The immunotoxic effects of tributyltin on non-specific biodefense system in rainbow trout (Oncorhynchus mykiss)

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ABSTRACT

The immunotoxic effects of tributyltin (TBT) exposure on the respiratory burst activities of neutrophils in head kidney (HK) and peripheral blood (PBL), and lysozyme activities of plasma were examined in rainbow trout (Oncorhynchus mykiss). After fish were exposed to TBT at the concentration of 5, 10 and 20\(\mu\)g/L for 5 days, and 5\(\mu\)g/L for 14 and 28 days, leukocytes obtained from HK and PBL were evaluated for the active oxygen production by flow cytometry (FCM). The contents (%) of neutrophils population in total leukocytes collected from HK of fish exposed to 20\(\mu\)g/L of TBT for 5 days was clearly increased compared with the control group. However, respiratory burst activity of neutrophils in the exposure group decreased compared with the control group. The increase of neutrophils from fish exposed to 5\(\mu\)g/L of TBT for relatively long term exposure (14 and 28 days) was observed only in PBL. These results indicate that high level exposure of TBT stimulated immune function of rainbow trout temporally, and neutrophils were actively produced in HK, however neutrophils themselves showed weak productivity of active oxygen. It was observed that lysozyme activities decreased in the group exposed to 10 \(\mu\)g/L for 5 days, while TBT concentration in blood increased in accordance with the TBT concentration in water ranging from 0 to 10\(\mu\)g/L. We supposed that immunotoxic effects of TBT were apparent both as immunostimulative and immunosuppressive, and these immune responses might be dependent on the TBT concentration of exposure.

Key Words: rainbow trout, immunotoxicity, tributyltin, flow cytometric analysis, respiratory burst activity
1. INTRODUCTION

Many synthetic chemicals have adverse effects on terrestrial and aquatic organisms, and the immune system is one of the target organs of these effects. Immunotoxic influences of chemicals have been studied and understanding of them has developed considerably over the last decade\(^9\). Since a large number of chemicals ultimately flow into the aquatic environment, aquatic organisms are always exposed to and affected by these chemicals. Fish are suitable aquatic animals as biomarkers in the toxicological evaluation of chemicals, including immunotoxic effects\(^9\). Fish immunology has been actively developed from the view point of comparative immunology and from the serious disease problems in fish culture. Recently, the effects of chemicals on the immune system also have been investigated, however many problems concerning immunotoxic assessment in fish remain to be solved.

Immunotoxic effects of various chemicals on the immune system include general inhibition and stimulation of the system. For example, fish collected from a river heavily contaminated with various chemicals and from a relatively non-polluted river were analyzed for phagocytic activities of kidney macrophages, and it was reported that the activity was significantly reduced in the fish obtained from the contaminated area\(^9\). On the other hand, respiratory burst activity in phagocytes was increased in the fish exposed to paper mill effluent, showing concentration- and time-dependent effects\(^6\).

Tributyltin (TBT) is a common pollutant in the aquatic environment that has been used as a biocide in antifouling paints to prevent the attachment of organisms to boat bottoms. Because of its unexpected toxicity, TBT’s use in antifouling paints has been restricted since 1990’s in Japan. Consequently, average TBT concentrations in the coastal area of Japan were reduced by several ng/L in recently years. However TBT contamination has already spread worldwide and TBT residue often has been detected near shipyards and in harbor areas. In addition, TBT concentrations are still relatively high in the sediment of coastal areas, showing the low biodegradability by microorganisms\(^6\). TBT exposure causes endocrine disruptions that include imposex phenomena in gastropods, developmental abnormalities and immunotoxicity in a wide range of animals. Regala et al.\(^7\) demonstrated that injection of high dose of TBT to channel catfish suppressed phagocyte respiratory burst activity. O’Halloran et al.\(^8\) described that TBT exposure in rainbow trout inhibited lipopolysaccharide-induced proliferation of lymphocytes prepared from both head kidney and spleen, and reported that splenic immune cells, especially B-lymphocytes, were selectively affected by TBT. Furthermore, TBT accumulated in the serum and plasma of various fish, especially cultured Japanese flounder (Paralichthys olivaceus) purchased from a fish market at Fukuoka, Japan, showed high concentration of TBT in plasma compared with cultured red sea bream (Pangrus major) from the same area\(^9\).

