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Modulation of cell adhesion and viability of cultured murine bone marrow cells by arsenobetaine, a major organic arsenic compound in marine animals

*,[1]Teruaki Sakurai & [1]Kitao Fujiwara

[1]Laboratory of Environmental Chemistry, School of Life Science, Tokyo University of Pharmacy and Life Science, Horinouchi 1432-1, Hachioji, Tokyo 192-0392, Japan

1 In this study, we investigated the biological effects of trimethyl (carboxymethyl) arsonium zwitterion, namely arsenobetaine (AsBe), which is a major organic arsenic compound in marine animals using murine bone marrow (BM) cells and compared them with those of an inorganic arsenical, sodium arsenite, *in vitro*.

2 Sodium arsenite showed strong cytotoxicity in BM cells, and its IC_{50} was 6 μM. In contrast, AsBe significantly enhanced the viability of BM cells in a dose-dependent manner during a 72-h incubation; about a twofold increase in the viability of cells compared with that of control cells cultured with the medium alone was observed with a μM level of AsBe.

3 In morphological investigations, AsBe enhanced the numbers of large mature adherent cells, especially granulocytes, during a 72-h BM culture. When BM cells were cultured together with AsBe and a low dose (1 u ml^{-1}) of recombinant murine granulocyte/macrophage colony-stimulating factor (rMu GM-CSF), significant additive-like increasing effects were observed on the numbers of both granulocytes and macrophages originated from BM cells. However, AsBe did not cause proliferation of BM cells at all as determined by colony-forming assay using a gelatious medium.

4 These findings demonstrate the unique and potent biological effects in mammalian cells of AsBe, a major organic arsenic compound in various marine animals which are ingested daily as seafood in many countries.

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Keywords: Arsenobetaine; arsenical; arsenic compound; bone marrow cell; viability; granulocyte; macrophage; marine product; granulocyte/macrophage colony stimulating factor

Abbreviations: AB, AlamarBlue; AsBe, arsenobetaine; BM, bone marrow; Con A, concanavalin A; rMu GM-CSF, recombinant murine granulocyte/macrophage colony-stimulating factor; PP, Peyer's patch

Introduction

For centuries, arsenic has had the reputation of being a poison (Le *et al*., 1994). Epidemiological studies have proposed that an inorganic arsenical, arsenite, has high toxicity; its LD_{50} in mice is 0.035 g kg^{-1} by oral administration (Kaise *et al*., 1985), it has also been shown to be carcinogenic in experimental animals and humans (IARC, 1987). We previously reported that marine animals, which are ingested daily as seafood in many countries, contain very high concentrations of arsenic, such as fish (the average arsenic concentration is about 4 μg g^{-1}), crustacea (including crab, lobster and shrimp; 30 μg g^{-1}), snails (78 μg g^{-1}), clams (4 μg g^{-1}), sea slugs (8 μg g^{-1}), sea urchins (23 μg g^{-1}) and cuttlefish (4 μg g^{-1}) (Kaise *et al*., 1988a). These arsenicals are generally in the form of water-soluble organic arsenic compounds and trimethyl (carboxymethyl) arsonium zwitterion, namely arsenobetaine (AsBe; see Figure 1), is a major organic arsenic compound in marine animals (Edmonds *et al*., 1977). It has been suggested that AsBe is the final metabolite in the arsenic cycle in marine ecosystems because AsBe is widely distributed in various species of marine animals.

The limit for arsenic in drinking water in Japan, 10 μg l^{-1}, 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 unfit for consumption, given that their contents are often 1000 times this concentration (Le *et al*., 1994). This finding has caused great concern with respect to the health of people who often ingest considerable amounts of seafood. It is, therefore, necessary to investigate the effects of AsBe in living systems; however, there have been few reports on them because sufficient amounts of pure AsBe for biological experiments had not been obtained. In 1985, our collaborator first reported the acute toxicity of AsBe using synthesized pure AsBe and found that it had no acute toxicity in murine models even over 10 g kg^{-1} when it was orally administered (Kaise *et al*., 1985). Subsequently, using this synthesized material we observed that the *in vitro* cytotoxicity of AsBe was very weak compared with that of inorganic arsenicals in cultured murine macrophages and splenocytes (Sakurai *et al*., 1996) additionally, AsBe did not induce chromosomal aberrations in human fibroblasts (Oya-Ohta *et al*., 1996). Irvin & Irgolic (1988) also documented that AsBe had no

