APPLIED ORGANOMETALLIC CHEMISTRY, VOL. 10, 727–732 (1996)

Immunotoxicity of Organic Arsenic Compounds in Marine Animals

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In the present study, we demonstrated for the first time the immunotoxic effects of organic arsenic compounds in marine animals, namely arsenocholine [AsCho; trimethyl(2-hydroxyethyl)arsonium cation], arsenobetaine [AsBe; the trimethyl(carboxymethyl)arsonium zwitterion] and the tetramethylarsonium ion (TetMA), to murine principal immune effector cells (macrophages and lymphocytes), comparing them with the effects of inorganic arsenicals *in vitro*. Inorganic arsenicals (arsenite and arsenate) showed strong cytotoxicity to both macrophages and lymphocytes. The concentration of arsenite that reduced the number of surviving cells to 50% of that in untreated controls (IC_{50}) was 3–5 μmol dm^{-3}, and the cytotoxicity of arsenate (IC_{50} = 100 μ-1 m mol dm^{-3}) was lower than that of arsenite. Compared with these findings, trimethylarsenic compounds in marine animals, AsCho and AsBe, were less toxic even at a concentration over 10 mmol dm^{-3} to both macrophages and lymphocytes; however, TetMA had weak, but significant, cytotoxicity to these cells (IC_{50} was about 6 mmol dm^{-3}).

Keywords: immunotoxicity; arsenocholine; arsenobetaine; tetramethylarsonium ion; macrophages; lymphocytes

INTRODUCTION

It is well known that marine organisms contain high levels of water-soluble organic arsenic compounds. Edmonds *et al.* isolated a major organic arsenic compound in marine animals, arsenobetaine [AsBe; the trimethyl(carboxymethyl)arsonium zwitterion; Fig. 1], from the tail muscle of the western rock lobster, and structurally characterized it in 1977.[1] It has been suggested that AsBe is the final metabolite in the arsenic cycle in marine ecosystems because AsBe is found only in various species of marine animals.[2–6]

Subsequently, other minor organic arsenic compounds, such as arsenocholine (AsCho)[5,7–10] and tetramethylarsonium salts (TetMA),[11,12] were found in some marine animals. AsCho, the trimethyl(2-hydroxyethyl)arsonium cation (Fig. 1), detected at low levels in shrimps[5,7–9] and conch,[10] is thought to be a possible candidate as the precursor of AsBe in the marine food chain. Marafante *et al.* and Kaise *et al.* examined the metabolism of AsCho in experimental animals and reported that it was converted to AsBe and rapidly excreted in urine.[13,14] TetMA (Fig. 1) was recently detected by Shiomi *et al.* from some species of marine animals such as the clam,[11] sea hare and sea anemone.[12]

Generally, arsenicals have high potential toxicity. This finding has been of great concern with respect to the health of people who ingest considerable amounts of seafood. It is therefore necessary to investigate the biological effects of organic arsenic compounds in marine animals from the viewpoint of food hygiene. We previously reported that the acute toxicity of AsBe,

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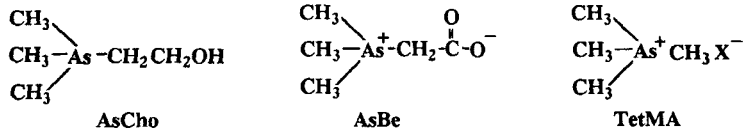

Figure 1 Primary structures of arsenocholine (AsCho), arsenobetaine (AsBe) and tetramethylarsonium ion (TetMA).

CCC 0268–2605/96/090727–06

Received 5 December 1995
Accepted 12 January 1996

AsCho or TetMA was lower than that of inorganic arsenicals using murine models.[14–17] However, further details of the biological effects, especially, the immunotoxic effects, of these organic arsenic compounds have not been studied.

In the present study, we investigated, for the first time, the immunotoxic effects of AsBe, AsCho and TetMA on murine principal immune effector cells, macrophages and lymphocytes, comparing them with the effects of inorganic arsenicals.

