Evaluation of mitogen-induced responses in marine mammal and human lymphocytes by in-vitro exposure of butyltins and non-ortho coplanar PCBs

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“Capsule”: Butyltins may affect the immune response in marine mammals.

Abstract

The effects of exposure to butyltin compounds (BTs: tributyltin; TBT, dibutyltin; DBT and monobutyltin; MBT) and non-ortho coplanar PCBs (IUPAC 77, 126 and 169) on marine mammals and human lymphocyte were evaluated. Peripheral blood mononuclear cells (PBMCs) isolated from Dall’s porpoises (Phocoenoides dalli), bottlenose dolphins (Tursiops truncatus), a California sealion (Zalophus californianus), a larga seal (Phoca largha) and humans (Homo sapiens) were exposed at varying concentrations of BTs and coplanar PCBs. Concanavalin A (Con A)-stimulated mitogenesis found significantly suppressed (*P* < 0.01) when the cells were exposed at 300 nM (89 ng/ml) of TBT and 330 nM of DBT (77 ng/ml), while MBT showed little cytotoxicity at treatment levels of up to 3600 nM (620 ng/ml). BTs concentrations in the liver of Dall’s porpoises from Japanese coastal waters ranged between 81–450 ng/g for TBT and 200–1100 ng/g (wet wt.) for DBTs, which is greater than the cytotoxic levels registered in this study. In contrast, non-ortho coplanar PCBs did not suppress cell proliferation at concentrations of up to 30 nM (10 ng/ml). The residue levels of coplanar PCBs in the blubber of Dall’s porpoises were 0.12–1.3 ng/g, which were one order of lower than those levels that do cell proliferation. When cells were exposed to a mixture of TBT/DBT and coplanar PCBs, the proliferation was significantly reduced to 33 nM DBT plus 34 nM CB-77 and 33 nM DBT plus 28 nM CB-169 mixtures, respectively. The investigations relating the contaminant-induced immunosuppression in marine mammals have been focused on persistent organochlorines such as PCBs, pesticides and dioxin compounds. However, this study suggested the possibility of BTs could also pose a serious threat to the immune functions in free-ranging marine mammals and humans. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Immunosuppression; Peripheral blood mononuclear cells (PBMCs); Mitogen-induced response; Marine mammal; Butyltins; Non-ortho coplanar PCBs

1. Introduction

During the last two decades, approximately seven serious mass mortalities of marine mammals have been reported (Simmonds, 1992). Nearly, 20,000 harbour seals and several hundred grey seals were found dead in the North Sea in 1988 (Dietz et al., 1989). Similarly, mass mortalities of 2500 bottlenose dolphins and 8000 Baikal seals were found in the US east coast waters (Kuehl et al., 1991) and in the Lake Baikal (Grachev et al., 1989), respectively. When we consider such mortalities, three common features were observed to all these incidents. First, the animals were severely infected...
by virus. Secondly, most of them occurred near industrial regions or semi-closed ecosystems. Thirdly, elevated concentrations of persistent toxic contaminants were found in the organs of the affected animals.

Immunotoxical studies have been conducted with marine mammals such as harbour seals under semi-field conditions. Immune parameters such as T cell mitogen response, NK cell activity and delayed-type hypersensitivity have been investigated, and found that the contaminant levels in free-ranging harbour seals inhabiting polluted areas may be high enough to cause immunosuppression (Swart et al., 1994, Ross et al., 1995). Similarly, Shaw (1998) documented the impairment of immune functions in Pacific harbour seal pups by the accumulation of non- and mono-ortho coplanar PCBs and \( p,p'\)-DDE. In in-vitro assays, exposure to a mixture of PCB congeners (CB-138, CB-153 and CB-180: 5 ppm each) significantly reduced splenocyte proliferation in beluga whales, which were in the range of those found in the tissues of wild marine mammals (De Guise et al., 1998). Considering all these observations, it is likely that the chemical pollution cannot be ruled out as a cause of debilitating diseases and mass mortality that has occurred in the past.

