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Tributyltin–sulfhydryl interaction as a cause of immunotoxicity in phagocytes of tunicates

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Abstract

We reported elsewhere that tributyltin (TBT) has detrimental effects on the immune system of the colonial ascidian *Botryllus schlosseri*, through interaction with calmodulin and alteration of Ca^{2+} homeostasis. Here, we studied the capability of TBT to react with intracellular thiols. After exposure to 0.1 μM TBT, a significant decrease in *B. schlosseri* hemocytes stained for total thiols and reduced glutathione (GSH) was detected. Exogenous sulfhydryl and sulfide compounds can prevent TBT-induced cell morphology alterations and decrease the percentage of tin-containing hemocytes, indicating the scavenging ability of thiol peptides. No effects were observed with disulfides, *N*-acetylcysteine, or the GSH fragment Cys-Gly. No interactions were observed with TBT and carmustine, whereas TBT and *N*-ethylmaleimide (NEM) showed a combined antagonistic action, suggesting direct interaction of TBT with thiol-containing compounds. Regulation of Ca^{2+} efflux from internal stores seems to depend on stimulation of the inositol 1,4,5-trisphosphate ($InsP_3$) receptor by oxidized glutathione (GSSG), which results from interactions of both TBT-GSH and TBT-GSH reductase.

Keywords: *Botryllus*; Disulfides; Glutathione; Hemocytes; Immunotoxicity; Morphofunctional alterations; Organotin compounds; Sulfhydryl compounds; TBT; Tunicates

1. Introduction

Among xenobiotics in aquatic ecosystems, organotin compounds are lipophilic, organometallic substances, used since the 1960s as powerful biocides in the formulation of antifouling paints. They are formed of a tetravalent tin atom bound to one to four alkyl or aryl groups and one to four monovalent anions or anionic groups, which determine both their physicochemical and biological properties. Tributyltin (TBT) is the most important and widespread compound, and its slow leaching from antifouling paints in both freshwater and seawater columns has caused such severe, long-term damage to aquatic biocenoses, mainly in the last two decades, that it was banned in 1990 by many countries. However, recent research has demonstrated that sediments collected from various TBT-contaminated stations contain even now the same concentrations detected 10 years ago, indicating the very low decay of this xenobiotic in natural environments (Maguire, 2000). In the most contaminated areas along Italian coasts, such as marinas and harbors, TBT concentrations may reach 1 μM in sediments (Chiavarini et al., 1991).

In marine predators at higher trophic levels, including sharks and cetaceans, bioaccumulation of butyltin compounds preferentially occurs in liver and kidneys, and seems to be associated with the presence of metal-binding molecules such as glutathione (Kannan et al., 1996). It has also recently been reported that TBT, in nanomolar concentrations, can decrease the cell content of glutathione in rat thymocytes (Okada et al., 2000). The tripeptide glutathione (γ-glutamyl cysteinyl glycine) is present in all animal cells at concentrations ranging from 0.5 to 10 mM (Shan et al., 1990) and contains a glutamyl group forming an unusual peptide bond, in which its γ-carboxyl group, instead of the α-carboxyl group, is involved. Reduced glutathione (GSH) is an electron-donating, sulfhydryl compound that participates in amino acid transport systems, activates

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enzymes, protects against lipid peroxidation by reducing reactive oxygen species (ROS), has important functions as an antioxidant, and detoxifies both electrophilic xenobiotics and potentially alkylating agents (Meister and Anderson, 1983).

