Reduction in Peripheral Lymphocytes and Thymus Atrophy Induced by Organotin Compounds *In Vivo*

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ABSTRACT. To clarify the involvement of apoptosis in the immunotoxicity of organotin compounds, we examined the induction of apoptosis in the peripheral lymphocytes and thymus of mice treated with triphenyltin (TPT), tributyltin (TBT) or dexamethasone (Dex). Application of TPT or TBT and Dex resulted in a transient reduction in peripheral lymphocytes at 3 to 6 hr, and thymus atrophy was observed at 6 and 24 hr after administration. Lymphocyte subpopulation analysis showed that TPT and TBT induced a greater reduction in B cells than in T cells. The maximum levels of organotin in the blood were about 450 ng TPT/ml in the TPT-treated mice, and 170 ng TBT/ml in the TBT-treated mice. When the isolated peripheral lymphocytes were incubated with the organotins at 500 ng/ml, TPT and TBT induced necrosis in over 70% of cells, while both organotins caused lower percentages of apoptosis as well as necrosis after 3 hr at 100 ng/ml. In the thymus, although *in vivo* treatment of mice with Dex caused apoptosis, neither apoptotic nor necrotic thymocytes were observed in the TPT- and TBT-treated mice, indicating that the thymus atrophy might be caused by the antiproliferative effects of these organotin compounds. Thus, our results did not support the idea that apoptosis played a decisive part in the immunotoxicity of the organotin compounds *in vivo*.

KEY WORDS: organotin compounds, peripheral lymphocytes, thymus atrophy.

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The toxicity of organotin compounds has attracted special interest because of their expanding utilization and pollution resulting from organotin compounds in the form of plastic stabilizers, catalytic agents, industrial agricultural biocides, anti-fouling paints and pesticides [10]. Although the production and use of these organotin compounds are regulated, environmental contamination still persists [11–13]. Recently, the areas reporting organotin compounds as environmental pollutants are shifting from advanced countries to the developing countries [25]. Human exposure to organotin compounds may come from consumption of organotin-contaminated meat and fish products because food chain accumulation and bioconcentration have been demonstrated in some marine species [3].

Organotin compounds are reported to cause serious health problems, particularly endocrine disruption in some marine species [9] as well as in experimental animals [8], and are suspected to have immunotoxic effects in mammals [3, 29]. Among the toxic effects of organotin compounds, immunotoxicity is particularly notable in many species of mammals [18]. Triphenyltin (TPT), tributyltin (TBT) and dibutyltin (DBT) have been shown to induce thymus atrophy [22, 23, 29] *in vivo*, and depletion of cortical thymocytes has been observed upon treatment with these compounds. Previous studies have shown that relatively low doses of DBT [19] and TBT [6] selectively inhibit the

proliferation of immature CD4(–)CD8(+)CD3(–/low) and CD4(+)CD8(+)CD3(–/low) thymocytes without signs of apoptotic cell death. However, various studies have shown that organotins at high doses induce apoptosis in rat thymocytes *in vitro* and *in vivo* [2, 5, 7, 15, 17, 20] and suggest that exposure to TPT and TBT actually triggers apoptosis, rather than being directly cytotoxic. Moreover, Stridh *et al.* reported that TBT can induce caspase activation and apoptosis in human peripheral lymphocytes *in vitro* and suggested that TBT-induced deletion of peripheral lymphocytes is likely a component in the overall risk for immunotoxic responses in exposed humans [24]. Therefore, the participation of apoptosis induced by these organotin compounds has been a major theme in toxicological evaluation of organotins, although the relative contribution of apoptosis to the reduction of peripheral lymphocytes and thymus atrophy in whole body *in vivo* experiments remains unclear.

In the present study, in order to evaluate the contribution of apoptosis or necrosis in the reductions of peripheral lymphocytes and thymus atrophy induced by organotin compounds such as TPT and TBT in mice, we analyzed apoptotic cells in peripheral lymphocytes or thymocytes *in vitro* and *in vivo* by flow cytometry and the distribution and metabolism of the organotin compounds *in vivo*.

