) was 6 mmol dm^{-3}.[5] Taken together, the tetramethylarsonim ion was believed to have significant toxicity in mammalian living systems, although other researchers indicated that this weak toxicity of the tetramethylarsonium halide salts might be dependent on the halogen atoms.

In this study, we first examined the detailed cytotoxic effects *in vitro* of tetramethylarsonium hydroxide (TetMA-OH), which was prepared from synthetic tetramethyarsonium iodide using an anionic ion-exchange resin column, on various murine immune effector cells, and found that TetMA-OH had no cytotoxicity in these cells *in vitro*.

EXPERIMENTAL

Reagents

Sodium arsenite was purchased from the Wako Pure Chemical Co. (Osaka, Japan). TetMA-OH was prepared from pure tetramethylarsonium iodide, which was synthesized by the procedures described in our previous reports,[10] using on anionic ion-exchange resin (Dowex 2 × 8, OH type; Muromachi Kagaku Kogyo Kaisha, Co., Tokyo) column, and was recrystallized twice from dried n-butanol. The structure of this prepared TetMA-OH was confirmed by fast atom bombardment mass spectrometric (FAB MS) analysis (see Fig. 1).

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 sterilized laboratory chow (LabDiet; PMI Feeds Inc., Richmond, IN, USA), 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 anesthesized with ethyl ether. Single-cell suspensions of splenocytes or thymocytes were prepared by teasing the spleen[5] or thymus[11] with a sterilized steel screen in Eagle's MEM medium (MEM medium; Nissui Pharmaceutical Co., Tokyo). A single-cell suspension of Peyer's patch (PP) lymphocytes was prepared by cutting the PP with a scalpel blade and then gently teasing it with two slide glasses in MEM medium containing 10% heat-inactivated fetal calf serum (FCS–MEM).[11] Peritoneal macrophages (PMs)[12] or alveolar macrophages (AMs)[13] were collected by washing the peritoneal cavity or by bronchial lavage, respectively, using Ca^{2+}-and Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.05% ethylenediamine tetra-acetate. Bone-marrow (BM) cells were prepared by flushing the femoral shafts using MEM medium.[11] These immune effector cells were washed twice with MEM medium and resuspended in FCS–MEM medium.

Assay for cytotoxicity

Lymphocytes (splenocytes, thymocytes and PP lymphocytes; 2.5×10^5 cells / 100 µl per well), macrophages (5×10^4 cells / 100 µl per well) or BM cells (5×10^5 cells / 100 µl per well) were incubated with arsenite (1–20 µmol dm^{-3}) or TetMA-OH (20 µmol –10 mmol dm^{-3}) on flat-bottomed 96-well tissue culture plates for 48 h (macrophages) or 72 h (lymphocytes and BM cells) at 37 °C in a CO_2 incubator with FCS–MEM medium. The viability of the cells was determined by measuring live cells using the AlamarBlue (AB) assay, which is similar to the MTT assay.[14] Briefly, six hours 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 (ref. 630 nm) was measured using a microplate reader, model 550 (Bio-Rad Laboratories, Hercules, CA, USA). Arsenicals themselves did not affect the absorbance of the AB solution, even at a concentration above 40 mM.

Assay for lymphocyte blastogenesis

Lymphocytes (2.5×10^5 cells / 100 µl per well) were incubated with arsenite (1–20 µmol dm^{-3}) or TetMA-OH (20 µmol–10 mmol dm^{-3}) on flat-bottomed 96-well tissue culture plates for 72 h at 37 °C in a CO_2 incubator in the presence of submitogenic concentrations of T lymphocyte mitogen, concanavalin A (Con A; Sigma; 2.5 µg cm^{-3}), or B lymphocyte mitogen, lipopolysaccharide (LPS, O111:B4; Sigma; 50 µg cm^{-3}), and the blastogenesis was determined by the AB assay.[5]

Statistics

Statistical evaluations in some experiments were performed by Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Effect of TetMA-OH on the viability and blastogenesis of lymphocytes