Immunotoxicity, one of the most important effects of TBT, affects the capacity for survival of animals. It was first described in mammals and teleosts as depletion of lymphocytes and inhibition of chemotaxis and phagocytosis in polymorphonuclear leukocytes and monocytes (Penninks and Seinen, 1983; Elferink et al., 1986). Recently, it has also been studied in hemocyte cultures of filter-feeding marine invertebrates such as mollusks and tunicates, both of which are very sensitive (Cima et al., 1995, 1998a). Both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms have been separately proposed to explain mammalian immunotoxicity (Aw et al., 1990; Chow and Orrenius, 1994). In our earlier papers we demonstrated that, in the colonial ascidian *Botryllus schlosseri*, a useful sentinel organism among coastal filter-feeding invertebrates, phagocytes exposed in vitro to 0.1–10 μM TBT show an immunosuppressive effect. Several immune responses are recognized as stress indexes, or biomarkers, including the amebocytic, phagocytic, apoptotic, and enzymatic indexes. These biomarkers express various morphofunctional alterations and represent a practical tool in evaluating the mechanism of action of toxicants. For TBT, the latter follows the Ca^{2+}-dependent pathway through direct interaction of butyltin compounds with calmodulin, which causes inhibition of calmodulin-dependent Ca^{2+}-ATPase and consequently a substantial increase in cytosolic Ca^{2+} concentration (Cima et al., 2002a). This irreversible process in turn triggers a cascade of events including (1) cell morphology alterations due to extensive depolymerization of cytoskeletal proteins (Cima and Ballarin, 2000); (2) inhibition of both phagocytosis and associated processes, such as the respiratory burst (Cima et al., 1995); and (3) induction of apoptosis (Cima and Ballarin, 1999). In mouse thymocytes, the increase in intracellular concentration of Ca^{2+} induced by TBT has been explained as a consequence of increased membrane Ca^{2+} permeability and release of Ca^{2+} from intracellular stores (Chikahisa and Oyama, 1992).

However, Chow and Orrenius (1994) consider that $>75\%$ of the decrease in F-actin content, observed in cultured rat thymocytes after TBT exposure, is due to Ca^{2+}-independent effects. As evidence, pretreatment with the alkylating agent *N*-ethylmaleimide (NEM) inhibits TBT-induced disassembly of F-actin, suggesting the involvement of alterations in the thiol group. Recently we have found that, in *B. schlosseri* cultured hemocytes, TBT can inhibit the activity of such antioxidant enzymes as glutathione transferase (GST) and glutathione peroxidase (GPX), probably through a change in their conformation due to direct interaction (Cima et al., 2002b), but until now possible interactions with GSH and/or proteins containing sulfhydryl groups have never been considered.

Therefore, the aim of our research was to reveal Ca^{2+}-independent mechanisms of TBT toxicity by studying the effects of various natural sulfhydryl substances on the morphology of *B. schlosseri* phagocytes coexposed in vitro to increasing concentrations of the xenobiotic.

2. Materials and methods

2.1. Animals

Wild colonies of *B. schlosseri* were collected from the Lagoon of Venice, Italy, where the average of TBT contamination in sediments was generally $\leqslant 30 \pm 5$ ng/g dry wt.; however, in the sampling site, the TBT concentration was even lower than the determinability limit of the analytical assay (Pavoni et al., 2002). The colonies were kept in aerated aquaria, attached to glass slides, and fed with Liquifry Marine (Liquifry Co., Dorking, England) and *Dunaliella* sp. microalgae.

2.2. Chemicals

Chlorobimane, *N*-acetyl-L-cysteine, NEM, glutathione fragments (Cys-Gly, γ-Glu-Cys), L-methionine, thimerosal, and TBT chloride were obtained from Sigma; cacotheline, L-cysteine, and L-cystine came from Fluka; GSH and GSSG were from Aldrich; and carmustine solution (Nitrumon) was from the Sintesa-Prodesfarma Group.

2.3. Hemocyte culture preparation

Blood was collected in glass micropipettes after tearing, with a fine tungsten needle, the tunic marginal vessels of colonies previously rinsed in filtered seawater (FSW) containing 0.38% sodium citrate (Sigma), pH 7.5, to prevent clotting. Blood was centrifuged at 780*g* for 10 min and pellets resuspended in FSW to obtain a final concentration of 8×10^6–10×10^6 cells mL^{-1}. Subsequently, 60 μL of the hemocyte suspension were placed in the center of a culture chamber made by gluing Teflon rings (15 mm i.d., 1 mm thick) to siliconized glass slides. Coverslips were gently pressed down over the Teflon rings, which had been smeared with Vaseline, to touch the drop of cell suspension. Culture slides were kept upside down for 30 min to allow cells to settle and adhere to the coverslips. Cell viability, assessed with the Trypan Blue dye exclusion test, exceeded 95%.