*Author for correspondence: E-mail: sakurai@ls.toyaku.ac.jp

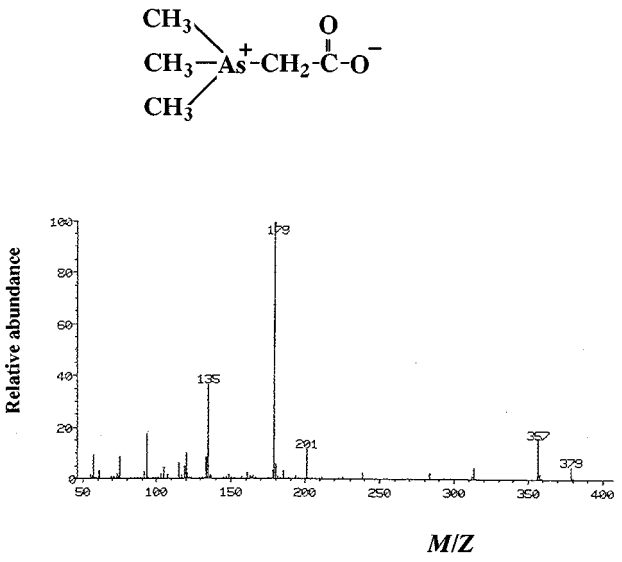

Figure 1 Primary structure of AsBe and the f.a.b.-m.s. of synthesized AsBe. The f.a.b.-m.s. was performed using a JMS DX-300 mass spectrometer (JEOL Co., Tokyo) equipped with f.a.b. ion source and xenon atoms at 6 KeV. The f.a.b.-m.s. of AsBe showed m/z 135 $[M\text{-}CO_2+H]^+$, m/z 179 $[M+H]^+$, m/z 201 $[M+Na]^+$, m/z 357 $[2M+H]^+$ and m/z 379 $[2M+Na]^+$.

embryotoxicity using rat models. Taken together, we believed that AsBe has no biological effects, including toxic effects, in living systems.

In this study, we examined whether AsBe has any biological effects on murine bone marrow cells (BM cells), which are known to be very sensitive to changes in environmental conditions, and compared these with the effects in murine lymphocytes. Interestingly, we found that AsBe modulated cell viability of BM cells *in vitro*, although it had no biological effects in lymphocytes at all.

Methods

Reagents

Sodium arsenite was purchased from the Wako Pure Chemical Co. (Osaka, Japan), trimethylarsine oxide was prepared from trimethylarsine using H_2O_2 as described elsewhere (Kaise *et al*., 1987), and they were twice recrystallized from water or benzene, respectively. AsBe was synthesized from trimethylarsine reacted with ethyl β-bromo-propionate in an atmosphere of carbon dioxide (Edmonds *et al*., 1977) and was twice recrystallized from acetone containing a trace of methanol (Kaise *et al*., 1985). It gave white prismatic crystals, m.p. 204°C, and its structure was confirmed by ^{1}H-n.m.r., ^{13}C-n.m.r., h.p.l.c-inductively coupled plasma mass spectrometry (h.p.l.c.-i.c.p.-m.s.) and fast-atom bombardment m.s. (f.a.b.-m.s.) (see Figure 1). The purities of these arsenicals were >99.9% as determined by t.l.c., h.p.l.c.-i.c.p.-m.s. and gas chromatography-m.s. (g.c.-m.s.). Lipopolysaccharide (LPS) contamination of AsBe was $<8.1\times10^{-7}$% (w w^{-1}) determined by the endotoxin specific limulus test. Glycinebetaine was purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). 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 10.

Cells

Cells were obtained from anaesthesized mice by ethyl ether. BM cells were prepared by flushing the femoral shafts using Eagle's MEM medium (MEM; Nissui Pharmaceutical Co., Tokyo) (Sakurai *et al*., 1992); thymocytes were prepared by grating the thymus using MEM medium. Peyer's patch lymphocytes (PP lymphocytes) were prepared by grating the PP using MEM medium containing 10% heat-inactivated fetal calf serum (FCS-MEM; LPS contamination of FCS was <6 pg ml^{-1} measured by the limulus test) (Sakurai *et al*., 1992). These cells were washed twice and resuspended in FCS-MEM medium.

Assay for viability of cells

BM cells (5×10^5 cells 100 μl^{-1} well^{-1}) or lymphocytes (thymocytes and PP lymphocytes; 2.5×10^5 cells 100 μl^{-1} well^{-1}) were incubated with arsenicals on flat-bottomed 96-well tissue culture plates for 72 h at 37°C in a CO_2 incubator using FCS-MEM medium. The viability of the cells was determined by measuring live cells by AlamarBlue (AB) assay, similar to MTT assay (Ahmed *et al*., 1994). Briefly, 6 h before the end of incubation, 10 μl well^{-1} 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, U.S.A.). The arsenicals themselves did not affect the absorbance of the AB solution even at concentrations over 40 mM.

Assay for thymocyte blastogenesis

Thymocytes (2.5×10^5 cells 100 μl^{-1} well^{-1}) were incubated with arsenicals 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 cell mitogen, concanavalin A (Con A; Sigma; 2.5 μg ml^{-1}), and the blastogenesis was determined by AB assay (Sakurai *et al*., 1996).