EXPERIMENTAL

Reagents

Arsenocholine (AsCho), arsenobetaine (AsBe) and tetramethylarsonium iodide (TetMA) were prepared by the procedures described in our previous reports.[14–17] Arsenite and arsenate were purchased from Wako Pure Chemical Co. (Osaka, Japan). Lipopolysaccharide (LPS; from *E. coli* 0111:B4) and concanavalin A (Con A) were obtained from Difco Laboratories (Detroit, MI, USA) and Sigma Chemical Co. (St. Louis, MO, USA), respectively.

Mice

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

Macrophages

Peritoneal macrophages (PMs) or alveolar macrophages (AMs) were collected from anesthetized mice 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 tetraacetate as described previously.[18,19] The cells were washed twice with warmed PBS (37 °C), resuspended in Eagle's MEM medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (FCS–MEM) and incubated for 2 h at 37 °C in a CO_2 incubator. After incubation, the cells were washed again with warmed PBS to remove any nonadherent cells; at least 97% of the washed cells were macrophages as by a Diff-Quick Stain kit (Kokusai Shiyaku Co., Hyogo, Japan) and by a nonspecific esterase stain text.

Lymphocytes

The spleens were removed from anesthetized mice and gently minced using an 80-gauge stainless steel screen in FCS–MEM. The cell suspensions were passed through a 130-gauge stainless steel screen to remove tissue fragments and they were then centrifuged (1500 rpm, 5 min). The pelleted cells were resuspended in a 0.83% (w/v) ammonium chloride solution to lyze erythrocytes, washed three times with PBS and resuspended in fresh FCS–MEM.

Assay for cytotoxicity

Single-cell suspensions of macrophages (5×10^4 cells/50 μl per well) or lymphocytes (splenocytes; 2.5×10^5 cells 50 μl per well) in FCS–MEM were incubated with various doses of arsenic compounds on flat-bottomed 96-well tissue culture plates for 48 or 72 h, respectively, at 37 °C in a CO_2 incubator. The cytotoxicity of cells was determined by measuring live cells by the AlamarBlue (AB) assay (similar to the MTT assay;[20] Iwaki Glass Co., Chiba, Japan). Six hours before the end of incubation 5 μL/well of AB solution 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).

Assay for lymphocyte blastogenesis

Lymphocytes (2.5×10^5 cells/well) were incubated with various doses of arsenic compounds on flat-bottomed 96-well tissue culture plates for 72 h at 37 °C in a CO_2 incubator in the presence of mitogenic concentrations of Con A (2.5 μg cm^{-3}) or LPS (50 μg cm^{-3}). The cell blastogenesis was determined by the AB assay.

RESULTS AND DISCUSSION

Peritoneal (PMs) and alveolar macrophages (AMs) isolated from CDF_1 mice were incubated

with various concentrations of arsenite (As^{3+}), arsenate (As^{5+}), AsChO, AsBe or TetMA for 48 h and the cytotoxic effects were investigated by the AB assay. As shown in Fig. 2, inorganic arsenicals were toxic to both PMs and AMs. The concentration of arsenite that decreased the number of surviving cells to 50% of that in untreated controls (IC_{50}) was about 5 μmol dm^{-3} for PMs and 4 μmol dm^{-3} for AMs, respectively. Morphological analysis showed that incubation with arsenite caused necrotic cell death in PMs; however, apoptotic cell death was induced on 30% of AMs by arsenite because some apoptotic bodies appeared during the culture period (Fig. 3). The cytotoxicity of arsenate was lower than that of arsenite because the IC_{50} of arsenate was about 1 mmol dm^{-3} for PMs and 400 μmol dm^{-3} for AMs, respectively. The morphological changes in macrophages caused by arsenate were similar to those caused by arsenite (data not shown).

In comparison with these findings, organic arsenic compounds in marine animals, AsCho, AsBe and TetMA, were less toxic even at concentrations over 10 mmol dm^{-3}. However, only TetMA induced slightly abnormal morpho-

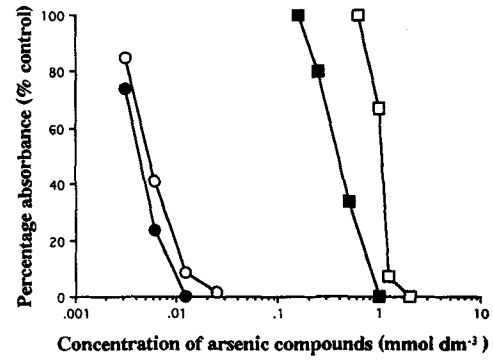

Figure 2 Cytotoxic effects caused by inorganic arsenicals in macrophages. Monolayers of PMs (○, □) or AMs (●, ■) isolated from CDF_1 mice were incubated with various concentrations of arsenite (○, ●) or arsenate (□, ■) for 48 h at 37 °C, and the cytotoxicity of cells was determined by measuring live cells by the AB assay. Results are expressed as the arithmetic mean of duplicate dishes from two similar separate experiments, and the S.D. was less than 4%.