MATERIALS AND METHODS

Chemicals: Triphenyltin chloride (TPT) and diphenyltin dichloride (DPT) were obtained from Kanto Chemical Co., Inc., (Tokyo, Japan). Tri-n-butyltin chloride (TBT) and

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dibutyltin dichloride (DBT) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Dexamethasone (Dex) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan) as the positive control for induction of apoptosis. All chemicals used were of the highest quality commercially available.

Animals: The experiments were performed in accordance with Japanese legislation concerning the protection of animals and the 'Guide for the Care and Use of Laboratory Animals' (NIH publication no. 86-23, revised 1985) after approval by the Committee of Animal Experimentation at Kitasato University School of Veterinary Medicine. ICR male mice 8 weeks of age were purchased from SLC Inc. (Hamamatsu, Japan). The mice were fed a standard cubed diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan), maintained on distilled water *ad libitum* and exposed to a 12-hr light (7:00 AM to 7:00 PM) and 12-hr dark (7:00 PM to 7:00 AM) cycle. The ambient temperature during the study was maintained at about 21°C.

Treatment: TPT or TBT was dissolved in corn oil (Sigma Aldrich Co., St. Louis, MO, U.S.A.) and administered orally (10 ml/kg for mice). The dosage of the organotin compounds in our experiments was 180 μ mol/kg, respectively (TPT, 69 mg/kg; TBT, 63 mg/kg). These dosages are equivalent to the dosage (196 μ mol/kg) of TBT reported to induce apoptosis *in vivo* [20]. We also injected Dex as an apoptosis inducer at a dosage of 3 mg/kg intraperitoneally.

Extraction and measurement of organotin compounds from samples: The phenyltin or butyltin compounds were extracted from the blood or thymus and purified by the method of Suzuki *et al.* [26]. The levels of each organotin compound in the samples were determined by the method of Suzuki *et al.* [26], using an HP Model 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a split/splitless injection port connected to an HP Model 5921A atomic emission detector equipped with a turbo make-up gas valve.

Total and differential leukocyte counts: Blood samples were collected via heart puncture under ether anesthesia into tubes containing EDTA at 3, 6, 24 and 48 hr after treatment. Total peripheral blood leukocyte counts were obtained by staining with Turk's solution and counting using a hemocytometer. Differential cell counts were performed on blood smears stained with Wright-Giemsa dyes.

Analysis of lymphocyte subpopulation by flow cytometry: Peripheral lymphocytes were isolated from the blood using Lymphoprep (Nycomed, Oslo, Norway). Single-cell suspensions of lymphocytes, prepared as described above, were washed twice in cold Hanks' balanced salt solution (HBSS). After washing, 1×10^6 cells were resuspended in 50 μ l of PBS with 1% bovine serum albumin (BSA) and 0.01% sodium azide. Anti-mouse CD4-PE, anti-mouse CD8-FITC, anti-mouse CD3-FITC or anti-mouse CD45R (B220)-PE (Immunotech, Marseille, France) were added to the cell suspensions at appropriate dilutions, and the cell suspensions were then incubated for 30 min at 4°C to detect the respective cell surface markers. The cell suspensions

were washed twice with PBS containing 1% BSA and 0.01% sodium azide, and then the numbers of stained cells were determined by counting about 10,000 cells on a fluorescence-activated cell sorter (FACS 440, Becton Dickinson, Bedford, U.S.A.).

Analysis of apoptotic lymphocytes by flow cytometry: We also observed the ratio of necrotic or apoptotic cells in isolated peripheral lymphocytes treated with Dex or organotin compounds. Isolated peripheral lymphocytes (1.0×10^6 cells/ml) suspended in RPMI-1640 medium were incubated with Dex or organotin compounds at 37°C for 1 or 3 hr. After incubation, the lymphocytes were stained with FITC-Annexin V and propidium iodide (PI) using an Annexin-V-FLUOS Staining Kit (Roche Diagnostics GmbH, Penzberg, Germany) and analyzed by a fluorescence-activated cell sorter (FACS 440, Becton Dickinson, Bedford, U.S.A.). The flow cytometry test can discriminate intact cells (Annexin-/PI-), early apoptotic cells (Annexin+/PI-), late apoptotic cells or necrotic cells (Annexin+/PI+) and necrotic cells (Annexin-/PI+).