2.4. Assays with tributyltin and sulfhydryl substances on cell morphology

After adhesion of hemocytes to coverslips, monolayers were incubated for 60 min at 25°C in 60 μL of TBT solutions, at concentrations ranging from 0.1 to 10 μM, previously demonstrated to be sublethal (Cima and Ballarin, 1999), obtained from a 10 mM TBT stock solution in 95% ethyl alcohol. In controls, an equal volume of FSW containing 0.1% of 95% ethyl alcohol was used instead of TBT solutions. Using the Trypan Blue test, this ethyl alcohol concentration was demonstrated to be widely sublethal, because the mortality was significant ($P<0.01$) beginning from 10%. In these experiments, some monolayers were coexposed to 10 and 20 mM L-cysteine or GSH.

In coincubation experiments, the chosen concentration of TBT was 1 μM rather than 0.1 μM, because the lower concentration resulted in a borderline effect (i.e., it resulted in inconsistently significant alteration of cell morphology) and because 1 μM corresponds to the half-maximal effective concentration (EC_{50}). Hemocytes were coincubated with 1 μM TBT and increasing concentrations (10, 20, 50 mM) of exogenous, sulfhydryl and sulfide compounds (i.e., L-cysteine, L-cystine, *N*-acetylcysteine, GSH, GSSG, L-methionine, and GSH fragments). GSH fragments were represented by cysteinyl-glycine (Cys-Gly) and L-cysteinyl-glutamic acid (γ-Glu-Cys). The pH of all solutions was adjusted to 7.5.

After exposure, hemocytes adhering to coverslips were washed several times in FSW, fixed in 1% glutaraldehyde (Fluka) plus 1% sucrose in FSW for 30 min at 4°C, rehydrated in phosphate-buffered saline (PBS: 0.15 M NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 8 mM Na_2HPO_4), and stained in 10% Giemsa for 5 min. Then the coverslips were rinsed in distilled water, mounted on glass slides with an aqueous medium (Acquovitrex, Carlo Erba), and observed by light microscopy (LM; Leitz Dialux 22). The percentage of hemocytes showing an ameboid shape, counted at magnification of 1250 × in 10 fields per coverslip, was expressed as the amebocytic index.

2.5. Cytochemical assays for thiol, tin, and calcium contents

The specific Mercury Orange dye was used for estimation of total thiol contents inside cultured hemocytes, which was expressed as the percentage of cells positive to the reaction, that is, showing brick-red spots in their cytoplasm (Asghar et al., 1975). After exposure to TBT, monolayers were fixed in glutaraldehyde, rinsed in PBS, dehydrated in an ethyl alcohol concentration series (80%, 95%, 100% each for 5 min), and transferred in 50 μM Mercury Orange solution in toluene for 5 min. Then they were rinsed in toluene, mounted in synthetic resin (Eukitt, Kindler GmbH & Co.), and observed under LM at a magnification of 1250 × .

The percentage of cultured hemocytes stained after treatment with chlorobimane, a fluorescent dye specific for GSH with λ_{max} of 461 nm, according to the method of Cookson et al. (1998), was estimated. Briefly, after treatment with TBT, monolayers are washed in FSW, stained for 10 min at 37°C in 40 μM chlorobimane solution in FSW obtained from a 20 mM stock solution in ethyl alcohol, and then rinsed in FSW. Living cells were immediately observed under LM with an ultraviolet light filter block at a magnification of 1250 × . Positive sites appeared fluorescent blue.

Tin contents were evaluated inside the cells according to the staining method of Gutzeit (1929). After aldehyde fixation, hemocyte cultures were incubated in 0.25% cacotheline aqueous solution for 60 min at 37°C, and then rinsed in distilled water and mounted in Acquovitrex. This golden-yellow solution is very stable and specifically reacts with tin, forming a violet precipitate, which is easily recognizable inside the cells.

Cytosolic Ca^{2+} increase was revealed as a dark blue precipitate using Von Kossa's substitution method (Callis, 2002). Briefly, aldehyde-fixed monolayers are immersed in 5% silver nitrate and exposed to ultraviolet light for 5 min. Cells were then rinsed in distilled water, treated for 2–3 min in 5% aqueous sodium thiosulfate solution, washed in distilled water, and mounted with Acquovitrex.

