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Tributyltin affects phagocytic activity of *Ciona intestinalis* hemocytes

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Organotin compounds have been used in marine anti-fouling paints as biocides. Because tunicates are vulnerable to these compounds in their natural habitats, we used *Ciona intestinalis* to establish an assay for phagocytosis *in vitro* of yeast by hemocytes after exposure to different concentrations (0.0015, 0.015, 0.15 and 1.5 μM) of four organotin compounds: tributyltin (TBT), triphenyltin (TPT), dibutyltin (DBT) and diphenyltin (DPT). To evaluate the phagocytic activity, we used a method based on fluorescence excitation of yeasts pre-treated with eosin-Y. The percentage of phagocytosis decreased from 45.1 ± 3.49 to 22.4 ± 5.14 at 1.5 μM of TBT ($P < 0.001$); it was significantly reduced in presence of the ionophore A23187. TPT, DPT and DBT did not show significant effects on phagocytosis. Because the effect of TBT was irreversible, differences between the inhibitory mechanisms of ionophore and TBT are suggested. These results indicate that for future analyses, tunicates should become excellent models for dissecting events such as phagocytosis that are associated with immunosuppression after exposure to xenobiotics.

Key words: Ascidian; Hemocytes; Phagocytosis; Stress; Dibutyltin; Tributyltin; Diphenyltin; Triphenyltin.

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Introduction

Tributyltin compounds (TBT) are used in marine anti-fouling paints as a biocide (Evans and Smith, 1975). The toxicological implications of increased levels of TBT in estuarine and coastal waters have prompted analyses using aquatic invertebrates as models for detecting effects of xenobiotics. Research using bivalve mollusks reveals that TBT affects chromosomes (Dixon and Prosser, 1986), sex ratio and gamete production (Roberts *et al.* 1987) as well as survival of embryos and larvae (Beaumont and Bud, 1984; Roberts, 1987; Mansueto *et al.*, 1993). The hemocytes of aquatic invertebrates function in immunodefense against parasites and pathogens by mechanisms that include phagocytosis, encapsulation, inflammation and wound repair (Bang, 1975; Ratcliffe *et al.*, 1985). Hemocytes are contained in and circulate freely within hemolymph and interstitial spaces where they are exposed to ambient changes in temperature, salinity and any foreign materials, some of which may be pathogenic, that crosses the gill epithelium.

Phagocytosis requires several vital cell activities, such as non-self-recognition, cell spreading, locomotion, binding and ingestion of particulate matter and intracellular degradation of the offending pathogen (Fisher, 1986). These events that involve interrelated mechanisms, essential to the immunodefense

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responses, require the simultaneous participation of the cytoplasm, cytoskeleton, membranes and the nucleus. Loss of their functions can be expected to interfere with immunodefense capabilities. Hemocytes can be used to assess the effects of chronic exposures, bioaccumulation and sub-lethal toxicity of TBT (Fisher *et al.*, 1990).

Ciona intestinalis is a filter-feeding tunicate that has figured prominently in understanding immunological responses of their hemocytes (Parrinello *et al.*, 1977; Wright and Ermark, 1982; Parrinello and Arizza, 1992). For example, almost half of the hemocytes phagocytose erythrocytes (Rowley, 1981) or bacteria (Smith and Peddie, 1992). To asses their potential after exposure to a xenobiotic, we examined the phagocitic activity of *Ciona* hemocytes after exposure to TBT, triphenyltin (TPT), dibutyltin (DBT) and diphenyltin (DPT). In addition, the effects of the ionophore A23187 and TBT have been compared with respect to phagocytosis.

Materials and Methods

Tunicates

C. intestinalis were collected from the gulf of Mazara and then maintained in healthy condition until use, usually within 2 days, in an aerated and refrigerated seawater tank at 15°C.