Determination of the numbers and the species of adherent cells originated from cultured BM cells

BM cells (5×10^5 cells 100 μl^{-1} well^{-1}) were incubated with samples on flat-bottomed 96-well tissue culture plates for 72 h at 37°C in a CO_2 incubator using FCS-MEM medium, then, the wells were washed twice with warmed phosphate buffered saline (PBS) to remove any nonadherent cells. The

species (granulocytes, macrophages or others) of the remaining adherent cells were determined by the cellular morphology (using a Diff-Quik stain kit; Kokusai Shiyaku Co., Hyogo), Mac-1 (CD11b) antigen expression (by immunohistochemical staining; see below), phagocytic ability (using LATEX beads; Difco Laboratories, Detroit, MI, U.S.A.) and the cellular non-specific esterase staining (Sakurai *et al*., 1992). The numbers of these adherent cells on the wells were counted under a microscope equipped with an eyepiece micrometer which was 40 mm^2 square.

Immunohistochemical staining

The adherent cells fixed by 3% paraformaldehyde on 96-well tissue culture plates were covered with 3% H_2O_2 for 1 h at room temperature, rinsed twice with PBS, and this was followed with 10% goat serum for 30 min at room temperature for blocking. The wells were then incubated with primary rat anti-mouse Mac-1 (CD11b) monoclonal antibody (MCA711; IgG2b; Serotec Co., Oxford) or rat IgG2b isotypic control (Cedarlane Co., Ontario, Canada), followed by secondary biotinylated goat anti-rabbit IgG (Caltag Lab., Burlingame, CA, U.S.A.), and were immunostained with peroxidase-conjugated streptavidin and 3,3′-diaminobenzidine/4HCl substrate kit (Nichirei Co., Tokyo). Mac-1 (CD11b)-positive cells appeared as dark brown cells.

Assay for colony-forming activity of BM cells

The colony-forming activity of BM cells was determined using a single layer methylcellulose system as described in our previous paper (Sakurai *et al*., 1992). Briefly, mouse BM cells (5×10^5) suspended in 1 ml of FCS-MEM medium containing 1.0% methylcellulose with or without AsBe and/or recombinant murine granulocyte/macrophage colony-stimulating factor (rMu GM-CSF; Boehringer Mannheim GmbH, Mannheim, Germany) were placed in a 35-mm diameter plastic dish and incubated for 7 days at 37°C in a CO_2 incubator. After the incubation, cellular aggregates of more than 50 cells were scored as colonies.

Arsenic analysis

BM cells (1×10^7/well) on flat-bottomed 12-well tissue culture plate were incubated with 10 mM AsBe 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 M NaOH and transferred into polypropylene tubes. Cell lysates in the tubes were heated at 80°C for 2 h in a water bath, cooled and neutralized with 1 M 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 (Kaise *et al*., 1988b). Briefly, 0.6 M HCl and 2% $NaBH_4$ in 0.2 M NaOH were continuously pumped through the mixing coil of the arsine generator at 6 ml min^{-1}. The generated arsines were collected in a U-shaped tube by cooling in liquid nitrogen and flashed into the g.c.-m.s. equipped with a cryofocus system using selected ion monitoring. AsBe was identified and quantified as trimethylarsine.

Statistics

All results are expressed as the arithmetic mean ± s.d., and data analysed by one-way analysis of variance (ANOVA) followed by a *post hoc* Scheffe's *F*-test (Suzuki *et al*., 2000), or an unpaired *t*-test (Table 1). Probabilities less than 5% ($P<0.05$) were considered significant.

Results

Effect of AsBe on the viability of lymphocytes

Figure 2 shows the effect of AsBe on the viability of thymocytes in the presence or absence of a T cell mitogen, Con A, compared with that of an inorganic arsenical, arsenite. Arsenite strongly decreased the viability of thymocytes incubated with or without Con A, and its IC_{50} was 6 or 5 μM, respectively. However, an organic arsenic compound in marine animals, AsBe, was less toxic in thymocytes even at concentrations over 10 mM. Similar results were observed using PP lymphocytes; the percentage of the viability of PP lymphocytes incubated with 10 mM AsBe compared with that of control cells was 78.9 ± 8.8%, and we previously reported that AsBe had no cytotoxicity in splenocytes even over 10 mM (Sakurai *et al*., 1996). These data imply that AsBe is less toxic in murine lymphocytes.

Effect of AsBe on BM cells

In contrast, AsBe showed an interesting biological effect on BM cells. Figure 3 shows the effect of AsBe on the viability of BM cells compared with that of arsenite. Arsenite showed strong cytotoxicity in BM cells with an IC_{50} of 6 μM; however, AsBe conversely increased the viability of BM cells in a concentration-dependent manner. About a 1.6 fold increase in the viability of BM cells compared with that of control cells cultured with medium alone was observed when the cells were incubated with over 5 mM AsBe for 72 h. This increasing effect was not observed when cells were incubated with trimethylarsine oxide or glycinebetaine, which were added as constituents of AsBe, at any concentration (Figure 3), and the simultaneous addition of trimethylarsine oxide and glycinebetaine also did not influence the viability of BM

Table 1 Effect of AsBe on the numbers of adherent cells originated from cultured BM cells

Incubation time	*Number of adherent cells*	
24 h	Medium	39.5 ± 0.7
	AsBe	114.0 ± 4.2*
72 h	Medium	85.5 ± 7.8
	AsBe	151.1 ± 14.8*

BM cells isolated from CDF_1 mice were incubated with AsBe (10 mM) or medium alone on 96-well tissue culture plates for 24 or 72 h at 37°C. After the incubation, wells were washed twice with warmed PBS to remove any nonadherent cells, and the numbers of remaining adherent cells were counted. Results are expressed as arithmetic mean ± s.d. of two BM cultures each performed in triplicate ($n=6$). *$P<0.05$ comparison with BM cells incubated with medium alone.