Since the surface of the fish body is always exposed directly to viruses and
bacteria in surrounding waters, non-specific biodefense mechanisms such as phagocytic activity and bactericidal factors like lysozyme are considered to be more important than specific biodefense mechanisms including the humoral and cellular immune systems. Recently, detection of respiratory burst activity in fish phagocytes using flow cytometric analysis also has been developed.

In order to investigate the effects of tributyltin on the non-specific immune system, especially respiratory burst activity of neutrophils, flow cytometric (FCM) analysis was carried out. Lysozyme activity in plasma was also measured in rainbow trout exposed to different TBT concentrations.

2. MATERIAL AND METHODS

2.1 Fish

Juvenile rainbow trout (3-5g) were obtained from the Samegai trout farm and reared in a 250L flow-through tank for 1 year, and were maintained at 15-19°C under a 12h light-dark cycle. The fish were fed daily with standard trout pellets (Nippon, Japan; without TBT). Rainbow trout (100-200g) used in the experiment were acclimatized in a tank for at least 2 weeks before the start of experiment. In principle, two fish are held separately using partition in a 50L tank. Fish were fed daily at 1-2% of body weight during the experiment. Four to ten fish in each experimental group were analyzed in this study.

2.2 Tributyltin preparations and exposure

Tributyltin chloride (95% purity) purchased from Wako Chemical Co. Ltd (Osaka, Japan) was diluted in dimethylsulfoxide (DMSO) to a concentration of 100-250μg/ml. TBT was added to the experimental tanks at the final concentrations of 0, 5, 10, and 20μg/L. The total amount of DMSO per tank was 0.005% v/v. A half volume of rearing water containing TBT was exchanged every two days, and water containing DMSO only was used for the control group. In the short period, i.e. 5 days experiment, TBT concentration was set at 5, 10, and 20μg/L. Five μg/L of TBT was set for the 14 to 28 days experiment allowing a relatively long term exposure. During the experiment, temperature of rearing water was maintained at 17-19°C under a 12 light-dark cycle.

2.3 Leukocytes preparation

Head kidney (HK) and peripheral blood (PBL) leukocytes were prepared by the hemolytic method as described by Moritomo et al. with some modifications. Briefly, fish were anesthetized and their PBL was collected from the caudal vein with a heparinised syringe. HK was removed and scraped out on stainless mesh with Minimum Essential Medium (MEM) supplemented with 0.1U/ml heparin, pH7.2. The suspension of cells and fragments was centrifuged for 10 min at 1,800rpm. Subsequently, 2ml of distilled water was added to the pellets and gently mixed with a pipette for 5 seconds. After hemolytic treatment, 8ml of MEM was immediately added, and the pellets were rinsed twice with MEM by centrifugation. As to PBL, 4ml of MEM was added to 1ml of blood. After centrifugation, the leukocytes-enriched fraction
that appeared between erythrocytes and MEM was separated and leukocytes were prepared by the same hemolytic method. Viability of both cells in head kidney and blood was determined by trypan blue exclusion and the number of cells was adjusted to $1 \times 10^6$ cells/ml.