Collecting hemocytes

Hemolymph containing hemocytes was obtained by cutting the tunic and puncturing the heart, according to previously described methods (Parrinello *et al.*, 1992). To prevent any coagulation, hemolymph was collected according to Smith and Peddie (1992) in ice-cold marine anticoagulant (0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 10 mM EDTA, 0.45 M NaCl, pH 7.0). Hemocyte suspensions were removed individually from each tunicate, centrifuged separately in a swing out rotor at 700 ***g***, 4°C for 10 min and resuspended in marine solution (MS) (12 mM $CaCl_2 \cdot 6\ H_2O$, 11 mM KCl, 26 mM $MgCl_2 \cdot 6\ H_2O$, 45 mM Tris, 38 mM HCl, 0.45 M NaCl, pH 7.4). Hemocyte numbers were adjusted to a concentration of 3×10^6. Viability was estimated by adding a solution of eosin-Y, 0.5% diluted 1:10 in the hemocyte suspension to determine cell viability.

In vitro phagocytosis assay

The method reported by Cammarata and Arizza (1994) was used. *Saccharomyces cerevisiae* (baker's yeast, type II, Sigma Chemical Co., St Louis, MO, U.S.A.) was prepared in distilled water (d. w.) as a 0.25% w/v solution ($\sim 1 \times 10^8$ yeasts/ml) autoclaved for 15 min, washed twice by centrifugation at 200 ***g*** at 4°C for 5 min and incubated for 2 hr at 20°C with a solution of eosin-Y at a final concentration of 0.05%. Yeasts were washed four times and resuspended at a final concentration of 0.125% w/v in MS and stored at −20°C for a maximum of 2 weeks. For the experiments, yeasts were washed once and resuspended in MS. Aliquots of 200 μl of hemocyte suspensions (6×10^5) were mixed with 100 μl of yeast and incubated in 1-ml test plastic tubes with gentle stirring for 90 min at 20°C. Then, 50 μl of a quenching solution (Trypan Blue 2 mg/ml crystal violet 2 mg/ml in 0.02 citrate buffer, pH 4.4 containing NaCl 33 mg/ml) was added. A drop of this suspension was then smeared onto slides and examined under an ultraviolet light microscope (Diaplan, Leika, Wetzlar, Germany). Hemocytes were counted at magnification ×800 ≥200 ± 35 hemocytes for slide. Results are expressed as percentage of hemocytes containing phagocytosed yeast over the total number of hemocytes.

Exposure to organotins and the ionophore A23187

DBT, TBT, DPT and TPT (Schering AG, Bergkamen, Germany) were prepared in an aqueous solution at nominal concentrations of 0.045, 0.45, 4.5 and 45 μM, wrapped in aluminum foil and stored at 4°C. Concentrations were confirmed using a Perkin-Elmer atomic absorption spectrophotometer equipped with a graphite furnace. Stock solutions were diluted 1:10 in MS, and this solution was then used in the assays.

Ionophore A23187 (Sigma Chemical Co.) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 3.0 mM. To obtain suitable concentrations, aliquots of organotin or ionophore stock solutions were added to obtain the desired experimental concentrations: 0.0015, 0.015, 0.15 and 1.5 μM for organotin compounds and 0.1 and 1.0 μM for the ionophore A23187. These were then added to the the yeast suspensions just before mixing them with hemocytes. In other experiments, hemocytes were pre-incubated for 10, 20 and 30 min with 1.5 μM of TBT or 10 and 30 min with 1.0 μM of A23187. Hemocytes were then washed twice and resuspended in MS before yeast particles were added.

Statistical analysis

Standard deviations were calculated based upon 10 to 30 experiments. Statistical signifi-

cance was determined by Student's *t*-test (Sokal and Rohlf, 1981). Differences were considered to be significant at $P < 0.05$.

Results and Discussion

Characteristics of phagocytic hemocytes

The hemolymph from the sea squirt, *C. intestinalis*, contains several types of hemocytes: stem cells, hyaline, granular and refractile amoebocytes and vacuolated (signet ring, morula, compartment, orange) cells (Wright, 1980). Of these cell types, only hyaline and granular amoebocytes are capable of phagocytosing erythrocytes *in vitro* (Rowley, 1981). The method we used to evaluate the phagocytic activity was based on the treating yeast with the fluorochrome eosin-Y and by quenching the target particles not ingested or those that adhered to hemocytes. Figure 1 shows phagocytes from *C. intestinalis* hemolymph that contain fluorescent yeast; usually at least two yeasts were contained in washed phagocytes. When hemocytes were counted after the phagocytosis assay, 45.1% of them was observed to be phagocytic (Fig. 2).

