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Immunotoxicity of Butyltins in Tunicates

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This study was designed to investigate the effects of butyltins on yeast phagocytosis *in vitro* by haemocytes of the colonial ascidian *Botryllus schlosseri*. This species has been reported to be very sensitive to organotins. Results show that, in analogy to reports on mammalian leukocyte reactivity, butyltins exert inhibitory effects on phagocytosis in a concentration-dependent manner, mainly by influencing cellular calcium homeostasis by interacting with the calcium pump. As the immunosuppressant activity of organotins described in mammals and teleosteans also occurs in an invertebrate marine species, the latter may assume a role as a sensitive biosensor of butyltins as immunotoxins in aqueous ecosystems.

Keywords: tributyltin (TBT); dibutyltin (DBT); monobutyltin (MBT); haemocytes; ascidians; *Botryllus*; phagocytosis; immunotoxicity; Ca^{2+}-ATPase; oxidative phosphorylation; polymorphonuclear leukocytes (PMNs)

INTRODUCTION

Butyltin compounds fall into two major classes. Low-molecular-weight compounds such as monobutyl- (MBT) and dibutyl-tin (DBT) are used in the plastics industry as PVC stabilizers and DBT has mutagenic potential.[1] Higher-molecular-weight compounds such as tributyltin (TBT) are used as biocides, especially in the aquatic environment, where they have been used as antifouling paints on boats, fishing nets and cages, and as wood preservatives.[2,3] The biological interest in the latter class of compounds is due to their high toxicity to aquatic species and their introduction in aquatic environments, having deleterious effects on both freshwater and marine ecosystems, particularly on filtering invertebrates. These range from retarded growth and shell thickening in molluscs,[4] development of imposex in bivalve molluscs,[5] altered limb regeneration and photobehaviour in crustaceans,[6,7] to embryotoxicity in tunicates.[8,9]

Moreover colonial ascidians are reported to be very susceptible to butyltins, so that mortality occurs even after exposure to concentrations higher than 100 ng l^{-1} of TBT.[10]

TBT has been shown to interfere with teleostean and mammalian immune systems, causing atrophy of the thymus and lymphoid tissues.[11,12] Inhibition of phagocytosis and cytolysis of polymorphonuclear leukocytes (PMNs) after inhibition of chemotaxis and respiratory burst, with resultant depression of cell-mediated immune responses, have also been demonstrated in the rabbit.[13] Therefore, our interest in the study of ascidian defence reactions led us to investigate the effects of this class of compounds on yeast phagocytosis *in vitro* by haemocytes of the colonial ascidian *Botryllus schlosseri*. Results demonstrate the immunotoxicity of butyltins in an invertebrate marine species and give evidence that, in analogy to reports on mammalian leukocyte reactivity, they exert their effects by influencing cell calcium homeostasis.

MATERIALS AND METHODS

Animals

Colonies of *B. schlosseri* from the Venetian lagoon were reared in our laboratory, attached to glass slides, immersed in aquaria, and fed with Liquifry Marine (Liquifry Co., Dorking, Surrey, UK) and algae.

Haemocyte collection

Blood cells were obtained by tearing with fine tungsten needles the peripheral tunic vessels of colonies previously rinsed in filtered seawater (FSW) containing 10 mM L-cysteine adjusted to pH 7.0 to prevent clotting. They were then

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washed by centrifugation at 780*g* for 15 min and the pellets were resuspended in FSW for a final concentration $(8–10) \times 10^6$ cells ml^{-1}.

Haemocyte cultures

Culture chambers were made by glueing Teflon rings (15 mm i.d., 1 mm thick) on siliconized glass slides. A 50 µl drop of the haemocyte suspension was placed in the centre of each well and washed coverslips were laid over the Teflon rings, smeared with vaseline and gently pressed down to touch the drop of the cell suspension. The culture slides were kept upside-down for 30 min to allow the cells to settle and adhere to the coverslips.