2.4 Flow cytometric analysis for neutrophil contents in total leukocytes and their respiratory burst activity

The cells were transferred to a test tube (Falcon 2059, Becton Dickinson) and were analyzed by flow cytometry (FACScalibur, Becton Dickinson). Forward scatter (FSC) is usually used as an approximate indicator of cell size, and side scatter (SSC) generally correlates with the amount of granularity of a cell or the complexity of the cytoplasm. The dot plots of forward vs. side scatter (FSC/SSC dot plots) of total HK and PBL cell suspensions were displayed and recorded 10,000 events (cells) for each sample, using the method of Moritomo et al. According to some previous studies, both HK and PBL cell suspensions were shown by FSC/SSC dot plot with three gated typical populations including neutrophils, lymphoid cells and monocytes (see Fig.1a, Region(R) 1-3). Each population was calculated by the provided instrument software (Cell Quest Pro, Becton Dickinson) and the contents (%) of neutrophils in total leukocytes were expressed according to the following equation: $R1 \div (R1 + R2 + R3) \times 100$.

In order to evaluate the respiratory burst activity of phagocytic cells, measurement of intracellular $H_2O_2$ at the single cell level was carried out by the stimulation of cells using phorbol myristate acetate (PMA, Sigma) at the final concentration of 0.1 $\mu$g/ml in a test tube at room temperature for 20 min. Subsequently, dihydrorhodamine 123 (DHR, Sigma) was added to the cell suspension at the final concentration of 1 $\mu$g/ml and incubated under dark condition for 10 min. Since DHR is oxidized by intercellular $H_2O_2$ into rhodamine, its fluorescence was detected on the FL-1 green channel. Cells stained by DHR after stimulation of PMA were composed of mainly two distinct cell populations including R4 (non-phagocytic cells) and R5 (phagocytic cells) (Fig.1b).

Based on the intensity of FL-1 and SSC properties or FCM analysis using Cell Quest Pro, the population of phagocytic cells consisted mainly of neutrophils. However, the population of monocytes did not appear in R5. The population of phagocytic cells was gated. Its histogram of rhodamine fluorescence (FL-1) was displayed and its respiratory burst activity was estimated by the mean FL-1 value (Fig. 1c). To distinguish dead cells, propidium iodide was added at the final concentration of 0.1 $\mu$g/ml before the measurement.

2.5 Measurement of lysozyme activity

Lysozyme activities of plasma were determined by using Micrococcus lysodeikticus (Sigma) as a substrate in 0.01M phosphate buffer (PB, pH7.0). Substrate suspension was adjusted about 0.5 in absorbance at 530nm. Plasma was prepared from PBL by centrifugation at 3,500 rpm for 15 min at 4 $^\circ$C. Plasma was diluted with PB to ten times and the diluted solution was added to
3 ml of substrate suspension. The mixed solutions were immediately measured in absorbance at 530nm after incubation for 10 min at 28°C. Finally, lysozyme activities were calculated from the decrease of turbidity, and the activities of the control group were expressed as 100%.

2.6 Analytical methods of tributyltin

TBT concentrations in rearing water and the blood were analyzed by the method described Harino et al. One hundred µl of surrogate acetone solution containing each 1µg/ml of MBTCl-d₈, DBTCl-d₈, TBTCI-d₁₅, MPTCl-d₈, DPTCl-d₈ and TPTCl-d₁₅ as a standard was added to 50ml of each water sample. One gram and a half of NaCl, 1ml of acetic acid-sodium acetate buffer (pH5.0) and 0.5ml of 2% NaBEt₃ were added to the analyte and treated for 10 min with occasional shaking in order to form the derivative of TBT. The analyte was extracted twice with 10 ml of Hexane. After the hexane layer was dried with anhydrous Na₂SO₄, it was transferred to a flask and concentrated to a trace level by a rotary evaporator.

