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EFFECTS OF BUTYLTIN COMPOUNDS ON PHAGOCYTIC ACTIVITY OF HEMOCYTES FROM THREE MARINE BIVALVES

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Abstract—Effects of tributyltin (TBT), dibutyltin (DBT), and monobutyltin (MBT) on the in vitro phagocytic activity of hemocytes from three marine bivalve species, *Mytilus edulis, Mya arenaria,* and *Mactromeris polynyma,* were determined using flow cytometry. Phagocytosis was reduced with increasing doses of TBT and DBT. The toxicity of butyltins on hemocytes decreased in the order DBT > TBT > MBT, and comparison of the relative sensitivity of the three species showed that blue mussels (*M. edulis*) were more tolerant of butyltin compounds than both clam species. Toxicity mechanisms of butyltins are discussed and compared to those of other metals.

Keywords—Butyltins Immunotoxicity Marine bivalves Phagocytosis

INTRODUCTION

Environmental problems linked to organotin compounds began more than 30 years ago, when new butyltin-based pesticides were introduced into both terrestrial and aquatic environments [1]. Industrial uses of organotin compounds include not only antifouling paints but also wood preservation, chemical stabilization of plastics, and crop protection against parasites [2]. Contamination of estuarine and coastal waters by organotin compounds has occurred worldwide for almost 20 years, and some highly industrialized northern countries (France, Great Britain, Canada, and the United States) have passed legislation to reduce the use of these products and their release into the environment. In many locations where regulations have been enforced, major improvements in water quality have been observed as tributyltin (TBT) pollution has decreased in seawater and surface sediment over the last 10 years [3]. Despite these improvements, some factors still limit a full recovery of heavily contaminated areas, such as the long-term persistence of TBT in sediments, the diffusion and dispersion of TBT from hulls of large vessels, and, more importantly, the lack of control measures in many developing countries [4,5]. The main concern of environmental marine chemists and toxicologists is now the widespread distribution of butyltin compounds at low concentrations in many shallow and deep coastal waters and sediments.

Multiple studies have demonstrated harmful effects of butyltin compounds on various aquatic organisms [6]. Oyster shell thickening [7], high mortality of mussel larvae [8], and particularly impairment of gastropod reproduction by the development of imposex [9] have been clearly established. Organotin compounds attack the defense system of cells and immuno-, neuro-, and cytotoxicity have been observed in mammal cells [3]. In invertebrates, some studies have demonstrated the effect of contaminants on immune functions of oysters [10] and clams [11], and such alterations could lead to impaired bacterial clearance and increased disease susceptibility [12].

The circulatory system of bivalves is an open system, and hemolymph is found in interstitial space, where it is exposed to any substance that crosses the gill epithelium [10]. The hemolymph contains hemocytes, which are part of the immune system. Some of these hemocytes have the capacity to phagocytose, an essential cellular defense mechanism against pathogens and parasites [13]. Phagocytosis seems to be the most significant defense mechanism in mollusks [11].

The objective of this study was to determine the immunotoxicological effects of TBT, dibutyltin (DBT), and monobutyltin (MBT) on bivalve hemocytes by measuring their phagocytic activity in blue mussel (*Mytilus edulis*), soft-shelled clam (*Mya arenaria*), and Stimpson surf clam (*Mactromeris polynyma*).

MATERIALS AND METHODS

Mussels and soft-shelled clams were collected during the summer on the south shore of the St. Lawrence Estuary near Metis Beach (48°40′N, 68°00′W), a remote location exempt from any direct sewage outputs or harbor activities. Stimpson surf clams were obtained from two remote sites on the north shore of the St. Lawrence Estuary, in front of Rivière-au-Tonnerre (50°15′N, 64°45′W) and Longue-Pointe de Mingan (50°14′N, 64°17′W). Organisms were kept in a tank with flowing seawater (7–8°C) for 5 to 7 d before hemolymph was collected.

Collection of hemocytes

Hemocytes were collected by withdrawal of hemolymph from the adductor muscle in individual bivalves using 5-ml syringes and 21-gauge needles. Cells of many individuals within the same species were pooled for blue mussels and soft-

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shelled clams and then filtered through nylon wool into a Pasteur pipette, but the Stimpson surf clam cells were neither pooled nor filtered to prevent aggregation. The total volume of hemolymph collected for each species was approx. 30 ml (enough to complete 27 tubes). Cell counts were recorded using a Neubauer hemocytometer. Cell suspensions (900 L) were transferred into test tubes, and cell concentration was around 0.5×10^6 cells/ml of hemolymph. All assays were repeated in triplicate.