Analysis of DNA fragmentation: DNA fragmentation was analyzed as described previously with some modifications [14, 27]. Briefly, thymus tissues from the mice were gently glass-ground to dissociate the cells, suspended in cold HBSS and then gently passed through a stainless steel mesh to obtain single-cell suspensions of thymocytes. Cells (5×10^6) in HBSS were centrifuged, and the resulting pellet was suspended for 10 min at 4°C in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.5% Triton X-100). Samples were then centrifuged at $14,000 \times g$ for 5 min, and the supernatants containing the fragmented DNA were digested with 200 μ g/ml of RNase (Sigma Aldrich, St. Louis, MO, U.S.A.) for 1 hr at 37°C. The samples were then treated with 200 μ g/ml Proteinase K (Merck, Darmstadt, Germany) for 30 min at 50°C. The supernatants were precipitated overnight using isopropyl alcohol. DNA fragments were separated on 2.0% agarose gels, visualized with ethidium bromide and photographed.

Statistical analysis: The differences between the averages of 4 treated animals and the untreated control animals were compared using Dunnett's test after one-way ANOVA, and a p value of less than 0.05 was considered statistically significant. Data from studies with only two groups were analyzed by the Student's *t* test for equal variance or Welch's *t* test for unequal variance after Bartlett's test.

RESULTS

Concentration of TPT or TBT and their metabolites in the blood of mice: First, we analyzed the concentrations of organotins including their main metabolites in the blood at 0.5 to 6 hr after oral administration. In the TPT-treated mice, the levels of TPT in the blood reached a maximum level of 445 ng/ml at 1 hr after administration, and diphenyltin (DPT), a metabolite of TPT, was also detected in the same samples (Table 1). On the other hand, the levels of TBT in the blood were about 100 ng/ml at 3 hr after administration, at which

Table 1. The concentrations of organotin compounds in the blood of mice administered TPT or TBT

		TPT (ng/ml or g)	DPT (ng/ml or g)
TPT	0.5 hr	129.5 ± 98.0	ND
	1 hr	445.5 ± 148.2	256.1 ± 46.1
	3 hr	299.0 ± 189.3	2.1 ± 5.1
	6 hr	388.9 ± 163.0	ND
TBT	0.5 hr	85.1 ± 38.8	64.4 ± 4.5
	1 hr	83.8 ± 15.9	49.1 ± 21.3
	3 hr	104.4 ± 32.5	32.6 ± 7.8
	6 hr	174.2 ± 40.4	56.5 ± 42.9

TPT or TBT was administered orally (180 μ mol/kg), and the organotin compounds in the blood were analyzed by the GC-MS method. Values are indicated as means $\pm$ SD (n=4). ND: Not detected.

point a transient reduction in peripheral lymphocytes could be observed.

Effects of TPT or TBT on peripheral lymphocytes: Figure 1 shows the effects of TPT, TBT and Dex administration on the number of peripheral lymphocytes in mice. Significant transient decreases in peripheral lymphocytes were observed at 3 hr to 6 hr in the Dex-, TPT- and TBT-treated mice. In the TBT-treated mice, this transient decrease was smaller than that in the TPT-treated mice, and an increase induced by dehydration was observed after 48 hr. As shown in Fig. 2, flow cytometric analysis of the reduction rates of T-cells and B-cells in the peripheral lymphocytes of the mice treated with the TPT or TBT showed that the numbers of B-cells were dramatically reduced to about 30% and 24% of the control while those of T-cells were about 55% and 56% of the control after TPT or TBT treatment at 6 hr, respectively. It should be noted that the organotins induced greater reductions in B-cells than in T-cells.