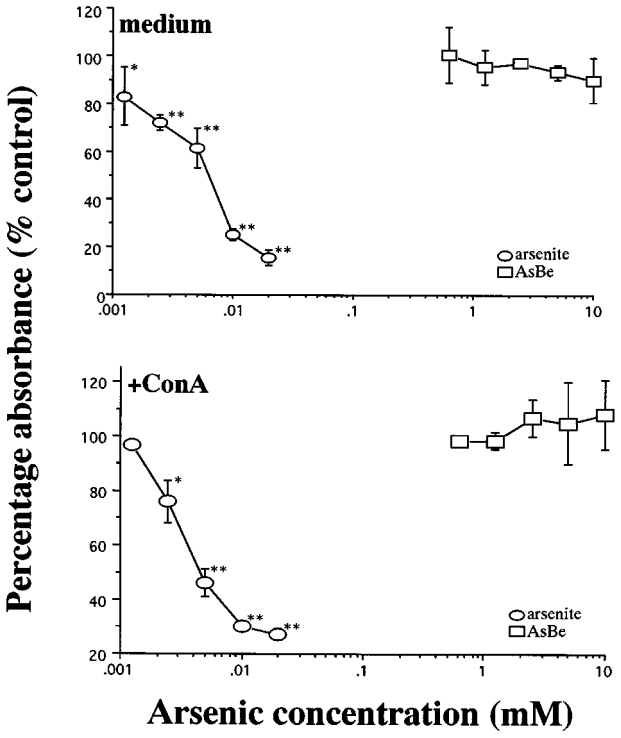

Figure 2 Effect of arsenicals on the viability of thymocytes. Thymocytes isolated from CDF_1 mice were incubated with various doses of arsenite, AsBe or medium alone in the presence or absence of Con A (2.5 μg ml^{-1}) for 72 h at 37°C, and the viability of the cells was determined by AB assay. Results are expressed as arithmetic mean ± s.d. of two thymocyte cultures each performed in duplicate dishes ($n=4$). *$P<0.05$ comparison with control thymocytes incubated with medium alone. **$P<0.001$.

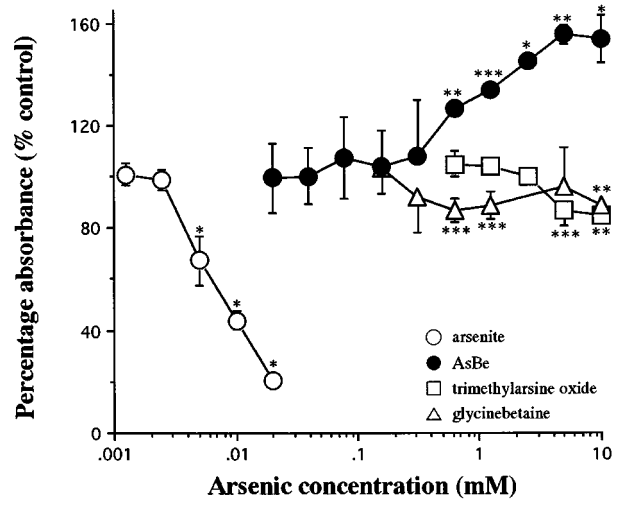

Figure 3 Effect of arsenicals on the viability of BM cells. BM cells isolated from CDF_1 mice were incubated with various doses of arsenite, AsBe, trimethylarsine oxide, glycinebetaine or medium alone for 72 h at 37°C, and the viability of cells was determined by AB assay. Results are expressed as arithmetic mean ± s.d. of two BM cultures each performed in duplicate dishes ($n=4$). *$P<0.001$ comparison with control BM cells incubated with medium alone. **$P<0.01$. ***$P<0.05$.

cells (data not shown). In the case of long-time (7-days) culture, AsBe was also effective on the number and the viability of BM cells; the numbers of survived BM cells incubated with medium alone (control) for 7 days were 87.0 ± 7.6 square^{-1} ($n=3$). In contrast, the numbers of survived cells incubated with 10 mM AsBe were 451.7 ± 37.1 square^{-1} ($n=3$, $P<0.001$ by unpaired t-test). In addition, the viability of BM cells incubated with AsBe compared with that of control cells (100%) was 640.0 ± 20.0% ($n=3$, $P<0.01$ by unpaired t-tests) at day 7 determined by AB assay.