Contamination of hemolymph

The chemicals used were tributyltin chloride ($[CH_3(CH_2)_3]_3SnCl$), dibutyltin dichloride ($[CH_3(CH_2)_3]_2SnCl_2$), and monobutyltin trichloride ($CH_3(CH_2)_3SnCl_3$). Compounds were dissolved in 100% ethanol, and a 0.1-M stock solution was prepared for each toxicant. Successive dilutions of the stock solution were made to reach concentrations down to 10^{-7} M, and 10 L of each dilution was then added to separate tubes (three tubes for each dilution). For the Stimpson surf clam, only 1 L of 1 to 10^{-6} M was added to the tubes. Two controls were performed, one with no contaminant and one with 10 L (1 L for the Stimpson surf clam) of 100% ethanol, and then the volume was completed to 1 ml with centrifuged hemolymph. The cell suspensions were thus exposed to 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M of MBT, DBT, and TBT.

Determination of phagocytic activity

Yellow-green latex Fluospheres (1.58 μm in diameter) (Molecular Probes, Eugene, OR, USA) were added to the cell suspensions at a ratio of 1:100 (hemocytes:beads). After 18 h of incubation in darkness at room temperature, each sample (1 ml) was poured onto a 3% bovine serum albumin gradient and centrifuged at 100 *g* for 8 min at 4°C to remove free beads. The cell pellets were resuspended in 1 ml of hematall, an isotonic diluent (Fisher Scientific, Nepean, ON, Canada). Samples were then analyzed with a FACScan (Becton Dickinson, San Jose, CA, USA) flow cytometer with an air-cooled argon laser, providing an excitation at 488 nm. Hemocyte populations were defined on the basis of their forward and right angle scatter properties. Fluorescence emission was measured at 520 nm. A total of 10,000 events were acquired for each sample and stored in the list data format. Data were then analyzed with the fluorescence frequency distribution histogram of the hemocyte population. Data collection and analysis were performed with the LYSYS-II program.

Statistical analysis

To compare the means (three means, each of three pseudoreplicates) between contaminants and between biological species, analysis of variance (ANOVA) was used. Normality was verified using the Kruskal-Wallis test, and variance homogeneity was verified with the F_{max} test. For every biological species (soft-shelled clams, Stimpson surf clams, and mussels), ANOVA tests were conducted to determine the significance of differences between contaminants (TBT, DBT, and MBT) for every dose. For all significant results ($p \leq 0.5$), an a posteriori test was performed to determine which contaminants were different from one another. Then, for each contaminant (TBT, DBT, and MBT), ANOVA tests were conducted to determine the significance of differences between biological species (soft-shelled clams, Stimpson surf clams, and mussels) for every dose. For all significant results ($p \leq 0.05$), an a posteriori test was performed to determine which species were different from one another.