Lymphocytes (splenocytes, thymocytes and PP lymphocytes) were incubated with arsenite (1–20 µmol dm^{-3}) or TetMA-OH (20 µmol–10 mmol dm^{-3}) in the presence or absence of the T lymphocyte mitogen (Con A; 2.5 µg cm^{-3}), or the B lymphocyte mitogen (LPS; 50 µg cm^{-3}), for 72 h at 37 °C in a CO_2 incubator, and the viability of the cells was determined by AB assay. As shown in Fig. 2 arsenite strongly decreased the viability of splenocytes incubated with or without Con A or LPS; their IC_{50} values were about 2 µmol dm^{-3} (with Con A), 2.5 µmol dm^{-3} (with LPS) or 5 µmol dm^{-3} (medium alone), respectively. However, an organic arsenic compound found in some marine animals, TetMA-OH, was less toxic even at concentrations above 10 mmol dm^{-3} in splenocytes with or without mitogens. In Fig. 3 arsenite also exhibited a strong cytotoxicity in thymocytes in the presence or absence of ConA, and its IC_{50} was about 5–6 µmol dm^{-3}. In contrast, TetMA-OH had no cytotoxicity in the thymocytes, even above 10 mmol dm^{-3}. A similar result was also observed in the PP lymphocytes (data not shown).

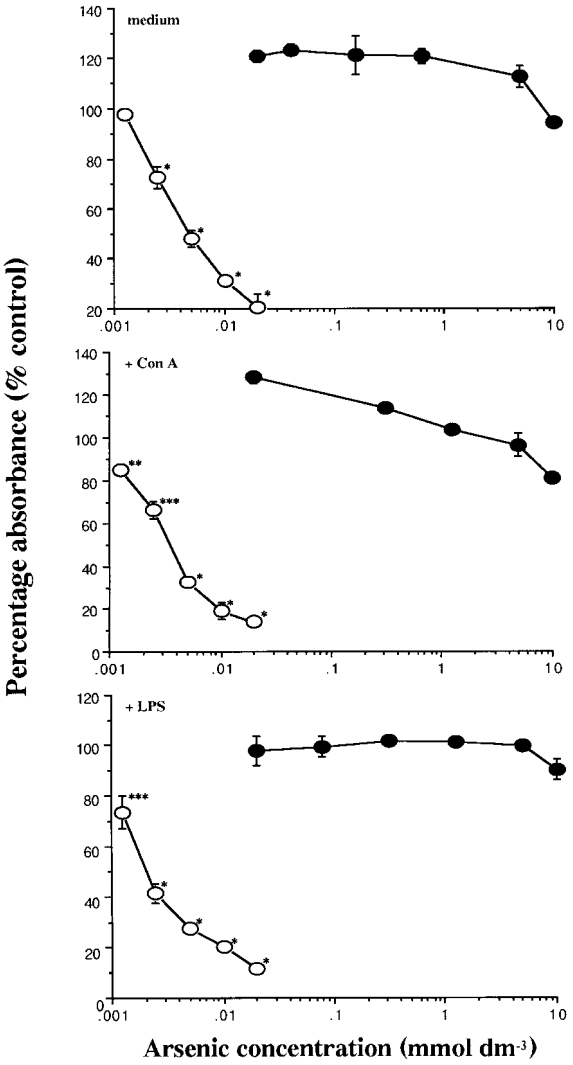

Figure 2 Effect of arsenic compounds on the viability and blastogenesis of murine splenocytes. Splenocytes isolated from CDF_1 mice were incubated with various concentrations of arsenite (○) or TetMA-OH (●) in the presence or absence of Con A (2.5 μg cm^{-3}) or LPS (50 μg cm^{-3}) for 72 h at 37 °C, and cell viability was determined by AB assay. Results are expressed as arithmetic mean ± S.D. of duplicate dishes. *$P < 0.001$ in comparison with splenocytes incubated with medium alone; ** $P < 0.05$; *** $P < 0.01$.

Effect of TetMA-OH on the viability of macrophages or BM cells

Subsequently, we examined the cytotoxic effects of TetMA-OH compared with those of arsenite using other immune effector cells, such as macrophages and BM cells. These cells have been known to be very sensitive to changes in environmental conditions.[15] Macrophages or BM cells were incubated with arsenite (1–20 μmol dm^{-3}) or TetMA-OH (20 μmol–10 mmol dm^{-3}) for 48 h (macrophages) or 72 h (BM cells) at 37 °C in a CO_2 incubator, and the viability of the cells was determined by AB assay. As shown in Figs 4 and 5, TetMA-OH showed absolutely no cytotoxicity, even at concentrations above 10 mmol dm^{-3} in either macrophages (PMs and AMs) or BM cells, although arsenite expressed a strong cytotoxicity in these cells; its IC_{50} was about 5 or 9 μmol dm^{-3}, respectively.