Phagocytosis assay

After adhesion of the haemocytes to the coverslips, the FSW containing debris was discarded and replaced with an equal volume of a suspension of autoclaved (15 min at 120 °C) ordinary baker's yeast in FSW (yeast/haemocyte ratio = 10:1). Cultures were kept upside-down for 60 min at room temperature. Haemocyte monolayers were then washed by dipping the coverslips several times in a large volume (100 ml) of FSW, fixed in a solution of 1% glutaraldehyde and 1% sucrose in FSW at 4 °C for 30 min, and stained with 10% Giemsa for 10 min. The coverslips were finally mounted on glass slides with an aqueous medium (Acquovitrex, Carlo Erba) and cells were observed with a Leitz Dialux 22 light microscope.

Haemocytes were counted at a magnification of ×1250 (at least 200 cells per coverslip in ten fields) and the phagocytic index, i.e. the percentage of haemocytes with ingested particles, was determined.

Effects of butyltins, ouabain and 2,4-dinitrophenol (DNP)

Tributyltin (TBT), dibutyltin (DBT) and monobutyltin (MBT), as the mono-, di-, and trichloride respectively (Sigma), were first dissolved in 95° ethanol at 10 mM and then diluted, at final concentrations of 0.1, 1 and 10 µM, in the FSW used to suspend the yeast. In controls, 0.1% 95° ethanol was added to FSW. Ouabain and DNP, dissolved in FSW, were used at concentrations of 1, 10 and 100 mM, and of 10 and 100 µM, respectively. Ouabain and DNP were also used in mixtures together with TBT as reported in the Results section. The pH of all solutions was maintained at 7.0. Viability was assessed by Trypan Blue exclusion.

Assay for Ca^{2+}-ATPase and internal Ca^{2+}

Histochemical assays for Ca^{2+}-ATPase activity were carried out on *B. schlosseri* blood smears according to Chayen *et al.*[14] In controls for specificity, glycerophosphate was used in place of ATP. The Ca^{2+}-ATPase index, i.e. the percentage of cells showing dark brown precipitates, was evaluated as a measure of Ca^{2+}-ATPase activity. Internal Ca^{2+} was revealed as a dark blue precipitate, following the substitution method of Von Kossa.[15]

Statistical analysis

All experiments were repeated three times ($n = 3$). Data are expressed as means ±SD; they were analysed using the χ^2 test.

RESULTS AND DISCUSSION

Phagocytosis of yeast cells *in vitro* by *B. schlosseri* haemocytes is negatively affected by organotin compounds present in the incubation medium. The phagocytic index, which ranges between 12 and 15 in controls, is significantly reduced by all three compounds when assayed at a concentration of 10 µM ($P < 0.01$ for MBT and $P < 0.001$ for DBT and TBT); DBT and TBT significantly ($P < 0.001$ and $P < 0.05$ respectively) inhibit at 1 µM also, DBT being significantly more effective than TBT ($P < 0.01$) (Fig. 1).

The viability of haemocytes, as assessed by Trypan Blue exclusion, exceeds 95% after 2 h of incubation in FSW not containing organotins; a significant decrease in haemocyte viability is observed after only 1 h exposure at concentrations higher than 100 µM for DBT and TBT, and 1 mM for MBT.

Some morphological changes occur in the presence of active doses of butyltins. haemocytes become spherical, but without cytolysis, suggesting a strong interaction with cytoskeletal constituents.

Ca^{2+}-ATPase activity, which differs from phosphatase activity as evidenced histochemically, is associated with phagocytosis in *B. schlosseri* haemocytes (Fig. 2a). When activity is assayed after

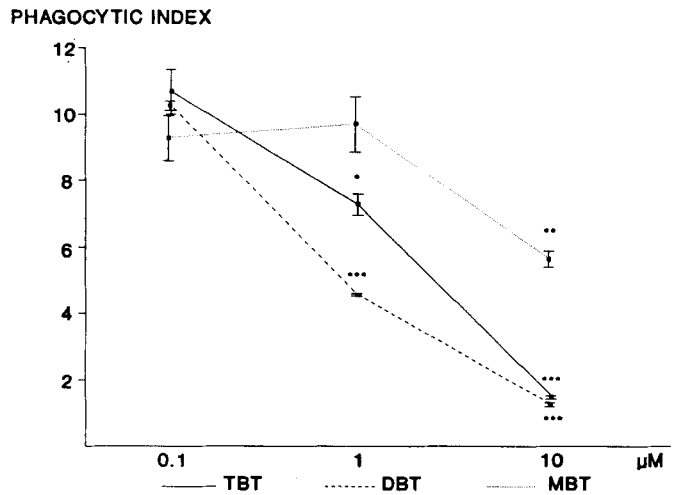

Figure 1 Effects of MBT, DBT and TBT on yeast phagocytosis by *B. schlosseri* haemocytes. Asterisks indicate the levels of significance with respect to controls: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