In morphological investigations, AsBe (10 mM) increased the number of adherent cells from 24 h compared with control cells cultured with medium alone (Figure 4 and Table 1). After the 72-h incubation with AsBe, many large spreading adherent cells appeared, about twice the number compared with that of the control incubated with medium alone (Figures 4 and 5 and Table 1). The form of the nuclei and cytoplasm of these spreading adherent cells showed that 81.9 ± 10.3% ($n=3$) were polymorphonuclear cells (granulocytes) and remaining 18.1 ± 2.5% ($n=3$) were mononuclear cells (macrophages and other cells), 71.7 ± 3.7% ($n=3$) of these spreading adherent cells were Mac-1^{low+} (granulocytes),

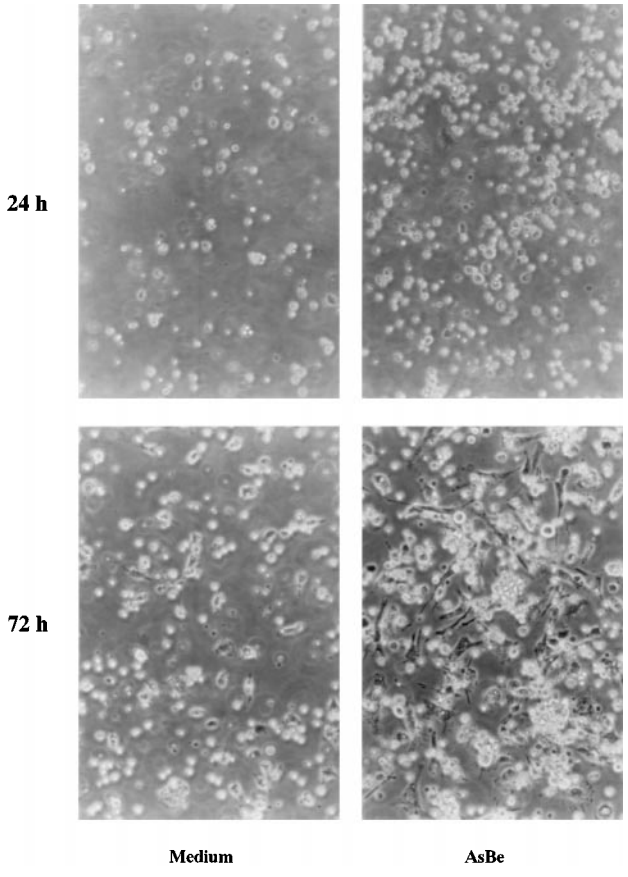

Figure 4 Effect of AsBe on the adherent ability of BM cells. BM cells isolated from CDF_1 mice were incubated with 10 mM AsBe or medium alone on 96-well tissue culture plates for 24 or 72 h at 37°C. After the incubation, the wells were washed twice with warmed PBS to remove any nonadherent cells, and the remaining adherent cells on the culture plates were observed. This experiment has been repeated three times, yielding the same results, and one representative experiment is given. The magnification of the microphotographs is ×200.

22.9±3.1% ($n=3$) were Mac-1^{high+} (macrophages) and 5.4±0.6% ($n=3$) were Mac-1^{-} (other cells) as determined by immunohistochemical staining using an anti-mouse Mac-1 monoclonal antibody. Additionally, 96.2±1.4% ($n=3$) of adherent mononuclear cells had potent phagocytic ability (macrophages) determined by using LATEX beads, and 92.3±5.5% ($n=3$) of these cells were also positively stained by cellular non-specific esterase staining which is a marker of macrophages. Taken together, about 70–80% of spreading adherent cells which appeared after the 72-h incubation of BM cells in the presence of AsBe were granulocytes, about 15–20% were macrophages and remaining 5–10% were other mononuclear cells. However, as shown in Table 2, AsBe increased only the numbers of granulocytes originated from immature BM cells, about twice the numbers compared with that of the control, but it had no effect on the numbers of macrophages. These findings suggested that AsBe induced initial cell adhesion, and continuously increased cell survival of adherent spreading cells, especially granulocytes.

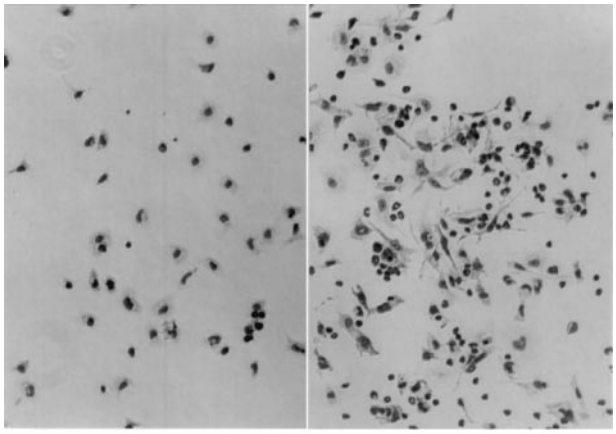

Figure 5 Diff-Quik staining of adherent cells generated from BM cells by the incubation with AsBe. BM cells isolated from CDF_1 mice were incubated with 10 mM AsBe or medium alone on 96-well tissue culture plates for 72 h at 37°C. After the incubation, the wells were washed twice with warmed PBS to remove any nonadherent cells, and the remaining adherent cells on the culture plates were stained using a Diff-Quik stain kit. This experiment has been repeated three times, yielding the same results, and one representative experiment is given. The magnification of the microphotographs is ×200.