One gram of blood sample from fish in a centrifuge tube was added to 100µl of previous surrogate solution as a standard. The mixture was extracted with 25ml of 1 M HCl-methanol/ethyl acetate (1/1) by shaking for 10min. After centrifugation for 10min, the supernatant was transferred to a flask and the residue was extracted with 25ml of 1 M HCl-methanol/ethyl acetate (1/1) and centrifuged again. The combined supernatants and 100ml of saturated NaCl solution were transferred to a separatory funnel and the analytes were extracted twice with 30ml of ethyl acetate/hexane (3/2) solution. The combined organic layer was then mixed with 100ml hexane and the mixture was left to stand for 20min. After removal of the aqueous layer, the organic layer was dried with anhydrous Na₂SO₄ and concentrated to a trace level by a rotary evaporator. The analytes were diluted with 5ml of acetic acid-sodium acetate buffer (pH5.0) and ethylated by addition of 1ml of 10% NaBEt₃. The lipids were saponificated with 40ml of 1M KOH-ethanol solution by
shaking for 1h. Twenty-five ml of distilled water and 40ml of hexane were added to the solution and the mixture was shaken for 10min. After removal of the aqueous layer, the analytes were extracted again with 40ml of hexane and the combined organic layer was dried with anhydrous Na2SO4. After concentration to 1ml, the solution was cleaned in a florisil Sep-Pak column (Waters Association Co. Ltd.), and the analyte was eluted with 5% diethyl ether/hexane. TeBT-d36 and TePT-d20 were added as an internal standard and the final solution was concentrated to 0.5ml.

A Hewlett-Packard 6890 series gas chromatograph equipped with a mass spectrometer (5973 N) was used for the analysis of organotins. Separation was carried out on a capillary column coated with 5% phenyl methyl silicone (J&W Scientific Co., 30m length×0.25mm i.d., 0.25µm film thickness). The column temperature was held at 60°C for the first 2 min, then increased to 130°C at 20°C /min, to 210°C at 10°C /min, to 260°C at 5°C /min and to 300°C at 10°C /min, where it was held for 2min. Interface temperature, ion source temperature and ion energy were 280°C, 230°C and 70eV, respectively. Selected ion monitoring was carried out under this programme. Splitless injection of sample was employed. Organotin concentration is expressed as Sn⁺.

Concentrations of TBT in rearing water were analyzed at each sampling time (Table 1).

2.7 Statistical analysis

Values are presented as mean ± standard error of untransformed data in the figures and tables. Differences between means values of control and TBT exposed groups were evaluated by student’s t-test. Level of significance was p < 0.05.

3. RESULTS AND DISCUSSION

3.1 Mortality of fish exposed to TBT

During the experiment, mortality of the fish exposed to 5µg/L of TBT for 14 days was 4%. Eight % of fish showed abnormal swimming behaviors such as side swim and lying on the same place or surface of water, whereas most other fish ate food well. These abnormal fish were excluded from this experiment, but analyzed independently and discussed elsewhere. It is well known that even extremely low levels of TBT are toxic to aquatic organisms and the 96 hr-LC50 of TBT in rainbow trout (BW 1.5g, under 15°C) was 3.6µg/L. Peters et al(1) demonstrated that stress induced by so-

<table>
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<th>Concentration (µg/L)</th>
<th>Exposed period (days)</th>
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<th>Before water change (Samples)</th>
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<td>28</td>
<td>5.1±1.3 (12)</td>
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cial conflict caused immune suppressive changes in rainbow trout. Moreover, steroid hormones such as cortisol, 17β-estradiol, testosterone and 11-ketotestosterone suppressed the antibody producing cells in rainbow trout. Stress induced by social conflict being characteristic in rainbow trout is considered to affect the results of immunological approach, but it was not observed in this experiment. No fish died in the group exposed to TBT 5, 10 and 20μg/L for 5 days, although abnormal eyes and internal hemorrhage from them were often observed at 10μg/L or above of TBT concentration.