In the experimental studies, butyltins (BTs) showed strong immunotoxicity, such as thymus atrophy, reduction in spleen weight and cytotoxicity to bone marrow and red blood cells at relatively low exposure concentrations (Boyer, 1989). Seinen and Penninks (1979) reported that \( ^{3}\text{H}\text{-thymidine incorporation into DNA in the rat thymus cells decreased at a concentration of 20 ng/ml DBT chloride, and it was completely inhibited at the concentration of 100 ng/ml DBT-Cl in the culture medium. Because BTs have been detected at parts per million levels in coastal cetaceans (Kannan et al., 1997; Tanabe et al., 1998), it is assumed that immunosuppression might have occurred in marine mammals. Based on these backgrounds, we examined the effects of the in-vitro exposure to BTs and coplanar PCB congeners on peripheral blood mononuclear cells (PBMCs) of marine mammals in this study. The human lymphocytes were also applied to this experiment to compare the results of immunological assay between marine and terrestrial mammals.

2. Experimental

2.1. Sample collection

Thirteen Dall’s porpoises were collected from Off Sanriku, the northeast coast of Japan, during January and February 1998 (Fig. 1). Details of the samples used in this study were shown in Fig. 1 and Table 1. The liver and blubber samples were packed in polyethylene bags and stored at −20 °C until chemical analysis. The blood samples from three Dall’s porpoises were collected in heparinized plastic tubes within 10 min following death of the animal, and kept under the ice (less than 4 °C) prior to the lymphocyte stimulation assay. The fresh blood samples were also collected from two bottlenose dolphins, one California sealion and one

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Table 1
Details of samples analysed in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Date of collection (1998)</th>
<th>Location</th>
<th>n</th>
<th>Sexa</th>
<th>Tissues analysedb</th>
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<tr>
<td>Dall’s porpoise (Phocoenoides dalli)</td>
<td>January–February</td>
<td>Off Sanriku</td>
<td>13</td>
<td>M: 10, F:3</td>
<td>Liver and blubber</td>
</tr>
<tr>
<td>Dall’s porpoise (Phocoenoides dalli)</td>
<td>January–February</td>
<td>Off Sanriku</td>
<td>3</td>
<td>M: 3</td>
<td>Bloodd</td>
</tr>
<tr>
<td>Bottlenose dolphin (Tursiops truncatus)</td>
<td>July–August</td>
<td>Aquarium (Fukuoka)</td>
<td>2</td>
<td>M: 2</td>
<td>Bloodd</td>
</tr>
<tr>
<td>California sealion (Zalophus californianus)</td>
<td>July</td>
<td>Aquarium (Shizuoka)</td>
<td>1</td>
<td>M: 1</td>
<td>Bloodd</td>
</tr>
<tr>
<td>Larga seal (Phoca largha)</td>
<td>July</td>
<td>Aquarium (Shizuoka)</td>
<td>1</td>
<td>M: 1</td>
<td>Bloodd</td>
</tr>
<tr>
<td>Human (Homo sapiens)</td>
<td>April</td>
<td>Matsuyama</td>
<td>2</td>
<td>M: 2</td>
<td>Bloodd</td>
</tr>
</tbody>
</table>

a M (male); F (female).

b Liver and blubber were analysed for butyltins and PCBs, respectively.

c The blood samples of three Dall’s porpoises were analysed for lymphocyte proliferation assay.

d Fresh samples (see Section 2).
larga seal housed in aquariums (Awashima Marine Park, Shizuoka and Marine World Umino-Nakamichi, Fukuoka). These samples were taken by venipuncture of the extradural veins and approximately 20–40 ml of whole bloods were collected from each animal. Additionally, two human blood samples (30 ml) were collected from volunteers with consent at School of Medicine, Ehime University.

2.2. Cell culture

Lymphocyte stimulation assays were carried out within 35 h of blood collection because mitogen-induced proliferation responses significantly decrease after that period (data not shown). The PBMCs were isolated by density gradient centrifugation on Ficoll-Histopaque-1077 (Sigma, USA) for 30 min at 400 × g. The PBMC layer was washed three times and resuspended in complete RPMI containing 10% heat-inactivated FCS (fetal calf serum), 100 unit/ml penicillin and 100 μg/ml of streptomycin, 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol. Con-A (Wako Pure Chemical, Japan) was used to stimulate the PBMCs. The PBMCs adjusted to 1 × 10⁵ cells/well, and Con-A (5 μg/ml) and varying concentrations of test chemicals were cultured in 96-well round-bottom culture plate at 37 °C in a humidified atmosphere of 5% CO₂, TBT–Cl, DBT–Cl, MBT–Cl and non-ortho coplanar PCBs, CB-77, 126, 169 were mixed into the culture medium at four to five concentrations, ranging from 0.30 to 3600 nM (0.062–890 ng/ml) of BT⁺ and 2.8 pM–34 nM (0.001–10 ng/ml) of the PCB congeners, respectively. All compounds were dissolved in DMSO, and the final DMSO concentration to be less than 0.5% in the medium. The eluates were prepared to be less than 0.5% in the medium.