Combined effect of AsBe and rMu GM-CSF on the numbers of granulocytes/macrophages originated from BM cells and the colony-forming activity of BM cells

Table 2 shows the combined effects of AsBe and rMu GM-CSF on the numbers of granulocytes/macrophages originated from cultured BM cells. GM-CSF is a potent cytokine which induces differentiation and proliferation of immature BM cells to a granulocyte/macrophage cell lineage. BM cells were incubated with AsBe (10 mM), rMu GM-CSF (1 or 10 u ml^{-1}), AsBe plus rMu GM-CSF or medium alone (control) for 72 h at 37°C, and the numbers and species of adherent cells originated from immature BM cells were determined. AsBe (10 mM) alone significantly increased only the numbers of granulocytes, about twice the number compared with the control. A low dose, 1 u ml^{-1}, of rMu GM-CSF also enhanced only the generation of granulocytes, about twice the control. When BM cells were cultured together with AsBe (10 mM) and a low dose of rMu GM-CSF (1 u ml^{-1}), significant additive-like effects were observed on the numbers of both granulocytes and macrophages originated from BM cells, especially on macrophages. In the presence of a high dose (10 u ml^{-1}) of rMu GM-CSF, it alone strongly induced the generation of both granulocytes (about three times that of the control) and macrophages (about twice that of the control); no additive-like effects were observed between AsBe and rMu GM-CSF.

We subsequently examined whether AsBe induced the cell differentiation and/or the proliferation of immature BM cells. BM cells were cultured with 10 mM AsBe and/or 1 or 10 u ml^{-1} rMu GM-CSF in FCS-MEM medium containing 1.0% methylcellulose for 7 days. If AsBe has the cell differentiating and/or proliferating effects on BM cells, cell colonies induced from one BM cell should have appeared in this gelatinous medium. As a result, many colonies appeared from BM cells cultured with rMu-GM-CSF; however, no colonies were observed in the incubation with AsBe alone (Table 3). When BM cells were cultured together with AsBe (10 mM) and 1 or 10 u ml^{-1} rMu GM-CSF, no additive-like effects were observed on the numbers of colonies (Table 3).

Effect of BM cell culture supernatants pre-treated with AsBe on the viability of other fresh BM cells

BM cells were incubated with 10 mM AsBe or medium alone for 24 h, and the culture supernatants containing AsBe were

Table 2 Effect of AsBe and rMu GM-CSF on the numbers of adherent cells originated from cultured BM cells*

Samples		*Number of adherent cells (square^{-1})*		
AsBe	*rMu GM-CSF*	*Granulocytes*	*Macrophages*	*Total*
–	–	60.0±15.6	12.3±0.5	72.3±15.8
10 mM	–	130.0±16.3**	16.7±3.9	158.7±12.0***
–	1 u ml^{-1}	136.3±15.6***	13.0±2.2	161.0±25.7**
10 mM	1 u ml^{-1}	211.3±15.9****,*****	37.0±5.0***,*****	260.0±17.2****,******
–	10 u ml^{-1}	186.3±9.7****	21.7±3.3**	224.3±9.9****
10 mM	10 u ml^{-1}	180.0±5.7****	23.7±7.4	206.7±8.7****

*BM cells isolated from CDF_1 mice were incubated with AsBe, rMu GM-CSF, AsBe+rMu GM-CSF or medium alone on 96-well tissue culture plates for 72 h at 37°C. After the incubation, wells were washed twice with warmed PBS to remove any nonadherent cells, and the numbers and the species of remaining adherent cells were determined. Results are expressed as arithmetic mean±s.d. of triplicate dishes. **$P<0.05$ comparison with BM cells incubated with medium alone. ***$P<0.01$. ****$P<0.001$. *****$P<0.01$ comparison with BM cells incubated with rMu GM-CSF alone. ******$P<0.05$.

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 that did not contain AsBe, but were thought to contain some factors which were secreted from the BM cells stimulated by AsBe 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 4, the addition of the culture supernatants collected after the first 24-h incubation with AsBe enhanced the viability of fresh BM cells, and it significantly increased the number of only granulocytes originated from fresh BM cells; however, the culture supernatants collected after the second 48-h incubation did not influence the viability of fresh BM cells. The other culture supernatants which were collected after 72-h continuous incubation with AsBe showed a potent enhancing effect on the viability of fresh BM cells and also significantly increased only the numbers of granulocytes originated from fresh BM cells.

