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Biomarkers for TBT Immunotoxicity Studies on the Cultivated Clam *Tapes philippinarum* (Adams and Reeve, 1850)

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The aim of this investigation was to quantify the effects of tributyltin (TBT) on the immune reactivity of haemocytes from the cultivated clam *Tapes philippinarum* (Adams and Reeve, 1850) using a series of *in vitro* bioassays. It is known that TBT has adverse effects on cellular immune functions like mobility, phagocytosis and lysosomal enzyme activity. As defining TBT-sensitive immunologic biomarkers in sentinel organisms is important in the field of ecotoxicology, the authors propose three indexes, amoebocytic (A.I.), phagocytic (P.I.), and lysosomal activity (L.A.I.), as sensitive and useful biomarkers to assess environmental risks due to TBT contamination.

Keywords: Biomarkers; Immunotoxicity; *in vitro* bioassays; *Tapes philippinarum*; Tributyltin.

Several xenobiotics alter immune functions and the immune system consequently assesses the toxic effects of chemicals (Dean *et al*., 1985). Immunological parameters have been used in various laboratory and field experiments to analyse the effects of toxicants on the immune response and disease resistance of mammals (Sharma, 1981). The interaction of environmental pollutants with the cellular and humoral components of the immune system may suppress or enhance immune responses. Therefore, the study and comparative analysis of immunotoxic effects are important in assessing the risk related to xenobiotics in the environment.

In recent years, the use of *in vitro* bioassays and toxicity tests has become fairly common in environmental toxicology studies (e.g., Alvarez and Friedl, 1992; Seibert *et al*., 1994), which are useful when looking for sentinel organisms in which the functional responses are indicative of environmental quality (Anderson, 1988).

For this purpose, bivalve molluscs are widely employed as bioindicators in programmes monitoring marine coastal ecosystems (Goldberg, 1986). Their sessile and filter-feeding habits make them good environmental biointegrators. Several immune responses are generally recognized as stress indexes, or biomarkers, and may represent a practical tool in evaluating the well-being of organisms exposed to various kinds of environmental conditions. Several methods of biological evaluation and possible quantification of exposure to specific chemicals in marine bivalves have been developed in laboratory studies and tested in field surveys in recent years, mainly due to the health implications and commercial interests related to shellfish fisheries and aquaculture. Among these, certain functional parameters of molluscan haemocytes have become significant, e.g., cell viability, phagocytosis, adherence, lysosomal enzyme activity, production of reactive oxygen metabolites (Moore and Lowe, 1977; Anderson *et al*., 1981; Cheng and Sullivan, 1984; Beckmann *et al*., 1992; Sami *et al*., 1992; Coles *et al*., 1994; Chen and Bayne, 1995; Pipe *et al*., 1995; Cajaraville *et al*., 1996).

In the present study, a number of simple, rapid and inexpensive *in vitro* bioassays using haemocytes of the clam *Tapes philippinarum* – widely cultivated in the northern Adriatic Sea – were used to evaluate the immunosuppressant activity of TBT, one of the most common organotin compounds in the marine environment wherever anthropogenic activities occur (WHO, 1990). Three particular indexes were established as TBT pollution biomarkers, since they provide different kinds of information related to different intracellular targets of TBT: (i) *amoebocytic index* (A.I.); (ii) *phagocytic index* (P.I.); and (iii) *lysosomal activity index* (L.A.I).

Materials and Methods

Specimens of the clam *T. philippinarum* (Eulamellibranchia, Veneridae) were collected from the lagoon of Venice. Haemocytes were syringed from the posterior adductor muscle. Blood cells were then kept for a few minutes in filtered sea water (FSW) containing 10 mM L-cysteine, adjusted to pH 7.0 to prevent clotting, and

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washed twice by centrifugation at 780×*g* for 10 min. A drop of 50 µl of the haemocyte suspension was placed in the centre of each culture chamber (a teflon ring, 15 mm internal diameter and 1 mm thick, glued to a siliconised glass slide covered with a coverslip), just touching the back of the coverslip. The culture slides were kept upside down for 30 min at 25°C to allow the haemocytes to settle and adhere to the coverslips.