Determination of total PCBs and non-ortho coplanar congeners were performed by a gas chromatograph with a mass selective detector (GC–MSD: Hewlett Packard 5890 Series II–5972 Series). A fused silica capillary column (30 m × 0.25 mm i.d.) coated with DB-1 (100% dimethyl polysiloxane, J&W Scientific, Folsom, CA) at 0.25 μm film thickness was used for the quantification. The PCB homologues were identified by selective ion monitoring (SIM) at m/z values of 254 and 256, 290 and 292, 324 and 326, 358 and 360, 392 and 394, 428 and 430 for the tri-, tetra-, penta-, hexa-, hepta- and octa-chlorobiphenyls, respectively. An equivalent mixture of Kanechlor 300, 400, 500 and 600 was used as the standard. PCB congeners were quantified from individual peak areas in the samples with the corresponding peak areas in the standard. Recoveries of PCBs and non-ortho coplanar congeners were examined by spiking 3.0 μg of total PCBs, 90 ng for CB-77, 91 ng for CB-126 and 30 ng for CB-169 into corn oil, and the percentages were 91, 113, 114 and 88%, respectively. The detection limits for the total PCBs and coplanar congeners were 0.1 and 0.03 ng/g, wet weight, respectively.

Butyltins were analysed by the method of Iwata et al. (1995) with some modifications. The liver tissues (1–2 g) were homogenized with 1 N HCl and 0.1% tropolone/acetone, and centrifuged at 3000 rpm for 15 min. The supernatant was transferred to 0.1% tropolone/benzene, and the moisture was removed by anhydrous Na₂SO₄. The BTs were propylated by adding propylmagnesium bromide as a Grignard reagent, and the excess of this reagent was decomposed by adding 1 N H₂SO₄. The extracts containing the BTs were passed through a Florisil packed wet column for cleanup. The BTs were measured using a gas chromatograph with a flame photometric detector (GC–FPD: Hewlett Packard Series II) and a fused silica capillary column (DB-1, described earlier). Monobutyltin trichloride, dibutyltin dichloride, tributyltin chloride of known amounts (0.16 μg each) were spiked into the liver of an Antarctic minke whale, containing trace levels of BTs for use as the external standard. Hexyltributyltin was added as the internal standard. A procedural blank was analysed with every set of six samples to check for interfering Na₂SO₄ and Soxhlet-extracted using diethyl ether:hexane (3:1) for 7 h. The fat content was determined after K-D (Kuderna-Danish) concentration of the extracts. An aliquot of the Soxhlet extract was refluxed in 1 N KOH-ethanol, followed by re-extraction into hexane, and purification by silica gel column chromatography. The eluates were concentrated and an aliquot was used for the determination of the PCB congeners except for the non-ortho coplanar PCBs. The remaining hexane extract was passed through a glass column packed with 125 mg activated carbon for separation of non-ortho coplanar PCBs (IUPAC 77, 126 and 169) from the other congeners.
compounds and to correct the sample values, if necessary. The average recovery rates of the BTs were 91%/C614, 121%/C614 and 115%/C65.2% for MBT, DBT and TBT, respectively. All the BTs concentrations in this study were presented as the corresponding ion, and they were not corrected for the recovery of the internal standard. The detection limits of MBT, DBT and TBT were 15, 4.0 and 2.0 ng/g wet weight, respectively.

2.4. Statistical analysis

For the proliferation assay, triplicates were averaged, and the mean and standard deviation were determined. A one-way analysis of variance (ANOVA) with Dunnett’s test was used to compare the different experimental groups to the control, using $P<0.01$ (#) for statistical significance.

3. Results and discussion

3.1. Cytotoxic levels of lymphocytes

An evaluation of mitogen-induced proliferation has been conducted in marine mammals and human lymphocytes using Con-A, PHA (Phytohaemagglutinin-M), PWM (Pokeweed mitogen) and LPS (Lipopolysaccharide from Salmonella typhimurium). Among them, Con-A, PHA and PWM were relatively active in the lymphocyte proliferation of these animals. Particularly, Con-A proved to be a most powerful mitogen (data not shown).