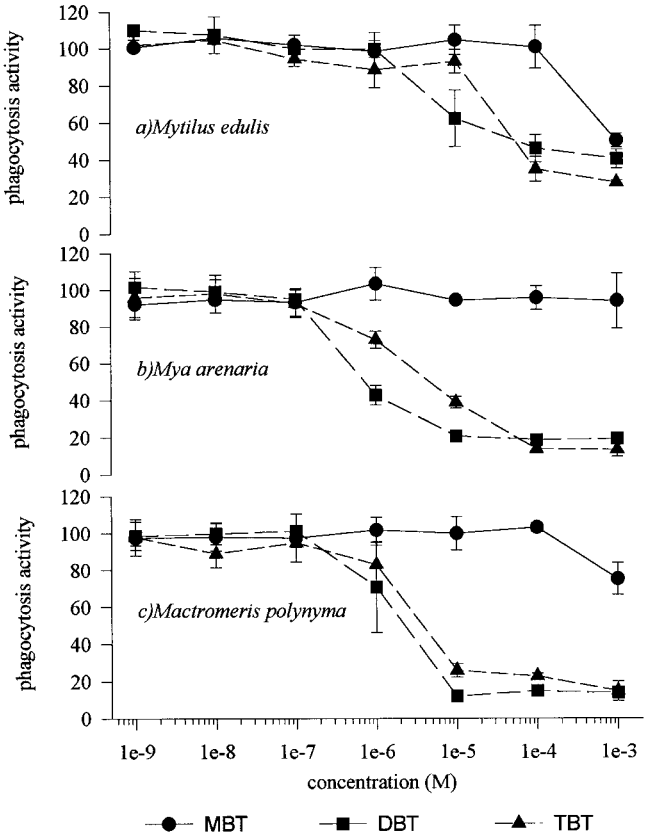

Fig. 1. Effects of organotins, monobutyltin (MBT), dibutyltin (DBT), and tributyltin (TBT), on the activity of phagocytosis of three bivalve hemocytes: (**a**) *Mytilus edulis*, (**b**) *Mya arenaria*, and (**c**) *Mactromeris polynyma*. Values are calculated as the percentage of the control and represent the mean value of three experiments. Vertical bars represent the standard deviation.

RESULTS AND DISCUSSION

Phagocytic activity of mussel hemocytes is presented in Figure 1a. At 10^{-9} M of DBT, a slight but significant stimulation was observed ($p \leq 0.05$). When the dose was increased from 10^{-8} to 10^{-6} M, organotin compounds had no apparent effect on the phagocytic activity, but further increases of TBT and DBT decreased phagocytosis progressively, whereas MBT did not induce any clear effect except at the highest concentration tested (10^{-3} M). The results for the soft-shelled clam are shown in Figure 1b. At concentrations between 10^{-6} and 10^{-5} M, DBT had a greater effect than TBT ($p \leq 0.05$), whereas MBT had no detected effect at any concentration studied ($p \leq 0.05$). Figure 1c represents results for the Stimpson surf clam. Here again, organotin compounds did not have any detectable effects on phagocytic activity at 10^{-7} M and less, and reduction of activity began at 10^{-6} M (although not statistically significant) and was strongly reduced at 10^{-5} M for TBT and DBT but not for MBT.

Similar concentrations of TBT and DBT have been reported to inhibit in vitro phagocytosis of rabbit polymorphonuclear leukocytes [14] and tunicate (*Botryllus schlosseri*) hemocytes [15]. In bivalves, TBT has been found to decrease chemiluminescence activity (used as a measure of phagocytic activity) in oyster hemocytes at a concentration of 1.5×10^{-6} M [10].

Table 1. Immunosuppression dosage at 50% activity of phagocytosis of organotins for three species of bivalves[a]

	Mytilus edulis (M)	*Mya arenaria* (M)	*Mactromeris polynyma* (M)
Monobutyltin	1.0×10^{-3}	—	—
Dibutyltin	5.0×10^{-5}	8.1×10^{-7}	2.3×10^{-6}
Tributyltin	5.0×10^{-5}	4.5×10^{-6}	3.8×10^{-6}

[a] Values were obtained graphically from Figure 1.

All these studies agree and show effective concentrations of TBT and DBT on macrophage phagocytosis ranging from 10^{-5} to 10^{-7} M.

The main finding of the present study is the apparently higher immunotoxicity of DBT over TBT in all three bivalve species. For mussels (Fig. 1a), the tendency for DBT to reduce the phagocytic activity more than TBT appeared at a toxicant concentration of 10^{-5} M, although this difference is lost at higher concentrations. The higher toxicity of DBT is also obvious for *M. arenaria* (Fig. 1b), in which 10^{-6} M DBT was much more toxic than 10^{-6} M TBT ($p \leq 0.05$). Again with *M. polynyma* (Fig. 1c), DBT was more toxic than TBT for concentrations $>1 \times 10^{-5}$ M ($p \leq 0.05$).

The usual toxicity sequence for butyltins is given in the order TBT > DBT > MBT and corresponds to a progressive dealkylation of butyltins by cytochrome P450, where each step of the process yields derivatives progressively less toxic [16]. This sequence has been confirmed by some toxicological studies at cellular levels [17,18]. Brüschweiler et al. [17] found that TBT was more cytotoxic than DBT, which was more cytotoxic than MBT on fish hepatoma cells. Moreover, Dooley and Kenis [18] observed that TBT was 35 times more toxic than DBT and 750 times more toxic than MBT when measuring the median effective concentration (EC50) on the luminescent bacterium *Photobacterium phosphoreum* with the Microtox® toxicity analyzer. However, a series of articles on the hepatotoxicity of butyltin compounds performed in vivo in mice suggested that dibutyltin chloride is more hepatotoxic than tributyltin chloride, which implies that DBT may be the active form of butyltin inside cells [19,20].