2.6. Assays with N-ethylmaleimide, carmustine, and thimerosal, and combined actions with tributyltin

Various concentrations of TBT (0.1, 0.5, 1, 10, 20, 50 μM) were added to cell cultures for 60 min to determine the concentrations capable of causing a 50% decrease in the number of chlorobimane-positive hemocytes and an equal increase in the number of hemocytes showing a rise in cytosolic Ca^{2+} content (ED_{50}). Then NEM, which forms specific bonds with thiol groups (Friedman, 1973), and carmustine, an inhibitor of glutathione reductase (Ahmad and Frischer, 1985), were used at concentrations ranging from 0.1 to 50 μM and from 1 to 200 μM in FSW, respectively, in cell cultures to detect the ED_{50} for GSH content decrease. Thimerosal, which interacts with calcium channels of intracellular Ca^{2+} stores, causing their depletion, was used at concentrations ranging from 0.1 to 10 μM in FSW, obtained from a 10 μM stock solution that had been dissolved in FSW, to detect its ED_{50} for cytosolic Ca^{2+} rise.

All these concentrations were nonlethal, as evaluated with the Trypan Blue dye exclusion test, and were used to obtain isodynamic mixtures of TBT and NEM, carmustine, or thimerosal, according to the method

reported by Musch (1996), in which the dose (D) is related to the effective dose (ED) of the substance, as the D/ED ratio. The isobologram outcome from the isodynamic mixtures of ED_{50} of both TBT and the other substances provided information on qualitative aspects of their combined action, particularly the mode of interaction with the molecular mechanisms that control GSH and Ca^{2+} contents inside cells.

2.7. Statistical analysis

All experiments were repeated three times ($n = 3$). The numbers of hemocytes positive to the various assays, at least 200 cells per monolayer in 10 fields, were counted. Data are expressed as a percentage $\pm$SD of the total number and analyzed using the χ^2 test with the FREQ procedure of the SAS statistical package (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Effects of tributyltin on hemocyte morphology

As reported elsewhere (Cima et al., 1998b), ascidian phagocytes exposed in vitro to 0.1–10 μM TBT undergo a series of morphological changes that involve disassembly of cytoskeletal proteins. The main feature of this toxic effect is that all ameboid-shaped cells (Fig. 1a) withdraw their long pseudopodia and become irreversibly spherical (Fig. 1b). This event can be quantified by evaluating the amebocytic index (Table 1). The presence of tin compounds inside cells after TBT exposure can also be detected with cacotheline, because violet-positive sites appear (Fig. 1c).

3.2. Total thiols and reduced glutathione in hemocytes

We studied the capability of TBT to oxidize intracellular thiols by assessing the fraction of phagocytes stained by Mercury Orange (Fig. 1d) and chlorobimane (Figs. 1e and f) to reveal total thiol and GSH contents, respectively. In both cases, we observed a

Table 1
Effects on amebocytic index of *B. schlosseri* hemocytes after 60 min exposure to various concentrations of TBT and to the co-presence of TBT and natural sulfhydryl compounds[a]

	Treatment	Amebocytic index
Filtered seawater (controls)		100
0.1 μM TBT		$50.41 \pm 0.87^{***}$
	10 mM L-cysteine	$52.22 \pm 0.91^{***}$
	20 mM L-cysteine	80.37 ± 2.41
	10 mM GSH	83.93 ± 1.67
	20 mM GSH	84.91 ± 1.08
1 μM TBT		$39.16 \pm 3.76^{***}$
	10 mM L-cysteine	$46.81 \pm 3.52^{***}$
	20 mM L-cysteine	90.86 ± 1.18
	10 mM GSH	$59.33 \pm 3.11^{**}$
	20 mM GSH	91.81 ± 2.14
10 μM TBT		$9.58 \pm 0.23^{***}$
	10 mM L-cysteine	$10.56 \pm 0.74^{***}$
	20 mM L-cysteine	$44.75 \pm 1.71^{***}$
	10 mM GSH	$30.65 \pm 1.06^{***}$
	20 mM GSH	$42.79 \pm 0.69^{***}$

[a] Asterisks: significant differences with respect to controls. $^{**}P < 0.01$; $^{***}P < 0.001$.