The proliferation activity of three Dall’s porpoises PBMCs in the presence of butyltin compounds and non-ortho coplanar PCBs were shown in Table 2. A significant decrease in the percentage of lymphocyte proliferation was found at concentrations of 300 nM for TBT (89 ng/ml) and 330 nM for DBT (77 ng/ml). The exposure to MBT resulted to a less significant decline at higher concentration levels (3600 nM; 620 ng/ml MBT), implying that MBT showed to less toxic than DBT and TBT. Similar observations were also reported in the values of LD50 among the BTs (TBT–Cl, 122–349 mg/kg, DBT–Cl, 112–219 mg/kg, MBT–Cl, 2020–2300 mg/kg; Snoeij et al., 1987). On the other hand, the suppression of lymphocyte proliferation did not observe with the exposures of non-ortho coplanar PCBs (2.8 pM–3.4 nM; 0.001–1 ng/ml) in Dall’s porpoises (Table 2). The proliferation activity of bottlenose dolphins when cultured with PCBs ($n=1$) and BTs ($n=2$) are presented in Fig. 2. All of the PCB congeners were less effective at concentrations of 2.8 pM–34 nM (0.001–10 ng/ml) in the culture medium. In contrast > 300 nM of TBT and DBT prevented the proliferation completely, which is in accord with the results of Dall’s porpoises. Besides, the effects on proliferation by the BTs and coplanar PCBs were also examined in a large seal, a California sealion and humans. The results also showed significant suppressions in the cell proliferation when exposed to TBT and DBT concentrations at > 300 nM, while exposure to MBT and coplanar PCBs has less effect (Figs. 3 and 4).

In the experimental studies, following the in-vitro BT treatment to immune cells, $^3$H-thymidine incorporation into the DNA of rat thymus cell has decreased at a concentration of 20 ng/ml of DBT–Cl, and was completely inhibited at a concentration level of 100 ng/ml (Seinen and Penninks, 1979). Whalen et al. (1999) suggested that TBT inhibited the tumour-killing capacity of NK cells at 200 nM. TBT concentration of 100 nM or greater in rat thymocytes increased the population of annexin V-positive live cells, suggesting the possibility of TBT-induced apoptosis or/and direct affects to cell membranes (Nakata et al., 1999). These inhibition levels were more or less similar to those levels obtained in this study. Although it is difficult to identify the cytotoxic

Table 2

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<td>1983c</td>
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<td>1478c</td>
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<td>20,832</td>
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<td>CB-77</td>
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<td>1921c</td>
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<td>34,082</td>
<td>29,129</td>
<td>CB-169</td>
<td>37,089</td>
<td>39,204</td>
<td>31,752</td>
<td>39,278</td>
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PBMC, peripheral blood mononuclear cell; TBT, tributyltin; DBT, dibutyltin; MBT, monobutyltin.

a Results are expressed in disintegrations per minute (dpm).
b 0.5% DMSO.
c $P<0.01$. 
mechanisms of PBMCs based on the present study, the effective concentrations of BTs to immune cells are in the range of several tens ppb of TBT and DBT, and its levels were less variable among mammalian species.

In-vitro studies of the exposure to PCBs congeners (IUPAC 138, 153, 180, 169) to beluga whale splenocytes showed reduced proliferation at concentrations of 20 and 25 ppm CB-138 (De Guise et al., 1998). Surprisingly, PCB 169 did not show any response to the exposed levels of 5–25 ppm, which are 500–2500 times greater than those tested levels. The lack of immunotoxicity of coplanar PCB congeners might be due to the possible low expression of the Ah receptor of beluga whale (Hahn et al., 1994). Few in-vivo studies suggested that the administration of 10 mg/kg CB-77 for 2 days prior and 2 days after immunization with SRBC (sheep erythrocytes) significantly decreased the percentage of thymus weight to body weight in C57BL/6 mice (Silkworth and Grabstein, 1982). Dose dependent inhibition of the splenic PFC (plaque-forming cell) response were observed when coplanar congeners such as CB-77, CB-126 and CB-169 were individually injected into B6C3F1 mice at concentrations of 120, 1.2 and 2.4 ng/g, respectively (Harper et al., 1995).