Tributyltin chloride (TBT) was purchased from Sigma and first dissolved at a 10 mM concentration in 95% ethanol. This stock solution was then diluted in FSW to the final concentrations of 0.05, 0.01 and 0.1 µM. After adhesion of the haemocytes, the monolayers were incubated in 50 µl of these test concentrations for 60 min at 25°C. In controls, an equal volume of FSW containing 0.1% of 95% ethanol was used instead of TBT solutions.

The A.I. was expressed as the percentage of haemocytes with amoeboid shape, and the P.I. as the percentage of haemocytes containing ingested yeast particles, preincubated in haemolymph for 30 min. This immunotoxicity assay was performed following a previously reported *in vitro* technique (Cima *et al.*, 1998b). The L.A.I. was expressed as the percentage of haemocytes showing histoenzymatic staining properties for ß-glucuronidase. A simultaneous azo-coupling detection method, following Hayashi *et al.* (1964), was used to detect enzymatic activity. Samples were kept at 37°C for 2 h in 20% naphtol AS-BI β-glucuronide (dissolved in DMF) in buffered hexazonium-p-rosaniline (0.1 M Na-acetate buffer, pH 5.0). The three indexes were calculated after 0, 15, 30, and 60 min of exposure to TBT.

All experiments were repeated five times ($n = 5$) with five independent samples. Haemocytes – 200 cells per coverslip distributed in at least ten fields – were counted under a Leitz Dialux 22 light microscope at a magnification of ×1250. Results were expressed as mean values ± SD. An ANOVA was performed with the SAS statistical package (SAS Institute, Cary, NC).

Results and Discussion

Highly reliable and reproducible tests exploiting immune functions exist for many animals and can be used as screening tools to evaluate the effects of various chemicals or investigate their mechanisms of action. This appears to be true for *T. philippinarum*.

Short-term haemocyte cultures of this species were set up in the bioassays in order to expose them to various TBT concentrations. At the end of the treatments, the viability of haemolymph cells, as monitored by the Trypan blue dye exclusion test, exceeded 90%, so that all exposure concentrations used were considered to be sublethal.

The number of cultured haemocytes never decreased, indicating that exposure to TBT does not interfere with the ability of the cells to adhere to the substrate. Cultured haemocytes changed to a common spherical morphology in the presence of increasing concentrations of TBT, since spreading haemocytes withdrew their pseudopodia.

As regards the kinetics of the three indexes, A.I., P.I. and L.A.I. were significantly inhibited by all the tested TBT concentrations after 30 min, except for L.A.I., which was significantly inhibited after only 60 min at the lowest TBT concentration (Fig. 1). The time course decrease of A.I., P.I. and L.A.I. up to 30 min was always higher than that of between 30 and 60 min. In the latter range, L.A.I. did not change significantly at the highest TBT concentration. P.I. showed a greater decrease compared to A.I. and L.A.I. at all tested concentrations.

These results suggest that the decreases in A.I. (Fig. 1a) are probably mediated by severe and irreversible alterations of cytoskeletal organization, causing remarkable changes in cell shape, as already observed in mammalian thymocytes (Chow and Orrenius, 1994) and