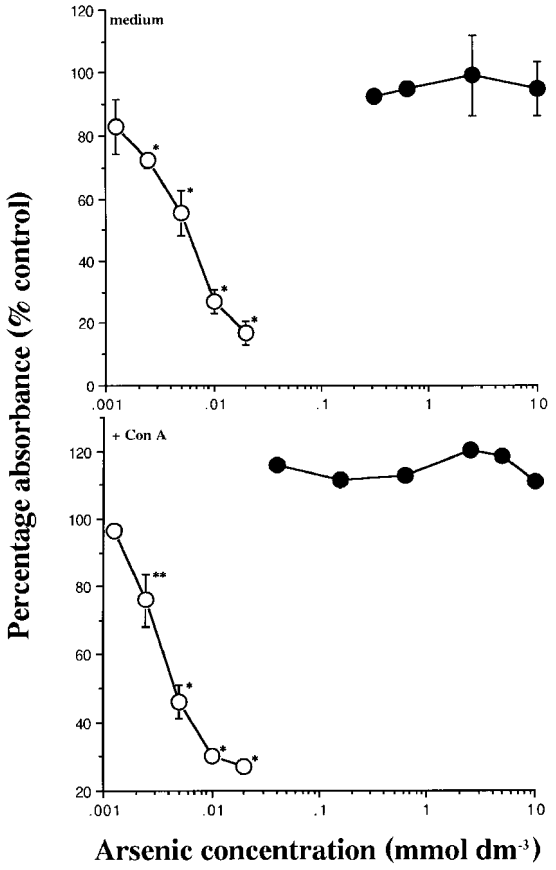

Figure 3 Effect of arsenic compounds on the viability and blastogenesis of murine thymocytes. Thymocytes isolated from CDF_1 mice were incubated with arsenite (○) or TetMA-OH (●) in the presence or absence of Con A (2.5 μg cm^{-3}) for 72 h at 37 °C, and cell viability was determined by AB assay. Results are expressed as arithmetic mean ± S.D. of duplicate dishes. *$P < 0.001$ in comparison with thymocytes incubated with medium alone; ** $P < 0.01$.

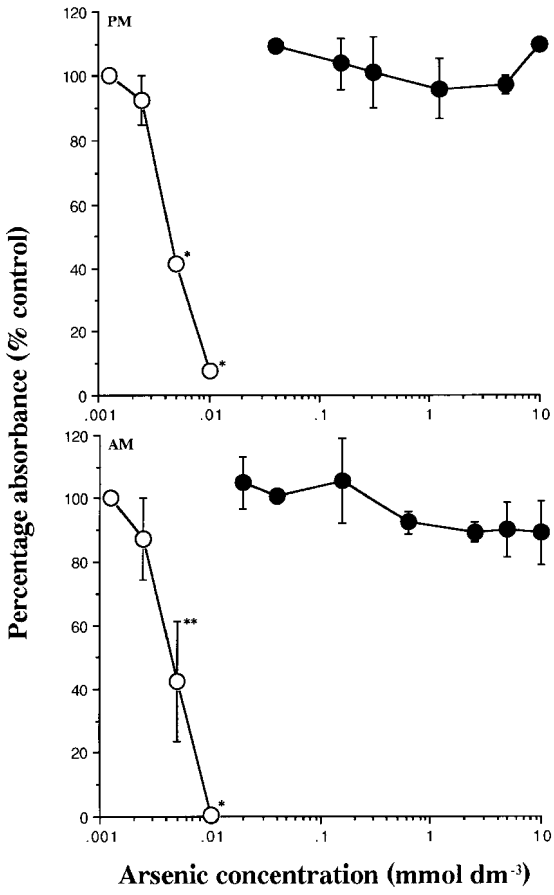

Figure 4 Effect of arsenic compounds on the viability of murine macrophages. PMs or AMs isolated from CDF_1 mice were incubated with arsenite (○) or TetMA-OH (●) for 48 h at 37 °C, and cell viability was determined by AB assay. Results are expressed as arithmetic mean ± S.D. of duplicate dishes. $*P < 0.001$ in comparison with macrophages incubated with medium alone; $** P < 0.01$.