Effects of exposure to TPT or TBT at the actual levels found in blood on isolated lymphocytes *in vitro*: Next, we tried to detect apoptotic lymphocytes in the blood of treated mice *in vivo*. Although the typical apoptotic thymocytes and DNA laddering were detected in the thymus from the Dex-treated mice, we could not detect the typical DNA laddering, annexin V (+) lymphocytes or PI (+) lymphocytes in any of the Dex-, TPT- or TBT-treated mice in the peripheral lymphocytes at 0.5 to 24 hr after administration (data not shown). Therefore, we observed the *in vitro* effects of these chemicals on isolated peripheral lymphocytes using the actual concentrations of organotins observed *in vivo*.

Figures 3 and 4 present the effects of the Dex (10^{-6} M = 392 ng/ml), TPT (100 or 500 ng/ml) and TBT (100 or 500 ng/ml) treatments on the isolated peripheral lymphocytes prepared from normal mice. The higher concentration of TPT (500 ng/ml) is equivalent to the maximum TPT concentration in the blood of the TPT-treated mice, and the lower concentration of TBT (100 ng/ml) is equivalent to the maximum TBT levels in the blood of the TBT-treated mice (Table 1). Dex treatment resulted in an increase in the annexin V (+) lymphocytes after 3 hr of incubation, indicating induction of apoptosis. The extreme increase in annexin

Fig. 1. Effects of organotin compounds on the peripheral lymphocyte counts in mice. The organotin compounds were administered orally (180 μ mol/kg), while dexamethasone (3 mg/kg) was given intraperitoneally. Values are indicated as means $\pm$ SD (n=4), *, **: Significantly different from the values before administration (p<0.05 or 0.01).

Fig. 2. Effects of organotin compounds on the peripheral subpopulations in mice at 6 hr after administration. The mice were administered the organotin compound (180 μ mol/kg) orally, and the cell counts of each subset were calculated from the results of FACS analysis. Values are indicated as means $\pm$ SD (n=4), *, **: Significantly different from the values before administration (p<0.05 or 0.01).

V (–) PI (+) lymphocytes resulting from the higher concentrations of TPT and TBT (500 ng/ml) indicates that the treatments with higher concentrations of TPT or TBT could induce necrosis in the isolated lymphocytes at 1 hr of incubation. On the other hand, treatment with the lower concentration of TBT (100 ng/ml) resulted in an increase of both annexin V (+) PI (–) cells and annexin V (–) PI (+) cells, indicating that the treatment with the lower concentration of TBT could induce apoptosis and necrosis in the peripheral lymphocytes. However, treatment with the lower concentration of TPT or DBT induced only apoptosis at 3 hr of incubation.

Effects of TPT or TBT on the thymus *in vivo*: With respect

Fig. 3. The effects of organotin treatments on lymphocytes prepared from normal mouse peripheral blood *in vitro* (1). The lymphocytes were incubated with the organotin compounds (392 ng/ml for Dex; 100 or 500 ng/ml for TPT and TBT) in RPMI-1640 medium at 37°C for 1 hr, and then the apoptotic or necrotic cells were analyzed by FACS using FITC-Annexin V and PI. □, Annexin (-) PI (-); ▨, Annexin (+) PI (-); ▩, Annexin (-) PI (+); ▤, Annexin (+) PI (+). Values are indicated as means $\pm$ SD (n=4). *, **: Significantly different from the values of the cells incubated under the same conditions without organotin compounds ($p < 0.05$ or 0.01).

to thymus atrophy, TPT or TBT and Dex caused a linear decrease in the weight of the thymus with time (6–24 hr) following administration (Fig. 5). In regard to reduction of thymocyte subpopulations in the thymus as a result of TPT or TBT, there was no significant difference in the percentages of CD4(+) CD8(+), CD4(+) CD8(-), CD4(-) CD8(+) and CD4(-) CD8(-) thymocytes between the control and organotin-treated groups (data not shown).