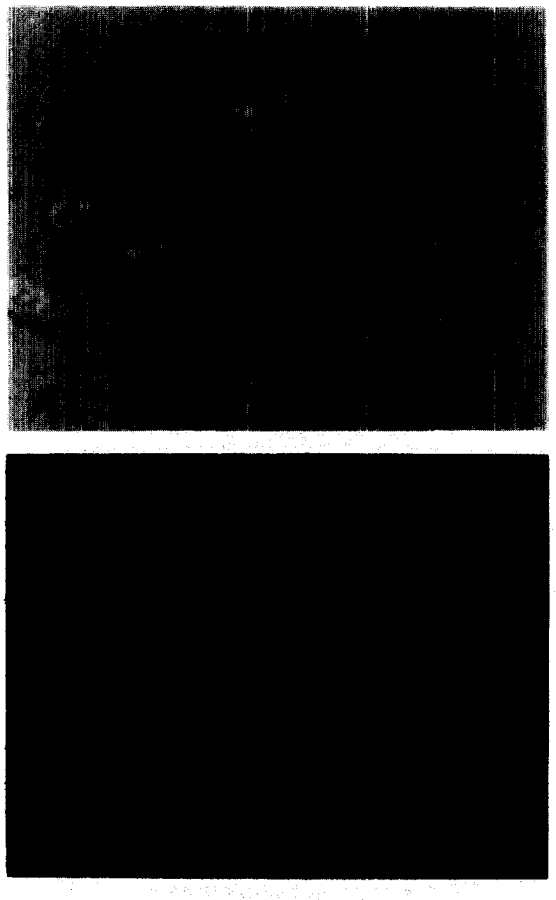

Fig. 1. Phagocytes of *Ciona intestinalis* after incubation with prestained yeasts. (A) Nomarsky contrast interference observation. Ph, phagocyte. (B) Ultraviolet light observation. Y, eosin-Y pre-treated yeast after phagocytosis. Bar, 3 μm.

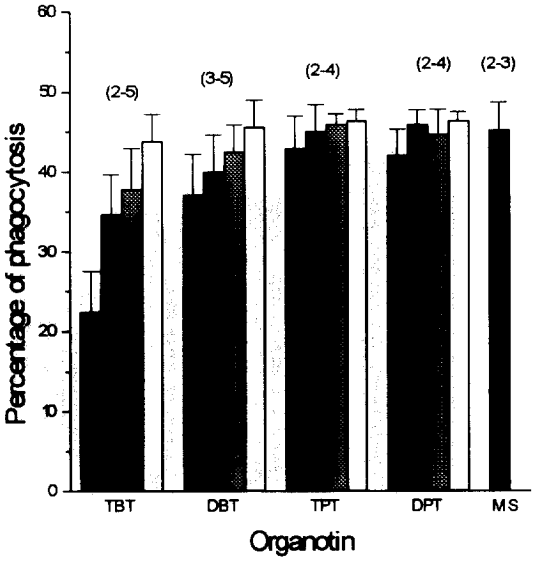

Fig. 2. Phagocytosis of *Ciona intestinalis* hemocytes after incubation (90 min) with different concentration of organotin compounds. 1.5 μM, ■; 0.15 μM, ■; 0.015 μM, ▨; 0.0015 μM, □ and MS, ■. In parenthesis, percent minimum and maximum value of hemocyte mortality after incubation with organotin is shown.

Effect of organotin compounds on phagocytosis

Organotin compounds affect defense-related hemocyte responses, interfere with locomotion, salinity regulation, chemotaxis and phagocytosis both in invertebrates (Fisher *et al.*, 1990) and vertebrates (Elferink *et al.*, 1986).