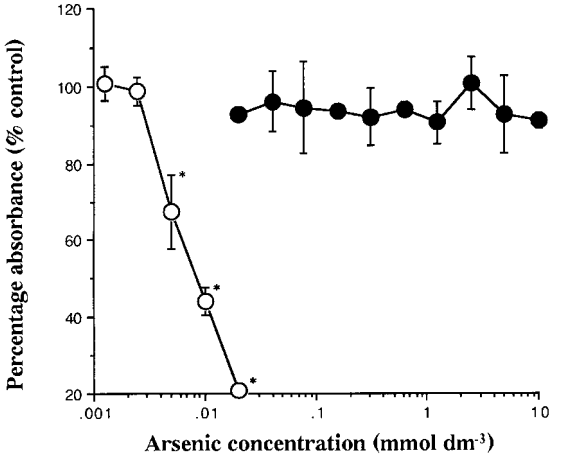

Figure 5 Effect of arsenic compounds on the viability of murine BM cells. BM cells isolated from CDF_1 mice were incubated with arsenite (○) or TetMA-OH (●) for 72 h at 37 °C, and cell viability was determined by AB assay. Results are expressed as arithmetic mean ± S.D. of duplicate dishes. $*P < 0.001$ in comparison with BM cells incubated with medium alone.

DISCUSSION

We have demonstrated that TetMA-OH, a minor organic arsenic compound found in some marine animals, had no cytotoxicity *in vitro* in some murine immune effector cells, including splenocytes, thymocytes, PP lymphocytes, macrophages and BM cells. We previously reported that a tetramethylarsonium halide salt, tetramethylarsonium iodide, showed weak but significant cytotoxicity in murine splenocytes *in vitro;* its IC_{50} was about 6 mmol dm^{-3}.[5] It was suggested that this weak cytotoxicity *in vitro* of tetramethylarsnium iodide might be due to the influence of the iodide ion.[16]

In our previous paper, tetramethylarsonium iodide exhibited a significant acute toxicity in murine models when orally administered, and its LD_{50} was 890 mg kg^{-1},[9] which was the highest LD_{50} of all the organic arsenic compounds. For example, for the major organic (trimethyl)arsenic compound in marine animals, arsenobetaine, the LD_{50} was >10 g kg^{-1},[2] and LD_{50} values for mammalian methyl metabolites of the inorganic arsenicals were much higher, eg. monomethylarsonic acid (>10 g kg^{-1}), dimethylarsinic acid (= 1.2 g kg^{-1}) and trimethylarsine oxide (>10 g kg^{-1}).[17] Although the acute toxicity *in vivo* of tetramethylarsonium iodide was not similar to the result of the cytotoxicity *in vitro*, the rank order of the LD_{50} values of the other organic arsenic compounds was similar to the results of the cytotoxicity *in vitro* using cultured murine tumor cells.[18] In our preliminary experiment, TetMA-OH also showed a significant acute toxicity in murine models after administration of a single oral dose; its LD_{50} was about 1.0 g kg^{-1} (unpublished data). The reason why the study of the cytotoxicity *in vitro* of TetMA-OH does not reflect its acute toxicity *in vivo* has not been clarified. We reported in a previous paper that about 70% of a single dose of orally

ingested tetramethylarsonium iodide was absorbed from the gastrointestinal tract in mice and then excreted into the urine without biotransformation within 72 h.[6] However, further detailed analysis of the tissue distribution and accumulation of tetramethylarsonium iodide, including its effects on the nervous system, have not been completed.

It is very interesting that tetramethylarsonium salts were detected from only some marine animals, such as the branchia of clam and the surface of the sea hare and sea anemone;[6,7] although the final putative biological metabolite in marine organisms (arsenobetaine) is widely distributed in various marine animals, including shellfish, sea anemone, sea urchin, sea slug, cuttlefish, clam, snail, crab, lobster, shrimp and other fish.[3] These data imply that tetramethylarsonium salts are produced via a minor metabolic pathway. We showed previously that trimethylarsine oxide was detected from the culture supernatants of micro-organisms collected from the branchia of a clam, *Meretrix lusoria*, when it was incubated with the major organic arsenic compounds in clams, i.e. arsenobetaine, but no tetramethylarsenicals were detected.[19] Hanaoka *et al.* reported that the bacteria in seawater could decompose arsenobetaine to trimethylarsine oxide and inorganic arsenicals, but could not convert it to a tetramethylarsenical.[20] On the other hand, Hanaoka *et al.* also stated that small amounts of tetramethylarsonium salts were present in marine sediments.[21,22] These findings imply that tetramethylarsonium salts detected from the sea clam, sea hare or sea anemone originated in the marine sediments and were trapped in the external tissues of these animals, such as the branchia and surface tissue.

We recently reported that some organic arsenic compounds in marine animals, such as arsenosugar, a major organic (dimethyl)arsenic compound in seaweed, and arsenobetaine showed unique immunopharmacological effects *in vitro* on some murine immune effector cells.[23] However, we suggest in the present study that TetMA-OH has no biological effects, either modulating or toxic on these cells *in vitro*.

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