Detection of apoptotic cells in thymocytes *in vivo*: As illustrated in Fig. 6, the flow cytometry analysis also revealed that treatment with TPT or TBT did not increase the number of apoptotic cells at 6 hr, as evaluated by the abundance of annexin V positive thymocytes, although Dex did induce an increase in the number of apoptotic thymocytes *in vivo*. Moreover, as shown in Fig. 7, typical DNA laddering, a hallmark of apoptosis, was detected only in the thymocytes obtained from Dex-treated mice (lane C), whereas the thymocytes from the TPT- or TBT-treated mice did not show this typical DNA fragmentation (lanes D and E). Histopathological analysis revealed that decreases in cellular density occurred in these thymuses without an increase in necrotic cells (data not shown).

DISCUSSION

In the present *in vivo* study, significant transient reductions in peripheral lymphocytes were observed in TPT- or TBT-treated mice (Fig. 1), and analysis of peripheral lymphocyte subpopulations by flow cytometry revealed that the

rates of reduction in B-cells for TPT and TBT were significantly higher than those in T-cells (Fig. 2), indicating that B-cells might be more sensitive to the organotins than T-cells in mice. In regard to the lymphocyte subpopulations, previous studies have reported that organotin compounds mainly suppressed T-cell dependent immunofunctions in rats [1, 21] and mice [16]. Although another study showed that DBT decreased *in vitro* survival, proliferation and differentiation of normal human B lymphocytes [4], the present results demonstrated that B-lymphocytes might also be an important target for induction of immunotoxicity by organotin compounds in mice.

Because it has been suggested that organotin-induced deletion of peripheral lymphocytes is likely a component in the overall risk for immunotoxic responses in exposed humans [24], we attempted to reveal the mechanism of the transient reduction in peripheral lymphocytes induced by TPT and TBT. Although we could detect transient decreases of peripheral lymphocytes in blood from Dex-, TPT- and TBT-treated mice, we could not observe the characteristics of apoptosis or necrosis, such as DNA laddering or staining patterns for annexin V and PI, in the peripheral lymphocytes isolated from these mice *in vivo* at any time after administration. On the other hand, we could detect apoptotic cells in the thymuses obtained from the same Dex-treated mice. From these results, we concluded that such an *in vivo* experiment was suitable for observation of the apoptotic or necrotic reactions induced by these chemicals in the thymus, but not in peripheral lymphocytes. Therefore, the

Fig. 4. The effects of organotin treatments on lymphocytes prepared from normal mouse peripheral blood *in vitro* (2). The lymphocytes were incubated with the organotin compounds (392 ng/ml for Dex; 100 ng/ml for TPT, TBT and DBT) in RPMI-1640 medium at 37°C for 3 hr, and then the apoptotic or necrotic cells were analyzed by FACS using FITC-Annexin V and PI. □, Annexin (-) PI (-); ▨, Annexin (+) PI (-); ▩, Annexin (-) PI (+); ▤, Annexin (+) PI (+). Values are indicated as means $\pm$ SD (n=4). *, **: Significantly different from the values of the cells incubated under the same conditions without organotin compounds ($p < 0.05$ or 0.01).

Fig. 5. Thymus atrophy induced by the organotin compounds at 6 and 24 hr after oral administrations in mice. The organotin compounds were administered orally (180 μ mol/kg), while dexamethasone (3 mg/kg) was given intraperitoneally, and the ratio of thymus weight to body weight was measured. Values are indicated as means $\pm$ SD (n=4). *, **: Significantly different from the values before administration ($p < 0.05$ or 0.01).

lymphocytes isolated from normal mice were exposed to Dex, TPT or TBT at the concentrations observed in the blood of mice administered these chemicals (Table 1), and

then the percentages of apoptotic or necrotic cells were analyzed by flow cytometry. When the isolated lymphocytes were exposed to 500 ng/ml of TPT, the ratio of necrotic cells

Fig. 6. The effect of organotin on thymocytes isolated from the mouse thymus at 6 hr after the *in vivo* treatments. The apoptotic or necrotic cells were analyzed by FACS using FITC-Annexin V and PI. □, Annexin (-) PI (-); ▨, Annexin (+) PI (-); ▩, Annexin (-) PI (+); ▤, Annexin (+) PI (+). Values are indicated as means $\pm$ SD ($n=4$). *, **: Significantly different from the values of the cells incubated under the same conditions without organotin compounds ($p<0.05$ or 0.01).