3.2 The effects of short term exposure to 20μg/L of TBT on the contents of neutrophils in total leukocytes and its respiratory burst activity

The contents (%) of neutrophils population in total leukocytes obtained from HK were measured by FCM analysis. The total recovery rates of 3 populations including neutrophils, lymphoid cells and monocytes of HK were 86.4±2.7%. As shown in FSC/SSC dot plots, neutrophils population in HK prepared from the exposure group was clearly increased compared with the control group (Fig.2). The contents of neutrophils in total leukocytes of control and 20μg/L of TBT exposure group (n=6) were 21.1±4.4% and 36.4±14.7%, respectively. Four of six analyzed fish showed the same results in the exposure group, and the neutrophil contents in most sensitive fish was 58%. Increase of neutrophils in HK which plays a role in hematopoietic function, i.e., tentative enhancement of immune system, might be attributed to the repairing of damages in body surface, gills and other tissues caused by TBT. Moreover, active oxygen production by neutrophils is an important mechanism for protection against many pathogens. On the other hand, during phagocytosis, toxic active oxygen such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals were also released in the fish bodies and damaged host tissue. Enhancement of the immune system would be favorable for the damaged body, although references to superoxide dismutase are essential. From the measurement of fluorescence intensities showing respiratory burst activities in phagocytic cells, following the method as described elsewhere, almost all cells consisted of neutrophils, and the mean value of FL-1 decreased in the exposure group (131±60) compared with the control group (279±110). A typical FL-1 histogram of the gated phagocytic cells (almost neutrophils) is shown in Figure 3. The neutrophils obtained from head kidney were analyzed by a histogram of FL-1, and they consisted of 3 populations which showed different fluorescence intensity as described Moritomo et al. Similar results were obtained in the control group, though only 2 populations were observed in the control group. On the basis of the FCM analysis in histogram, each population was analyzed using a marker (M) according to fluorescence intensity. One population which showed highest fluorescence intensity around 1,000 (M2) was not observed in the group exposed to 20μg/L of TBT, but another population showing lower fluorescence intensity (M1) was clearly noticed (Fig.3). However, mean
Immunotoxicity of TBT in fish

<table>
<thead>
<tr>
<th>Control</th>
<th>TBT 20μg/L</th>
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<tr>
<td><img src="image1" alt="Flow cytometric analysis" /></td>
<td><img src="image2" alt="Flow cytometric analysis" /></td>
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</table>

**Fig.2.** Flow cytometric analysis of the effect of exposure to TBT 20μg/L for 5 days on the contents of neutrophils in total leukocytes obtained from head kidney of rainbow trout. A typical FSC/SSC dot plot in the control group and the most affective aspect in the exposure group are shown. Six fish were analyzed in each experimental group. Dotted lines indicate each population of neutrophils.

**Fig.3.** Typical overlay of rhodamine fluorescence histogram in gated phagocytic cells in head kidney. Cells were exposed to 0 (solid line) and 20μg/L (dotted line) of TBT. According to fluorescence intensity, markers (M) were expressed, and indicate low and high FL-1 value as M1 and M2, respectively.

FL-1 value of M1 in the exposure group decreased significantly compared with the control group, and this suggests that hematopoietic head kidney produce actively immature neutrophils which possess weak active oxygen productivity. These results might cause the decrease of respiratory burst activity in neutrophils prepared from the fish exposed to high concentrations of TBT.

3.3. The effects of relatively long term exposure to 5μg/L of TBT on the contents of neutrophils in total leukocytes.

The contents (%) of neutrophils populations in total leukocytes obtained from HK and PBL (n=4 or 5) were measured by FCM analysis. The recoveries of the total of 3 populations for 14 and 28 days exposure were 86.3±2.2, 88.9±3.2 in HK, 73.1±1.5.4 and 80.6±11.0% in PBL, respectively.