exposure to TBT, DBT and MBT, it shows the same order of inhibition as found in phagocytosis (Fig. 3). This result suggests that inhibition of phagocytosis may be linked to inhibition of Ca^{2+}-ATPase activity. To check this hypothesis, the effects of TBT on Ca^{2+}-ATPase activity were compared with those induced by ouabain, as a reference inhibitor, at non-lethal doses (10–100 mM) which significantly inhibited phagocytosis ($P<0.001$; phagocytic index 6.19 ± 0.71 and 2.07 ± 0.61 respectively). Even though DBT gives a higher inhibition of phagocytosis than TBT, the latter has been chosen because of its wider distribution in the marine environment and the slight slope of its pattern of phagocytic index inhibition which enables a better differentiation among the effects of the different doses. In cross-experiments with isodynamic mixtures—i.e. maximum inactive dose of ouabain (1 mM) plus minimum active non-lethal amount of TBT (10 μM) and, in comparison, minimum active non-lethal amount of ouabain (10 mM) together with maximum inactive dose of TBT (1 μM)—a combined response of summation or an additive effect of TBT and ouabain is always seen (Fig. 4). In fact, by joining the tops of the three columns on the left and right, respectively, of Fig. 4, two isoboles can be drawn, denoting the close additivity of

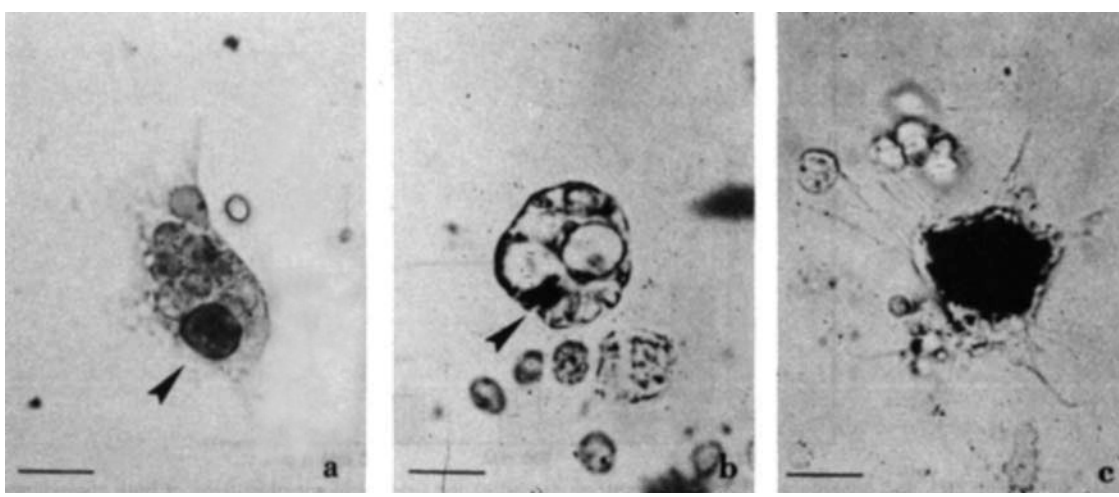

Figure 2 Phagocytizing haemocytes, showing (a) positivity for Ca^{2+}-ATPase (arrowhead), (b) localized internal calcium rise in normal conditions (arrowhead) and (c) diffuse internal calcium rise in the presence of TBT. Bars: 10 μm.