Fig. 7. DNA fragmentation in thymocytes at 3 or 6 hr after administration of dexamethasone (3 mg/kg, i.p.) or the organotin compounds (180 μ mol/kg, p.o.). Low-molecular-weight DNA was extracted from the cells of mice and electrophoresed on 2.0% agarose gels, as described in the Materials and Methods. Fragmentation of DNA was detected in the thymocytes of dexamethasone-injected mice (lane C) at 3 and 6 hr after administration, but not in those of the Control (lane B), TPT-treated (lane D) and TBT-treated (lane E) mice. Lane A is a 100 bp DNA ladder marker. One representative experiment of three replicates is shown.

that showed annexin V (-) PI (+) reached about 70% within 1 hr of exposure (Fig. 3), indicating that the transient reduction in lymphocytes of the TPT-treated mice was attributed

to necrosis induced by TPT *in vivo*. On the other hand, both apoptosis and necrosis were observed in the isolated lymphocytes within 3 hr of exposure to 100 ng/ml of TBT, and

DBT exposure at the same concentration could induce only apoptosis in the isolated lymphocytes (Fig. 4), suggesting that the transient reduction of peripheral lymphocytes observed in the TBT-treated mice was attributed to apoptosis as well as necrosis induced by TBT and its metabolite, DBT, *in vivo*. Thus, DBT, the main metabolite of TBT, might be involved in the transient reduction in peripheral lymphocytes *in vivo* as well as hepatotoxicity by TBT in mice [28]. However, the participation ratio of apoptosis and necrosis in regard to the transient reduction in peripheral lymphocytes might differ according to the concentrations of organotin and their metabolites in the blood.

Previous studies have suggested the participation of apoptosis in the thymus atrophy caused by organotins [7, 20], and the present study showed thymus atrophy at 6 hr–24 hr after TPT or TBT administration (Fig. 5). Therefore, we examined the induction of apoptosis in thymuses obtained from mice at 3 hr or 6 hr after administration of the organotins or Dex, the positive control, *in vivo*. As expected, DNA laddering characteristic of apoptosis was observed in the thymocytes obtained from the Dex-treated mice; however, the TPT- or TBT-treated mice did not show such DNA laddering (Fig. 7). Flow cytometric analysis for detection of apoptotic cells showed the same results (Fig. 6). Moreover, histopathological analysis showed decreases in cellular density were observed in these thymuses without an increase in necrotic cells (data not shown). Because the organotin levels in the thymus at 24 hr reached levels that could induce necrosis *in vitro* (778 ± 572 ng TPT/g for TPT-treated mice; $1,452 \pm 159$ ng TBT/g for TBT-treated mice), the necrotic cells might be detected after more than 24 hr. However, our results indicated that neither apoptosis nor necrosis may largely participate in thymus atrophy within 24 hr after organotin administration. Gennari *et al.* reported that the main mechanism of thymus atrophy by TBT at a lower dosage ($55 \mu\text{mol/kg}$) might be antiproliferative effects by DBT metabolized from TBT [6]. Therefore, we concluded that inhibition of thymocyte proliferation may be the main mechanism of the thymus atrophy induced by TPT or TBT even at such a high dosage.