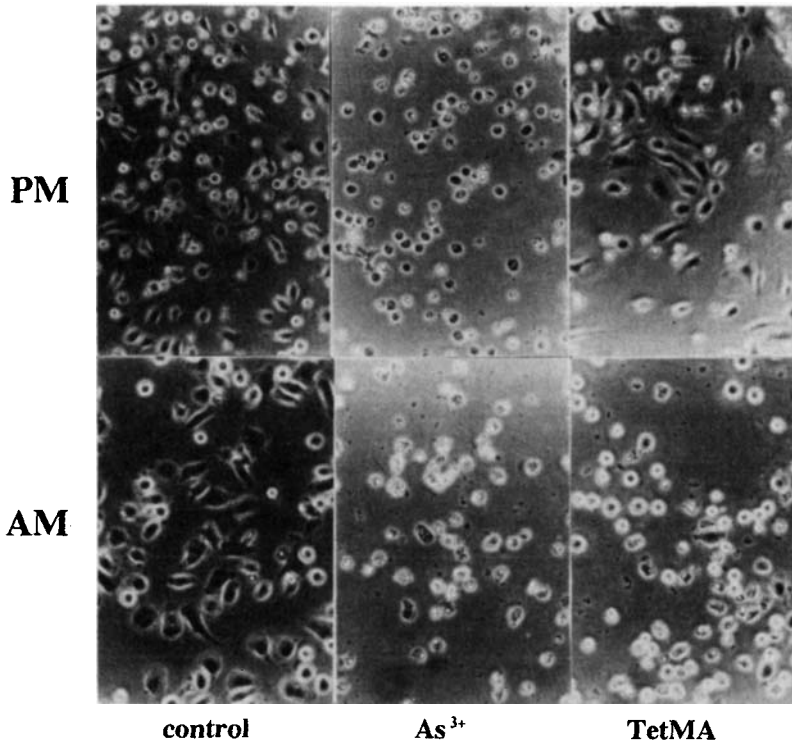

Figure 3 Morphological changes in macrophages by arsenic compounds. PMs or AMs isolated from CDF_1 mice were incubated with arsenite (12.5 μmol dm^{-3}), TetMA (10 mmol dm^{-3}) or medium alone for 48 h at 37 °C, and morphological changes in cells were observed. One representative experiment out of two performed is given.

logical changes on AMs at a high concentration, 10 mmol dm^{-3} (Fig. 3). In contrast, PMs were resistant to TetMA (Fig. 3). It is suggested that the sensitivity of AMs to arsenic compounds is greater than that of PMs. The characteristics of AMs are very different from those of PMs in many respects, such as their metabolic activity for energy requirement, cell-surface antigens and various functions.[21] It is possible that the difference in sensitivity to arsenic compounds between AMs and PMs may be dependent on the different characteristics of these two macrophages.