Table 3 Effect of AsBe on the colony-forming activity of BM cells

Samples		
AsBe	*rMu GM-CSF*	*Colonies dish*$^{-1}$
–	–	0.0±0.0
10 mM	–	0.0±0.0
–	1 u ml^{-1}	45.3±10.0*
10 mM	1 u ml^{-1}	44.0±14.9*
–	10 u ml^{-1}	93.3±13.3**
10 mM	10 u ml^{-1}	104.3±24.8*

BM cells (5×10^5) isolated from CDF_1 mice were suspended in 1 ml of FCS-MEM medium containing 1.0% methylcellulose with AsBe, rMu GM-CSF, AsBe+rMu GM-CSF or medium alone. The cell suspension was placed in a 35-mm diameter plastic dish and incubated for 7 days at 37°C. After the incubation, cellular aggregates with more than 50 cells were scored as colonies. One representative experiment out of three similar performed is given. Results are expressed as arithmetic mean±s.d. of triplicate dishes. *$P<0.01$ comparison with control BM cells cultured with medium alone. **$P<0.001$.

Cellular uptake of AsBe in BM cells

BM cells (1×10^7/well) on a flat-bottomed 12-well tissue culture plate were incubated with 10 mM AsBe for 72 h at 37°C, and the amounts of arsenicals in the BM cell lysates were determined by the fully automated continuous arsine generation system as described in Methods. As a result, 720 ng per 1×10^7 BM cells of trimethylarsenicals was determined from the BM cell lysates. Other kinds of arsenic compounds were not detected.

Discussion

Although certain arsenic compounds are toxic in animals and humans, others are not (Le *et al*., 1994). The toxicity of arsenic varies a great deal with the chemical species. An inorganic arsenical, arsenite, had been used as a traditional drug in Europe (namely Fowler's solution) and Asia (as a kampo medicine), but it was also known to have toxic side effects. Recently, there have been a few reports on the biological effects of arsenite. Meng described that arsenite at very low concentrations, <1 μM, significantly enhanced the DNA synthesis in phytohemagglutinin-stimulated human lymphocytes *in vitro*, but it was cytotoxic at over 1 μM (Meng, 1994). Chen *et al*. also reported a similar result in which arsenite had dose-dependent dual effects in human acute promyelocytic leukemia cell line NB4 cells, inducing partial differentiation at low concentrations (nM level) and preferentially inducing cell death at relatively high concentrations (μM level) (Chen *et al*., 1997; Shen *et al*., 1997). These reports imply that the toxicological or biological role of an inorganic arsenical, arsenite, in mammalian cells is very complex; therefore, it is suggested that the clinical applications of an inorganic arsenical such as arsenite may have many problems.

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, namely arsenosugars (Edmonds & Francesconi, 1981). They are further methylated and converted to trimethyl arsenic compounds, such as AsBe, in many species

Table 4 Effect of culture supernatants of BM cells incubated with AsBe on the viability of fresh BM cells*

Culture supernatants		*Viability* (percentage absorbance) (% control)	*Number of adherent cells* (square^{-1}) *Granulocytes*	*Macrophages*	*Total*
First 24 h	medium	105.9±5.4	18.3±4.8	38.3±14.3	58.7±7.9
	AsBe	130.3±3.3***	52.7±11.7****	17.7±5.8	72.0±8.3
Second 48 h	medium	111.7±3.1	14.3±2.5	35.0±5.0	55.7±4.5
	AsBe	113.6±0.0	22.0±2.8	13.7±5.0	44.7±2.4
Total 72 h**	medium	116.0±11.4	18.3±0.5	38.3±3.3	60.3±4.6
	AsBe	141.6±2.3***	56.7±15.6****	30.7±9.0	92.0±7.8****

*BM cells isolated from CDF_1 mice were incubated with AsBe (10 mM) 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 and the species of the adherent cells were determined. One representative experiment out of four similar performed is given. Results are expressed as arithmetic mean±s.d. of triplicate dishes. **BM cells isolated from CDF_1 mice were incubated with AsBe 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. ***$P<0.01$ comparison with fresh BM cells cultured with the supernatants of BM cells pre-incubated with medium alone for the same period. ****$P<0.05$.

of marine animals, including fish, crab, lobster and shrimp (Edmonds *et al*., 1977; Kaise *et al*., 1988a). AsBe has been thought to be a final metabolite of arsenicals in the marine ecosystem because it is widely distributed in various marine animals. We recently reported that arsenosugar enhanced the viability of only murine peritoneal macrophages *in vitro*, but it is conversely cytotoxic in other cells (Sakurai *et al*., 1997). In contrast, using the synthesized pure material, we demonstrated that the toxicity of AsBe in mammals is very weak. First, we reported that AsBe had no acute toxicity when it was orally administered to mice (Kaise *et al*., 1985) and also described that AsBe was not toxic *in vitro* in murine macrophages and splenocytes even at concentrations over 10 mM (Sakurai *et al*., 1996). Thus, we had been believed that AsBe had no biological effects, including toxic effects, in mammals. In this study, however, AsBe showed an interesting biological effect only on murine BM cells which are known to be very sensitive to changes of environmental conditions; AsBe significantly enhanced the cell viability of BM cells *in vitro* in a concentration-dependent manner. AsBe was effective from a μM level in BM cells (Figure 3), although it did not influence the other immune effector cells, such as thymocytes (Figure 2), PP lymphocytes, splenocytes and macrophages (Sakurai *et al*., 1996). The findings disclosed in this study represent the first report of a biological effect in mammalian cells of AsBe, a major organic arsenic compound in marine animals which are ingested daily as seafood in many countries.