In relatively long term exposure to TBT 5μg/L for 14 and 28 days, there were no effects on the contents of neutrophils in total leukocyte prepared from HK. On the other hand, those from PBL in the 14 days exposure group were higher than the control group and their contents were 10.4±4.5 and 4.5±1.2, respectively (Fig.4). This result correlated with the FSC/SSC dot plot and FL-1 histogram of the gated phagocytic
leukocytes obtained from both experimental groups showed a tendency to decrease (Fig.4). Since fish showing abnormal behaviors were observed on 6 days after start of TBT exposure, these were analyzed separately. As the results, the contents of neutrophils in total leukocytes in both HK and PBL were clearly increased compared with normal conditioned fish (Data not shown). As these fish were influenced more strongly by TBT, immunotoxic effects of TBT on these sensitive fish should be more investigated for detail discussion.

3.4. The effects of TBT on lysozyme activities in plasma and the TBT concentration in blood.

After fish were exposed to TBT at 0, 5 and 10 μg/L for 5 days, lysozyme activities in plasma were measured (n=9, 6 and 9, respectively), and TBT levels in blood were also analyzed for the same sample (n=4) (Fig.6). The lysozyme activity in 0, 5 and 10 μg/L of TBT was 100±20.0, 95.6±32.8 and 56.2±10.5%, respectively. Though there were no effects in the group of 0 and 5 μg/L TBT, significant differences were noticed between 5 and 10 μg/L exposure groups (p<0.05), and 0 and 10 μg/L exposure groups (p<0.01). TBT concentrations in the blood of fish exposed to TBT at 0, 5 and 10 μg/L for 5 days were 0.07±0.04, 5.5±3.8 and 9.4±0.9 μg/Kg, respectively. According, TBT was accumulated, showing dose-dependently, and lysozyme activity in the group exposed to TBT 10 μg/L was clearly inhibited. Since lysozyme is produced by phagocytes such as neutrophils, monocyte and macrophage, the effect of high level TBT on phagocytes was also
**Fig. 5.** Flow cytometric analysis of the effects of exposure to TBT 5µg/L for 14 days on the contents of neutrophils in total leukocytes obtained from PBL and its FL-1 histogram. Four or five fish were analyzed in each experimental group. Dotted lines indicate each population of neutrophils.

**Fig. 6.** The effects of different concentration of TBT exposure for 5 days on lysozyme activity and TBT concentration in blood. Lysozyme activity (□) and TBT concentration in blood (---) of same sample were shown. The values ± standard error represent the average of fish (n=6 or 9 for lysozyme activity and n=4 for TBT concentration in blood) in each experimental group. The Asterisk ** and * show significant differences between 0 and 10µg/L exposure groups, and 5 and 10µg/L exposure groups, for p<0.01 and p<0.05, respectively.
suggested.

TBT has a number of toxic effects on including immune system\(^8\), drug metabolizing enzyme system\(^7(16)\), histopathological changes\(^9\) and endocrine system\(^10(21)\) in aquatic organisms. The immunotoxic effects of TBT on the respiratory burst activity of phagocytic cells and lysozyme activity were investigated in this study. Respiratory burst activity has been shown to be a useful indicator of neutrophilic function in fish, and the method of detection of respiratory burst activity using flow cytometric analysis was also reported by Lidy Verburg-van Kemenade\(^20\).

TBT is incorporated by aquatic organisms through several pathways including uptake from contaminated diet and direct uptake from surrounding water. Consequently, high levels of TBT residue have been detected in aquatic organisms\(^20\). Therefore, chronic effects of TBT on immune system might be apprehensive. Field observation has also proved that aquatic pollutants could affect immune function in fish\(^24(20)\). Environmental chemicals have reduced resistance in the immune system and increased the prevalence of infections in the field\(^20\).

The present results using flow cytometry showed the immunotoxic effects of tributyltin on the non-specific immune system in rainbow trout. Although various kinds of environmental chemicals disperse in the aquatic environment and influence terrestrial and aquatic organisms, their toxic effects on immune systems are not clarified in detail. We suggest that FCM is a quite powerful tool in the immunotoxicological evaluation of various kinds of contaminants.

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5. REFERENCES


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