To assess any effects of combined chemicals, lymphocytes of bottlenose dolphins were exposed to a mixture of BTs and PCB coplanar congeners, and compared with the unexposed control (Fig. 5). When cells were exposed to mixtures of 30 nM TBT and 33 nM DBT and 2.8 pM–34 nM PCBs, proliferation was significantly reduced in mixtures of 33 nM DBT, and 34 nM CB-77 and 28 nM CB-169. In contrast, the mixture of 33 nM DBT and 31 nM CB-126 increased the proliferation. A mixture consisting of 5 ppm each of CB-138, 153 and 180 significantly reduced the splenocytes proliferation when compared with that of the control (De Guise et al., 1998). On the other hand, in-vitro exposure of rat leukocytes to low levels of methylmercury, PCDDs/DFs and PCBs mixtures had no suppressive effects on the immune functions analysed (Omara et al., 1998). While investigation should be conducted to examine any possible synergistic and/or antagonistic effects by mixture of these contaminants, this study might suggest the possibility of synergistic effects by the butyltins and coplanar PCBs in marine mammals.

3.2. Residue levels of PCBs and BTs in Dall’s porpoises and their implications for immunotoxicological potentials

PCBs and BTs were detected in the blubber and liver samples from all Dall’s porpoises, respectively (Table 3). PCBs concentrations in Dall’s porpoises were 6700 ± 1500 ng/g wet weight, and found that IUPAC-153 was the most prevalent congeners, followed by CB-138 and 180. CB-118 and 105 were predominant among mono-ortho PCB congeners, which were almost similar to those of other marine mammals (Minh et al., 2000). Non-ortho coplanar PCBs were detected with the mean
Fig. 3. Proliferation response of peripheral blood mononuclear cells (PBMCs) in California sealion (circle) and larga seal (square) following the treatment with non-ortho coplanar PCB congeners and butyltins. #: $P < 0.01$.

Fig. 4. Proliferation response of peripheral blood mononuclear cells (PBMCs) in humans following the treatment with non-ortho coplanar PCB congeners and butyltins. #: $P < 0.01$. 
levels of 0.50, 0.17 and 0.16 ng/g wet weight, for CB-77, 126 and 169, respectively (Table 3). Except for two individual porpoises, the animals showed less than 1 ng/g of non-ortho PCB congeners and butyltins.

Butyltin compounds were detected with a range of 260–1800 ng/g wet weight in the liver of Dall’s porpoises. Among the BTs, DBT was predominant (mean: 460 ng/g), followed by TBT (mean: 210 ng/g) and MBT (mean: 120 ng/g). The compositions of BTs were 15% for MBT, 58% for DBT and 27% for TBT in this study. The residue levels and compositions of BTs in Dall’s porpoises were almost similar to those reported for the same species collected from Japanese coastal waters in 1995 (Tanabe et al., 1998).

BTs residue levels in Dall’s porpoises were greater than the cytotoxic levels (approx. 100 ng/g of DBT and...
TBT) to Dall’s porpoise lymphocytes. On the other hand, non-ortho coplanar PCB concentrations in the blubber of Dall’s porpoises showed more than 10 times lower than those levels that suppress the proliferations (10 ng/g). The elevated concentrations of TBT and DBT (several µg/g) have been reported in marine mammals inhabiting Japanese coastal (Tanabe et al., 1998) and US (Kannan et al., 1997) waters. These findings may suggest that butyltins are more immunotoxic rather than PCBs in free-ranging marine mammals. On the other hand, PCB congeners, CB-138, 153 and 180 showed a significant reduction of splenocyte proliferations at concentrations of 5 ppm (De Guise et al., 1998), which are close to those measured in Japanese coastal cetaceans (Kim et al., 1996, Minh et al., 2000). The investigations of synergistic and/or antagonistic effects by individuals and mixture of these contaminants should be conducted to understand the realistic immunotoxicity by BTs and PCBs.

Butyltin compounds are environmental toxic contaminants with the potential for persistence in aquatic organisms. In particular, detrimental effects of BT were greater than that of TBT through the phagocytic activity of hemocytes in marine bivalves (Bouchard et al., 1999) and of carboxylesterases inhibition in fish (Al-Ghais et al., 2000). These results may suggest that DBT is one of the important immunotoxic compounds in marine biota combined with TBT. The immunotoxic effects of environmental contaminants have been speculated to be persistent organochlorines, such as PCBs, pesticides and dioxins in marine mammals so far. In addition, this study suggested that BTs such as TBT and DBT could also pose a serious threat to the immune systems of free-ranging marine mammals. An epidemiological investigation is warranted in order to elucidate the potential effects of BTs as well as persistent organochlorines.

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