As shown in Figures 4 and 5 and Tables 1 and 2, AsBe enhanced the initial adhesion and the viability of BM cells, and it also increased the continuous survival of Mac-1 (CD11b) positive large spreading cells, especially granulocytes originated from immature BM cells. The reasons why AsBe enhances the survival of immature BM cells are not yet precisely clarified. As shown in Table 3, AsBe had no effect at all on the proliferation of BM cells; although GM-CSF, which is a major cytokine for the maturity of BM cells, induced both cell proliferation and differentiation of these cells. These data imply that the mechanism of AsBe-induced modulation of viability of immature BM cells was different from mechanisms previously reported. Because the culture supernatants of BM cells pre-stimulated with AsBe had no effect on the viability of other fresh BM cells (Table 4), we postulated that the effect of AsBe in BM cells might be direct rather than due to autocrine mechanisms. This suggestion was supported by the experiments showing that AsBe was actually taken up into the BM cells; 720 ng of trimethylarsenicals per 1×10^7 BM cell was detected from BM cell lysates after 72-h incubation with 10 mM AsBe. AsBe first enhanced the adhesion of BM cells during the initial 24-h incubation (Figure 4) and continuously increased the survival of these cells, resulting in the maturation of these cells to large adherent cells, especially granulocytes, during the 72-h incubation (Figures 4 and 5). It is well established that the oxidation state of the arsenic molecule influences the type and severity of biological effects (Le *et al*., 1994; Zakharyan *et al*., 1995). For example, arsenical has a very high affinity for thiol groups when it has a trivalent oxidation state; in contrast, it can replace phosphate when it has a pentavalent oxidation state. It is suggested that the initial enhanced adhesion of BM cells induced by AsBe may depend on the conformational changes in cell surface proteins by the binding of AsBe on the cell surface thiol groups and/or phosphate. Further experiments are needed to clarify the chemical binding form and oxidation state of AsBe taken up into the BM cells.

It has also been reported that the biological effects of arsenic compounds depend on their chemical structures (Le *et al*., 1994; Zakharyan *et al*., 1995). In this study, significant modulating effects on the viability of BM cells were observed only with AsBe (Figure 3) but not with any other inorganic and organic arsenic compounds, such as sodium arsenite and trimethylarsine oxide (Figure 3). Glycinebetaine, the nitrogenous analogue of AsBe, did not have effects on BM cells (Figure 3), and the simultaneous addition of trimethylarsine oxide and glycinebetaine was also without effect. In this study, we demonstrated that AsBe was not methylated or demethylated in BM cells with a fully automated continuous arsine generation system using g.c.-m.s. Taken together, these findings suggest that the chemical structure of AsBe is a very important factor, at least in part, for the expression of the significant effects on the viability of immature BM cells. In our preliminary experiment, a weak effect on the survival of BM cells was observed with trimethyl (2-hydroxyethyl)-arsonium cation, namely arsenocholine, which has a chemical structure similar to that of AsBe and is thought to be a precursor of AsBe in marine animals (Kaise *et al*., 1992). Furthermore, this biological effect was not observed with any other methyl arsenic compounds, such as monomethylarsonic acid, dimethylarsinic acid and tetramethylarsonium hydroxide (Sakurai *et al*., 1999).

It is very interesting that this unique biological effect was found with AsBe, a major arsenic compound contained in large quantities in various marine animals which are ingested daily as seafoods in many countries. In immunocompromised hosts, such as individuals receiving drug therapy or irradiation and patients with acquired immunodeficiency syndrome, severe infectious disease are frequently caused because the number of leukocytes, including granulocytes and macrophages, which are essential immunological components for the initial response to infectious microorganisms as phagocytes, are decreased. Therefore, it is likely that AsBe has a possible application as a biological response modifier (BRM) to increase the number of granulocytes and macrophages by increasing the cell survival of immature BM cells without fatal toxic side effects. Additionally, as shown in Table 2, there were significant additive-like increasing effects on the numbers of granulocytes and macrophages originated from BM cells between AsBe and a low dose (1 u ml^{-1}) of rMu GM-CSF. GM-CSF is one of the promising cytokines to use as a BRM, but it also has severe inflammatory toxic side effects when it is used at high doses, thus, the combination of AsBe and a low dose of GM-CSF may be useful for the clinical application of these reagents. However, some researchers have reported that AsBe ingested upon consumption of seafood was rapidly excreted, within 36 h, into the urine unchanged by the human subjects (Buchet *et al*., 1980; Cannon *et al*., 1981; Le *et al*., 1994). Our collaborator also previously described that AsBe was detected in the urine in the non-metabolized form after oral administration using synthesized AsBe in murine models (Kaise *et al*., 1985). Thus, it is necessary to investigate the *in vivo* effect of AsBe on BM cells, including detailed examinations for drug design and administration routes.

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