Exposure of *Ciona* hemocytes to TBT reduced the phagocytic index in a dose-dependent fashion (Fig. 2). The level of 45.1 ± 3.5%, observed in controls, significantly ($P < 0.001$) decreased to 37.8 ± 5.1% in the presence of 0.015 μM TBT and to 22.4 ± 5.1% in hemocyte–yeast mixtures containing 1.5 μM TBT. DBT at the highest concentration, 0.15 μM, only reduced the phagocytic index to 37.1 ± 5.0% ($P < 0.05$), whereas no significant inhibition of phagocytosis was found when 1.5 μM DPT or TPT were assayed.

The effect of organotin compounds on hemocyte viability are not the cause for the reduction in the phagocytic index. The percentages of death cells were not higher than 8.0 ± 1.3% by incubating the hemocytes with 1.5 μM organotin compounds. Cell death (nearly 100%) was observed at a concentration 15 μM of organotin.

To examine whether the effect of TBT is

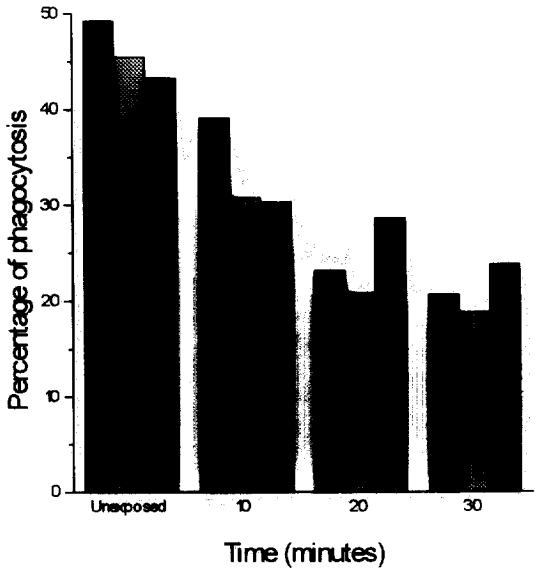

Fig. 3. Phagocytosis of *Ciona intestinalis* hemocytes from three individuals pre-treated with 1.5 μM of TBT for 10, 20 and 30 min. The cells were washed and resuspended in MS before the phagocytic assay. Individual 1, ■; individual 2, ▩; individual 3, ■.

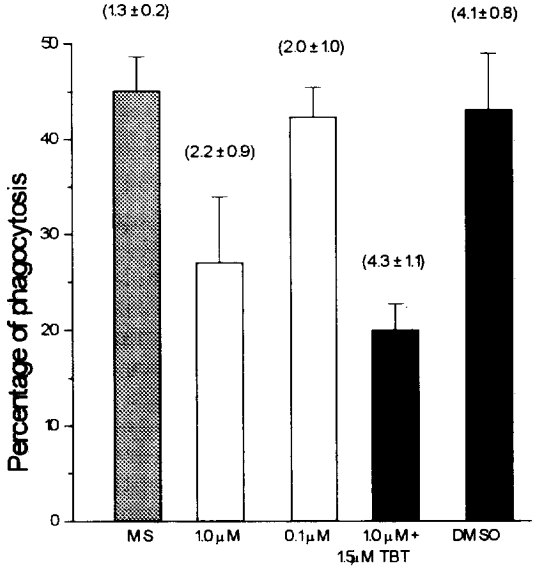

Fig. 4. Phagocytosis of *Ciona intestinalis* hemocytes with different concentration of calcium ionophore A 23187 (□). Phagocytosis of *Ciona intestinalis* in MS (▩) or in 1.0 % DMSO (■) used for ionophore dissolution; combined effects of ionophore and TBT (■). In parenthesis, percent of mortality and standard deviation of hemocytes after treatment is shown.

reversible, hemocytes were pre-incubated in MS containing 1.5 μM of this compound and then assayed for phagocytic activity. Repeated experiments showed that 10-, 20- and 30-min pre-incubation depressed phagocytosis irreversibly (Fig. 3).