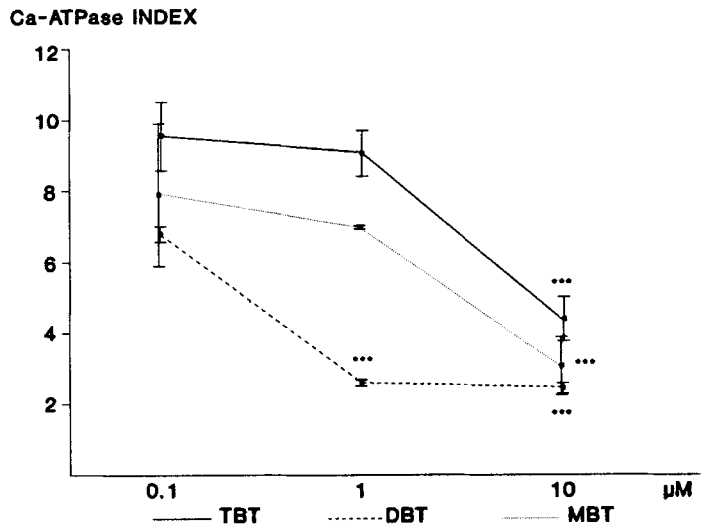

Figure 3 Effects of MBT, DBT and TBT on Ca^{2+}-ATPase activity of *B. schlosseri* phagocytes. Asterisks indicate significant differences with respect to controls: *** $P<0.001$.

action of TBT and ouabain. Moreover, the resulting enzymatic indexes in both cases do not significantly differ from that obtained in the presence of the maximum non-lethal dose of ouabain (100 mM; centre of Fig. 4). This suggests a common mechanism of action of TBT and ouabain, both interacting with the calcium pump probably through calcium channels[16,17] and calmodulin,[18] as demonstrated in mammals. These results are supported by the fact that phagocytes exposed to TBT or ouabain show an accumulation of Ca^{2+} ions, evidenced cytochemically as a single giant cytoplasmic dark blue precipitate, instead of the small localized spots observed in controls (compare Fig. 2b with Fig. 2c). The number of haemocytes with diffuse precipitates increases in parallel with the decrease of Ca^{2+}-ATPase index (Fig. 5).

As TBT is known to inhibit mitochondrial oxidative phosphorylation,[19] DNP was tested as a reference enzyme inhibitor at non-lethal doses (0.01–0.1 mM) for its effect on phagocytosis. A combined response of incomplete summation or a less-than-additive effect is observed in cross-

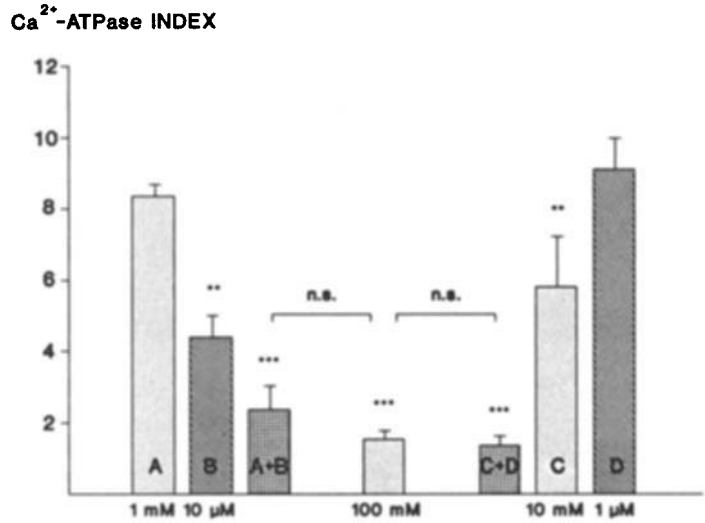

Figure 4 Effects of TBT and ouabain at different concentrations and of the isodynamic combinations of both compounds on Ca^{2+}-ATPase activity of *B. schlosseri* phagocytes. Asterisks indicate the levels of significance with respect to controls: ** $P<0.01$; *** $P<0.001$. ▨, ouabain; ▧, TBT; ▩, ouabain plus TBT.