In conclusion, our results demonstrated that apoptosis, which is one of the most interesting toxicological mechanisms of organotin compounds, may not be involved in the thymus atrophy induced by TPT and TBT in mice, and the cause of this phenomenon may be the antiproliferative effects on thymocytes of these organotin compounds. On the other hand, the transient reduction of peripheral lymphocytes observed in the TPT-treated mice might be induced by a direct necrotic reaction by TPT. In the case of TBT-treated mice, apoptosis might participate in the reduction of peripheral lymphocytes as well as necrosis induced by TBT. Thus, the thymus atrophy and transient reduction of peripheral lymphocytes by the organotin compounds *in vivo* were caused by different mechanisms.

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REFERENCES

1. Attahiru, U. S., Iyaniwura, T. T., Adaadi, A. O. and Bonire, J. J. 1991. Subchronic toxicity studies of tri-n-butyltin and triphenyltin acetates in rats. *Vet. Hum. Toxicol.* **33**: 499–502.
2. Aw, T. Y., Nicotera, P., Manzo, L. and Orrenius, S. 1990. Tributyltin stimulates apoptosis in rat thymocytes. *Arch. Biochem. Biophys.* **283**: 46–50.
3. Boyer, I. J. 1989. Toxicity of dibutyltin, tributyltin and other organotin compounds to humans and to experimental animals. *Toxicology* **55**: 253–298.
4. De Santiago, A. and Aguilar-Santelises, M. 1999. Organotin compounds decrease *in vitro* survival, proliferation and differentiation of normal human B lymphocytes. *Hum. Exp. Toxicol.* **10**: 619–624.
5. Gennari, A., Bol, M., Seinen, W., Penninks, A. and Pieters, R. 2002. Organotin-induced apoptosis occurs in small CD4(+)CD8(+) thymocytes and is accompanied by an increase in RNA synthesis. *Toxicology* **175**: 191–200.
6. Gennari, A., Potters, M., Seinen, W. and Pieters, R. 1997. Organotin-induced apoptosis as observed *in vitro* is not relevant for induction of thymus atrophy at antiproliferative doses. *Toxicol. Appl. Pharmacol.* **147**: 259–266.
7. Gennari, A., Viviani, B., Galli, C. L., Marinovich, M., Pieters, R. and Corsini, E. 2000. Organotins induce apoptosis by disturbance of $[\text{Ca}^{2+}]_i$ and mitochondrial activity, causing oxidative stress and activation of caspase in rat thymocytes. *Toxicol. Appl. Pharmacol.* **169**: 185–190.
8. Grote, K., Stahlschmidt, B., Talsness, C. E., Gericke, C., Appel, K. E. and Chahoud, I. 2004. Effects of organotin compounds on pubertal male rats. *Toxicology* **202**: 145–158.
9. Harrison, P. T., Holmes, P. and Humfrey, C. D. 1997. Reproductive health in humans and wildlife: are adverse trends associated with environmental chemical exposure? *Sci. Total. Environ.* **205**: 97–106.
10. Hoch, M. 2001. Organotin compounds in the environment—an overview. *Appl. Geochem.* **16**: 719–743.
11. Kannan, K., Tanabe, S. and Tatsukawa, R. 1995. Occurrence of butyltin residues in certain foodstuffs. *Bull. Environ. Contam. Toxicol.* **55**: 510–516.
12. Kannan, K., Guruge, K. S., Thomas, N. J., Tanabe, S. and Giesy, J. P. 1998. Butyltin Residues in Southern Sea Otters (*Enhydra lutris nereis*) Found Dead along California Coastal Waters. *Environ. Sci. Technol.* **32**: 1169–1175.
13. Kannan, K., Grove, R. A., Senthikumar, K., Henny, C. J. and Giesy, J. P. 1999. Butyltin compounds in river otters (*Lutra canadensis*) from the northwestern United States. *Arch. Environ. Contam. Toxicol.* **36**: 462–468.
14. Kashimoto, T., Ueno, S., Hayashi, H., Hanajima, M., Yoshioka, K., Yoshida, K., Mutoh, K. and Susa, N. 2005. Depletion of lymphocytes, but not neutrophils, via apoptosis in a murine model of *Vibrio vulnificus* infection. *J. Med. Micro.* **54**: 15–22.
15. Lee, J. J., Kim, Y. M., Park, S. K. and Lee, M. K. 2006. Effects of tributyltin chloride on L-DOPA-induced cytotoxicity in PC12 cells. *Arch. Pharm. Res.* **29**: 645–650.
16. Nishida, H., Matsui, H., Sugiura, H., Kitagaki, K., Fuchigami, M., Inagaki, N., Nagai, H. and Koda, A. 1990. The immunotoxicity of triphenyltin chloride in mice. *J. Pharmacobiodyn.* **13**: 543–548.
17. Nakatsu, Y., Kotake, Y., Komasa, K., Hakoza, H., Tagu-