Subsequently, we examined the effects of arsenic compounds on lymphocytes. Lymphocytes (splenocytes) isolated from CDF_1 mice were incubated with various concentrations of arsenic compounds for 72 h and cytotoxic effects were investigated by the AB assay. Inorganic arsenicals and TetMA showed potent cytotoxicity to lymphocytes (Fig. 4, medium), though trimethylarsenic compounds in marine animals viz. AsCho and AsBe) had no toxicity even at concentrations over 10 mmol dm^{-3} (data not shown). The rank order of the cytotoxicity was: arsenite (IC_{50} value 3 μmol dm^{-3}) > arsenate (100 μmol dm^{-3}) > TetMA (6 mmol dm^{-3}). These cytotoxic arsenic compounds also decreased lymphocyte blastogenesis. Lymphocytes were cultured with various concentrations of arsenic compounds in the presence or absence of optimal mitogenic concentrations of T-lymphocyte mitogen (Con A; 2.5 μg cm^{-3}), or B-lymphocyte mitogen (LPS; 50 μg cm^{-3}) for 72 h at 37 °C, and lymphocyte blastogenesis was investigated using the AB assay. As shown in Fig. 4, inorganic arsenicals strongly inhibited the mitogenic actions of both T- and B-lymphocytes, and TetMA also inhibited them weakly. However, AsCho and AsBe had no inhibitory effect, even over 10 mmol dm^{-3} (data not shown). Morphological analysis showed that the addition of inorganic arsenicals strongly blocked the cell–cell aggregation of lymphocytes in the presence of both Con A and LPS, and TetMA also blocked it slightly (Fig. 5)

Addition of 10 mmol dm^{-3} NaCl, 10 mmol dm^{-3} D-glucose and 10 mmol dm^{-3} glycinebetaine, to evaluate the effect of 10 mmol dm^{-3} arsenic compounds on the osmotic pressure of the culture medium, did not influence the morphology of either macrophages or lymphocytes.

We have shown in this study that the trimethylarsenic compounds in marine animals, AsCho and AsBe, showed no immunotoxicity to either macrophages or lymphocytes, although inorganic arsenicals had strong toxicity. This finding is advantageous from the viewpoint of food hygiene. It is believed that methylation of inorganic arsenicals results in a lowering of their general toxicity. We previously demonstrated that trimethylarsine oxide, which is a final metabolite of inorganic arsenicals in mammalia, had less acute toxicity (LD_{50} > 10 g kg^{-1}) follow-

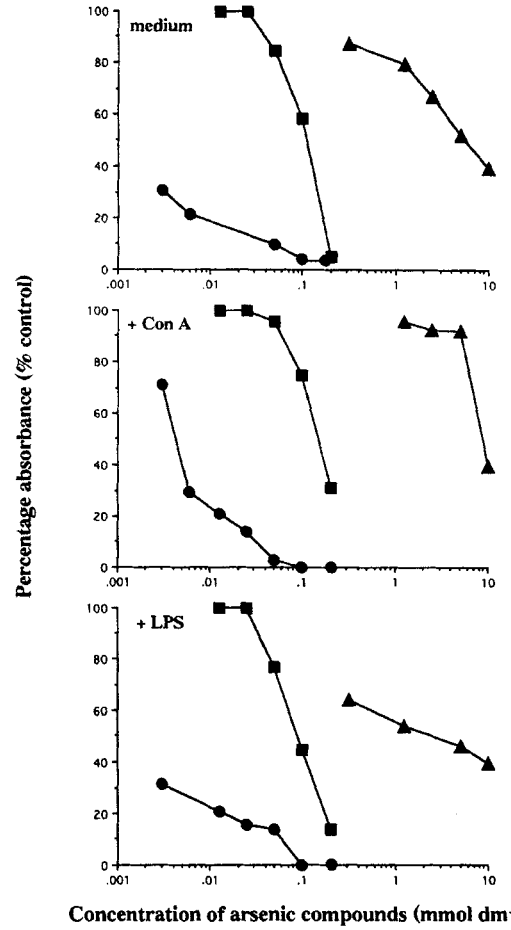

Figure 4 Effect of arsenic compounds on lymphocyte blastogenesis. Lymphocytes (splenocytes) isolated from CDF_1 mice were incubated with various concentrations of arsenite (●), arsenate (■) or TetMA (▲) in the presence or absence of Con A (2.5 μg cm^{-3}) or LPS (50 μg cm^{-3}) for 72 h at 37 °C, and lymphocyte blastogenesis was determined by measuring live cells by the AB assay. One representative experiment out of two performed is given. Results are expressed as the arithmetic mean of duplicate dishes from two similar separate experiments, and the S.D. was less than 15%.

ing oral administration in mice.[14] However, dimethylarsinic acid, which is a major metbolite of inorganic arsenicals in mammalia, showed strong acute toxicity ($LD_{50}=1.2$ g kg^{-1}).[14] Thus, it is also necessary to investigate the immunotoxic effects of dimethylarsenic-containing riboses in marine algae in terms of food hygiene because marine algae are consumed as food by people in some countries.