Role of Ca^{++} in suppressing phagocytosis

It is known that Ca^{++} plays an important role in phagocytic mechanisms, including spreading and locomotion of hemocytes, binding and ingestion of particulate matter (Stossel, 1973; Showell *et al.*, 1977). The effects of TBT on rat thymocyte (Chow *et al.*, 1992) and on trout hepatocytes (Reader *et al.*, 1993) has been correlated with increased Ca^{++} mobility through the plasma membrane and cytosolic Ca^{++}. It is known that the ionophore A23187 promotes passive transport of divalent cations across biological membranes and Ca^{++} enters the cytosol (Pressman, 1976; Campbell, 1985). Accordingly, this ionophore is widely used to increase the concentration of free Ca^{++} in the cytosol. When the effects of A23187 and TBT on hemocytes were compared, we found that both compounds inhibited phagocytosis in the presence of millimolar concentration of external Ca^{++} (MS). In fact, A23187 contained in the medium at 1.0 μM concentration did not significantly affect cell viability (death cells < 8%) and decrease phagocytic activity of hemocytes (Fig. 4). However, phagocytosis was significantly reduced ($P = 0.002$) when TBT was added to the reaction mixture that contained the final concentration of ionophore. The percentage of phagocytosis corresponded (Fig. 4) to that observed when TBT alone was used. (Fig. 2).

Differences in TBT and ionophore A23187 inhibitory mechanisms

Figure 5 shows that, at the used concentrations, the effect of A23187 on *Ciona* hemocytes is reversible, whereas the immunotoxic effect of TBT was irreversible (Fig. 3). Hemocytes pre-treated with 1.0 μM ionophore for 10 or 30 min maintained their phagocytic activity at control levels when assayed after washing with MS. This result points out the differences between the inhibitory mechanisms of TBT and supports that the immunotoxic effect could not be to an increased cytosolic Ca^{++} concentration as reported by Chow *et al.* (1992) and Reader *et al.* (1993). On the other hand, the toxic damage of TBT on *C. intestinalis* embryonic development appeared to be due to ultrastructural modifications of cytomembrane and mitochondria (Mansueto *et al.*, 1993).

Further analysis is required to understand the effect of TBT on phagocytic mechanism. Because hemocytes are representative cells that play a critical role in invertebrate immunodefense, analyses of cellular activities of these cells can be used as one indicator to evaluate the level of environmental stress

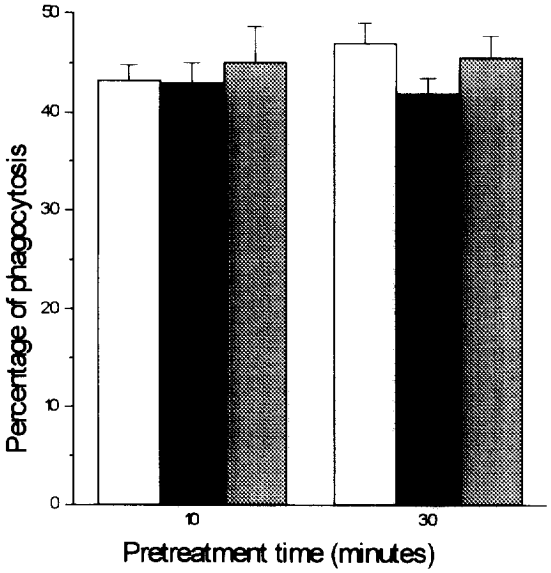

Fig. 5. Phagocytosis of *Ciona intestinalis* hemocytes pretreated for 10 and 30 min with MS (□), 1.0 μM A23187 (■) and 1 % DMSO (▒). The hemocytes were washed and resuspended in MS before the phagocytic assay.

caused by bioaccumulation and subletal toxicity of organotin compounds as immunotoxicants in aquatic organisms. Tunicates would add another dimension to the importance of tunicates as excellent models for examining the events associated with immunosuppression after exposure to xenobiotics.

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