- chi, R., Kume, T., Akaike, A. and Ohta, S. 2006. Glutamate excitotoxicity is involved in cell death caused by tributyltin in cultured rat cortical neurons. *Toxicol. Sci.* **89**: 235–242.
18. Penninks, A. H. 1993. The evaluation of data-derived safety factors for bis(tri-n-butyltin)oxide. *Food. Addit. Contam.* **10**: 351–361.
19. Pieters, R. H., Bol, M., Lam, B. W., Seinen, W. and Penninks, A. H. 1992. The organotin-induced thymus atrophy, characterized by depletion of CD4+ CD8+ thymocytes, is preceded by a reduction of the immature CD4– CD8+ TcR alpha beta-/low CD2high thymoblast subset. *Immunology* **76**: 203–208.
20. Raffray, M. and Cohen, G. M. 1993. Thymocyte apoptosis as a mechanism for tributyltin-induced thymic atrophy *in vivo*. *Arch. Toxicol.* **67**: 231–236.
21. Smiaowicz, R. J., Riddle, M. M., Rogers, R. R., Leubke, R. W., Copeland, C. B. and Ernst, G. G. 1990. Immunealterations in rats following subacute exposure to tributyltin oxide. *Toxicology* **64**: 169–178.
22. Snoeij, N. J., van Iersel, A. A., Penninks, A. H. and Seinen, W. 1985. Toxicity of triorganotin compounds: comparative *in vivo* studies with a series of trialkyltin compounds and triphenyltin chloride in male rats. *Toxicol. Appl. Pharmacol.* **81**: 274–286.
23. Snoeij, N. J., Penninks, A. H. and Seinen, W. 1988. Dibutyltin and tributyltin compounds induce thymus atrophy in rats due to a selective action on thymic lymphoblasts. *Int. J. Immunopharmacol.* **10**: 891–899.
24. Stridh, H., Cotgreave, I., Müller, M., Orrenius, S. and Gigliotti, D. 2001. Organotin-induced caspase activation and apoptosis in human peripheral blood lymphocytes. *Chem. Res. Toxicol.* **14**: 791–798.
25. Suzuki, T. 2003. Environmental chemistry of organotin compounds. *J. Food Hyg. Soc. Jpn.* **44**: 269–280.
26. Suzuki, T., Matsuda, R., Saito, Y. and Yamada, H. 1994. Application of helium microwave-induced plasma emission detection system to analysis of organotin compounds in biological samples. *J. Agric. Food Chem.* **42**: 216–220.
27. Szondy, Z., Reichert, U., Bernardon, J. M., Michel, S., Toth, R., Karaszi, E. and Fesus, L. 1998. Inhibition of activation-induced apoptosis of thymocytes by all-trans- and 9-cis-retinoic acid is mediated via retinoic acid receptor alpha. *Biochem. J.* **331**: 767–774.
28. Ueno, S., Susa, N., Furukawa, Y. and Sugiyama, M. 1994. Comparison of hepatotoxicity caused by mono-, di- and tributyltin compounds in mice. *Arch. Toxicol.* **69**: 30–34.
29. Vos, J. G., van Logten, M. J., Kreeftenberg, J. G. and Kruizinga, W. 1984. Effect of triphenyltin hydroxide on the immune system of the rat. *Toxicology* **29**: 325–336.