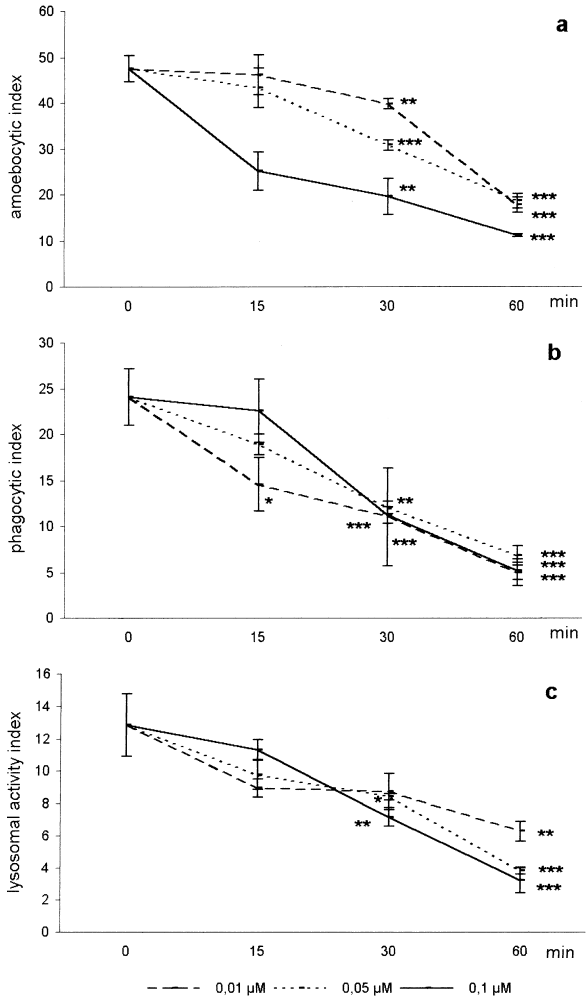

Fig. 1 Variations in (a) amoebocytic index, (b) phagocytic index, (c) lysosomal activity index of *Tapes philippinarum* haemocytes exposed to various TBT concentrations for 0, 15, 30, 60 min. Time 0: starting conditions of experiments; values refer to unexposed haemocytes which remained unaltered throughout experimental period. Significant differences with respect to controls are marked by asterisks. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

tunicate amoebocytes (Cima *et al*., 1998a). It has recently been demonstrated that *in vivo* exposure to metals and organic pollutants influences the mobility of molluscan haemocytes (Fisher and Tramplin, 1988).

Cytoskeletal alterations of haemocytes induced by TBT lead to significantly reduce phagocytic activity, due to the decreased adhesive ability. This event is reported as a significant decrease in P.I. (Fig. 1b). Beckmann *et al*. (1992) observed that diseased haemocytes of *Mya arenaria* with haematopoietic neoplasia are unable to adhere and ingest yeast, owing to modifications of the cytoskeletal organization. Moreover, significant differences in the phagocytic index were shown by *Mytilus galloprovincialis* specimens collected from variously polluted sites in the Bay of Biscay (Cajaraville *et al*., 1996). Fisher *et al*. (1990) showed that oyster haemocyte chemiluminescence, a presumed indicator of phagocytic ability, is reduced with increasing concentrations of TBT.

Haemolymph cells of several species of molluscs have been characterized on the basis of enzymatic properties (Bayne *et al*., 1979; Granath and Yoshino, 1983). The role of lysosomes in molluscan phagocytes involves degradation of phagocytized material, as synthesis of lysosomal enzymes occurs after stimulation with certain exogenous agents (Moore and Gelder, 1985; Carballal *et al*., 1997). It was observed that β-glucuronidase, a widespread lysosomal hydrolase, is significantly inhibited after TBT exposure (Fig. 1c). This is probably due to a disruption of lysosomal membrane stability, in agreement with the conclusions of Grundy *et al*. (1996) for haemocytes of *Mytilus edulis* exposed to environmental pollutants such as polycyclic aromatic hydrocarbons.

Conclusions

Study of the immune system is an indispensable starting-point for setting up toxicity tests based on the immune properties of sentinel organisms. Immunological biomarkers play an important role in marine mollusc biomonitoring prior to the occurrence of devastating disease outbreaks, and as early warning indicators of damage by pollutants. The search for new biomarkers capable of assessing the effects of TBT contamination in marine coastal ecosystems is essential for the cultivation of edible clams such as *T. philippinarum*, as well as for the protection of human health. The results of the present study indicate that the three indexes tested are useful in assessing the risk of TBT even at low concentrations. In particular, the P.I. was found to be the most sensitive and effective indicator in the short term, confirming the suitability of the phagocytosis bioassay as a short-term test for monitoring the aquatic toxicity of TBT. Nevertheless, the reliability of the proposed immuno-biomarkers needs to be fully evaluated on molluscs exposed to TBT both in the laboratory and in nature.

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