The comparative immunotoxicity of DBT and TBT has received very little attention, and only one published study [15] has investigated the effects of butyltins on in vitro phagocytosis of hemocytes from only one tunicate species. In that study, 10^{-6} M DBT induced a higher inhibition of phagocytosis than TBT at the same concentration. Our results with hemocytes of three different bivalves strongly support this finding.

Dose values for inhibition of 50% of the phagocytic activity (ID50) are presented in Table 1. No MBT values are available for the soft-shelled clam and the Stimpson surf clam because phagocytosis activity did not decrease below 50% for the range of tested concentrations. For mussels, the ID50 value for MBT was the highest dose tested in this experiment, and values for DBT and TBT were clearly higher than those for the soft-shelled clam and Stimpson surf clam. The ID50 of TBT shows a similar value for the soft-shelled clam and the Stimpson surf clam. The ID50 value for TBT is one order of magnitude higher for mussels compared with the two other species. Both clam species seem to have the same tolerance of TBT but not DBT, of which soft-shelled clams were more sensitive than Stimpson surf clams (Table 1). Similarly, Nelson et al. [21] found juvenile *M. edulis* (96-h median lethal concentration [LC50], 0.122 mg/L) to be more resistant to cupric chloride than *Spisula solidissima* (96-h LC50, 0.051 mg/L), another species of surf clam. *Mytilus edulis* (96-h LC50, 25.0 mg/L) is also more resistant to cadmium than *M. arenaria* (96-h LC50, 2.2 mg/L) [22].

The higher immunosuppression activity of DBT toward TBT might be associated with at least two factors acting simultaneously, the alteration of Ca^{2+} efflux by DBT and the particular membrane affinity of TBT. Results from a number of biochemical studies [3,23] indicate that immunotoxicity of butyltins in mammals is caused by an alteration of Ca^{2+} homeostasis, resulting in an alteration of cellular structure and functions. In their study on the hemocytes of tunicates, Cima et al. [15] observed a significantly more effective inhibition of phagocytosis by DBT than TBT and also found a more important and significant reduction of Ca^{2+} adenosine triphosphatase (ATPase) activity by DBT than TBT. The authors interpreted these results as a clear indication of a biochemical link between phagocytosis and Ca^{2+}-ATPase activity, but no attempt was made to explain why DBT was more efficient than TBT. Inhibition of Ca^{2+}-ATPase by TBT and DBT might result from a competition for enzyme active sites usually occupied by Ca^{2+}. Using a simple structure–activity approach, DBT appears to be a better competitor for calcium sites than TBT because the ionic form of DBT is a double-charged cation like Ca^{2+} and DBT is a much smaller moiety than TBT, which brings only one positive charge. The second factor to be considered is the possible partial retention of TBT at the surface or within the plasma membrane of hemocytes. The membrane affinity and toxicity of TBT has been clearly demonstrated by Porvaznik et al. [24] using human erythrocytes. The ability of TBT to induce major modifications in plasma membrane was recently established by means of anisotropy measurements of fluorescent probes embedded in the phospholipidic layer of isolated trout erythrocyte membranes [25]. Because TBT is more active than DBT at the membrane level by its insertion and retention within lipidic bilayers, less free TBT could be available in the cytoplasm and less TBT could interact directly with Ca^{2+}-ATPase. The partial retention of TBT in membranes coupled with better competition to Ca^{2+} sites by DBT seems to us a sound hypothesis to explain the higher immunosuppression of DBT over TBT. Mechanistic and kinetic studies are in progress in our laboratory to test this hypothesis and elucidate the toxic action of butyltins on bivalve hemocytes and to predict the effects of low levels of exposure of marine mollusks to organotins and the resultant effects in humans.

Although the concentrations used in this study were well above those observed in contaminated marinas and harbors, our results provide a good indication of the relative sensitivity of the different bivalve species to butyltins. Mussels are much less sensitive than soft-shelled clams and Stimpson surf clams. Our results also show that the trend for butyltin toxicity is different from the usual order: DBT > TBT > MBT. Even if DBT is generally considered much less toxic than TBT, it could have a higher immunotoxicity, at least in marine invertebrates.

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