The tetramethylarsenic compound, TetMA, showed weak but significant immunotoxicity to both macrophages and lymphocytes. In our previous report, TetMA had acute toxicity ($LD_{50}=0.9$ g kg^{-1}) after oral administration to mice.[17] It has not been clarified why further methylated metabolites of trimethylarsenic compounds have significant immunotoxicity. TetMA has only been detected in the branchia of a clam, *Meretrix lusoria*,[11] or in lower marine animals such as the sea hare, *Aplysia kurodai*,[12] and the sea anemone, *Parasicyonis actinostoloides*.[12] These data imply that TetMA is produced by a minor metabolic pathway.

In conclusion, we have demonstrated in this study that trimethylarsenic compounds in marine animal, AsCho and AsBe, have no immunotoxicity to either macrophages or lymphocytes although the tetramethylarsenic compound,

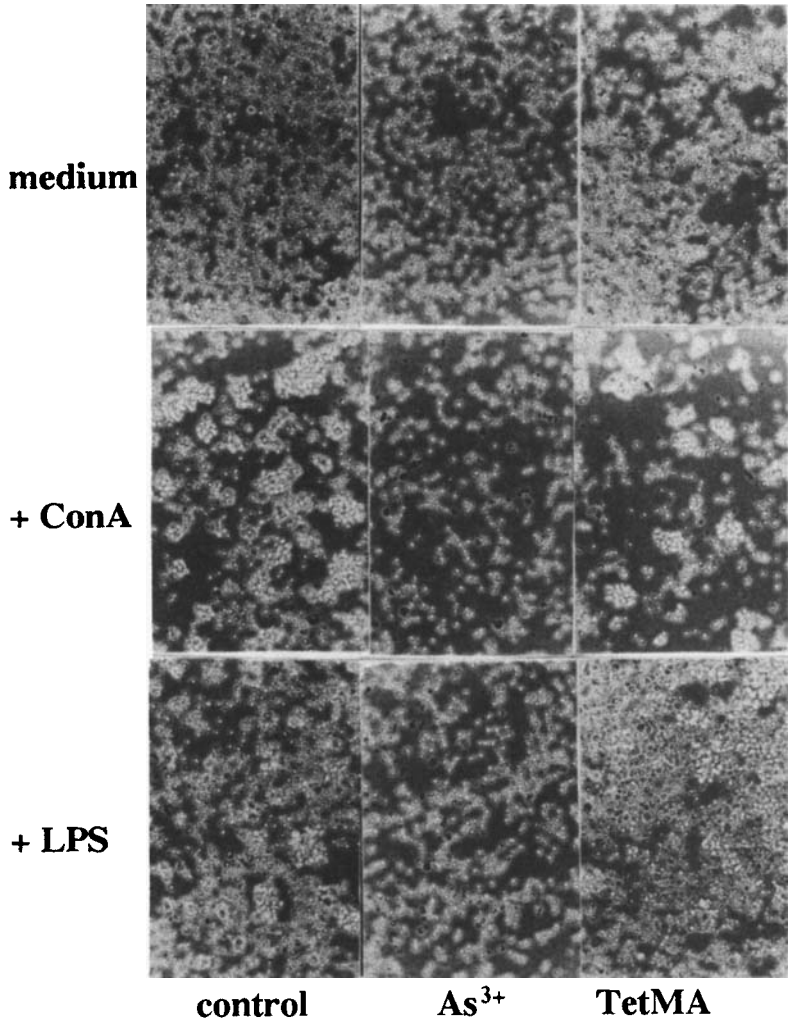

Figure 5 Morphological changes in lymphocytes caused by arsenic compounds. Splenocytes isolated from CDF_1 mice were incubated with arsenite (200 μmol dm^{-3}), TetMA (10 mmol dm^{-3}) or medium alone for 48 h at 37 °C, and morphological changes in cells were observed. One representative experiment out of two performed is given.

TetMA, showed weak cytotoxicity. Our study presents one of the first comprehensive analyses of the immunotoxic effects of organic arsenic compounds.

Acknowledgement This work was supported by the Sasakawa Scientific Research Grant from the Japan Science Society.

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