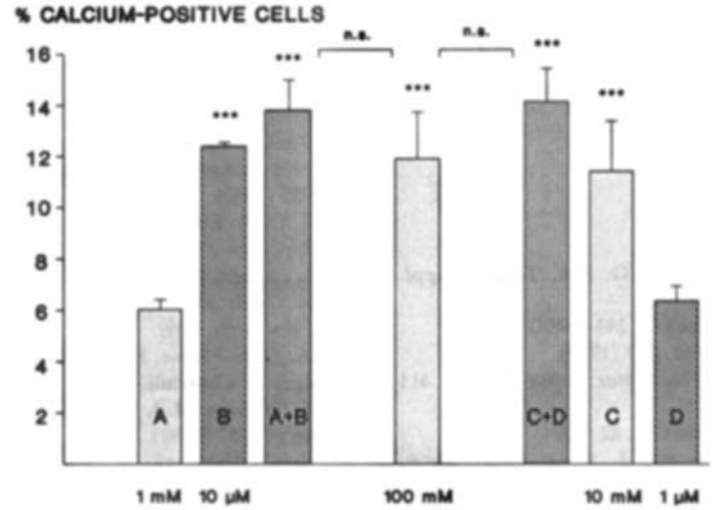

Figure 5 Percentage of *B. schlosseri* haemocytes showing the internal calcium rise associated with yeast phagocytosis after incubation in FSW containing different concentrations of TBT ▨, ouabain ⬚ or isodynamic combinations of both compounds ▩. Asterisks indicated significant differences with respect to controls: *** $P<0.001$.

experiments with isodynamic mixtures of TBT and DNP (Fig. 6). In other words, if the tops of the three columns on the left of Fig. 6 are joined, an isobole can be obtained suggesting a less-than-additive effect, while the isobole drawn from the respective columns on the right of Fig. 6 is indicative of synergy or potentiation. In fact, the resulting phagocytic indices in both cases do not differ significantly, but they do differ from that obtained in the presence of the maximum non-lethal dose of DNP (1 mM; centre of Fig. 6). The lack of symmetric patterns in isobolograms of interactions between TBT and DNP suggests at least a secondary and/or indirect action mechanism of the two compounds. Therefore, inhibition of phagocytosis by butyltins in our species appears to be closely linked to inhibition of Ca^{2+}-ATPase activity rather than to inhibition of mitochondrial oxidative phosphorylation.

Further research is necessary for better insight into the immunosuppressant activity of organotins, which may also involve inhibition of the

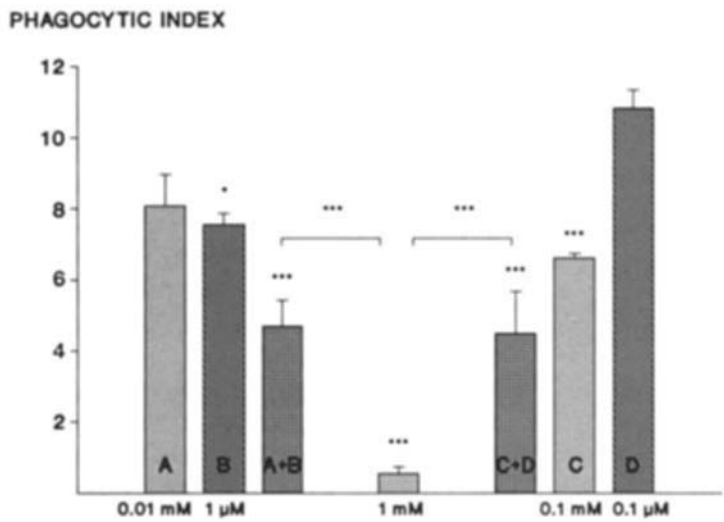

Figure 6 Effects of TBT and DNP at different concentrations and of the isodynamic combinations of both compounds on yeast phagocytosis by *B. schlosseri* haemocytes. Significance, unless otherwise indicated, refers to controls: * $P<0.05$; ** $P<0.01$; *** $P<0.001$. ⬚, DNP; ▨, TBT; ▩, DNP plus TBT.

hydrolysis of adenosine triphosphate and uncoupling of oxidative phosphorylation taking place in mitochondria.[20]

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