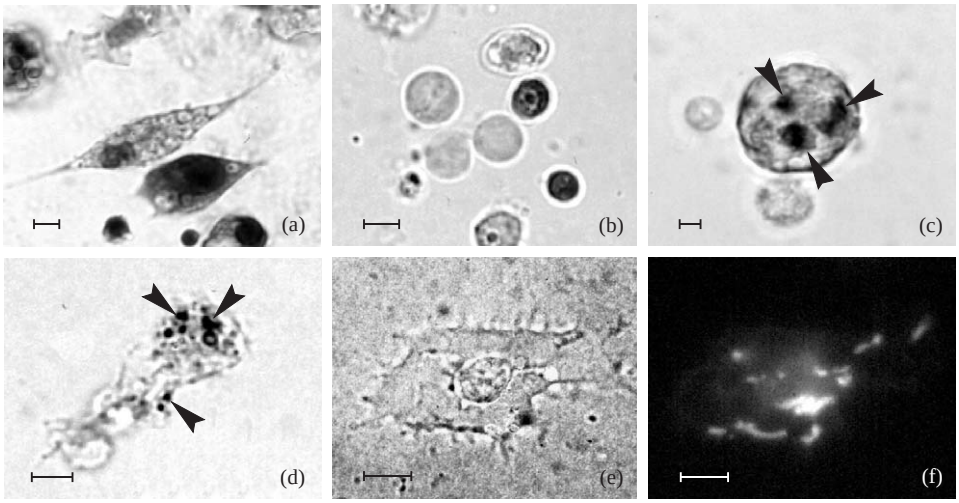

Fig. 1. Light micrographs of cultured hemocytes of colonial ascidian *B. schlosseri*. Cells were fixed in 1% glutaraldehyde plus 1% sucrose in filtered seawater. (a) Control hemocytes with normal ameboid shape, stained with 10% Giemsa. (b) Shrunken, rounded hemocytes after exposure to 10 μM TBT for 60 min, stained with 10% Giemsa. (c) TBT-treated hemocyte stained with cacotheline dye to reveal tin content (arrowheads). (d) Untreated hemocyte positive to Mercury Orange (arrowheads) for total thiol detection. (e) Untreated, aldehyde-fixed amebocyte, and (f) the former cell stained with chlorobimane for GSH content revealed as blue fluorescent spots. Bar length = 5 μM.

significant decrease ($P<0.05$) in the percentage of hemocytes positive to Mercury Orange and chlorobimane staining, that is, from $6.93\pm2.96\%$ in unexposed hemocytes to $2.84\pm0.13\%$, the latter after exposure to the minimal TBT concentration (0.1 μM).

3.3. Exogenous sulfhydryl and sulfide compounds and tributyltin

In preliminary experiments of coexposure of hemocytes to increasing concentrations of TBT (0.1, 1, 10 μM) and exogenous L-cysteine and GSH (10, 20 mM) (Table 1), both of these substances seemed able to restore the amebocytic index partly or completely, thus counteracting the negative effects of TBT on cell morphology. In particular, even though 10 mM L-cysteine never restored the amebocytic index, its highest concentration (20 mM) prevented damage induced by 0.1 and 1 μM TBT. Analogously, 20 mM GSH was able to counteract the effects of 0.1 and 1 μM TBT but unlike L-cysteine, 10 mM GSH showed positive effects even against 0.1 μM TBT.

When the ED_{50} of TBT on the amebocytic index (1 μM) was coincubated with increasing concentrations (20, 50 mM) of various exogenous sulfhydryl and sulfide compounds, the ability to restore the amebocytic index was not shared by all the substances assayed (Fig. 2a). Restoration was observed only for L-cysteine and GSH, the former being active beginning from 50 mM, the latter even from 20 mM. L-methionine also revealed restorative effects from 20 mM. Parallel experiments to evaluate the percentage of phagocytes containing tin, as revealed with cacotheline dye, after coexposure to TBT and sulfhydryl or sulfide compounds, showed trends that were nearly symmetrical with those of the amebocytic index at the same concentrations of TBT (Fig. 2b). Instead, disulfide compounds such as L-cystine and GSSG were unable to restore the amebocytic index,

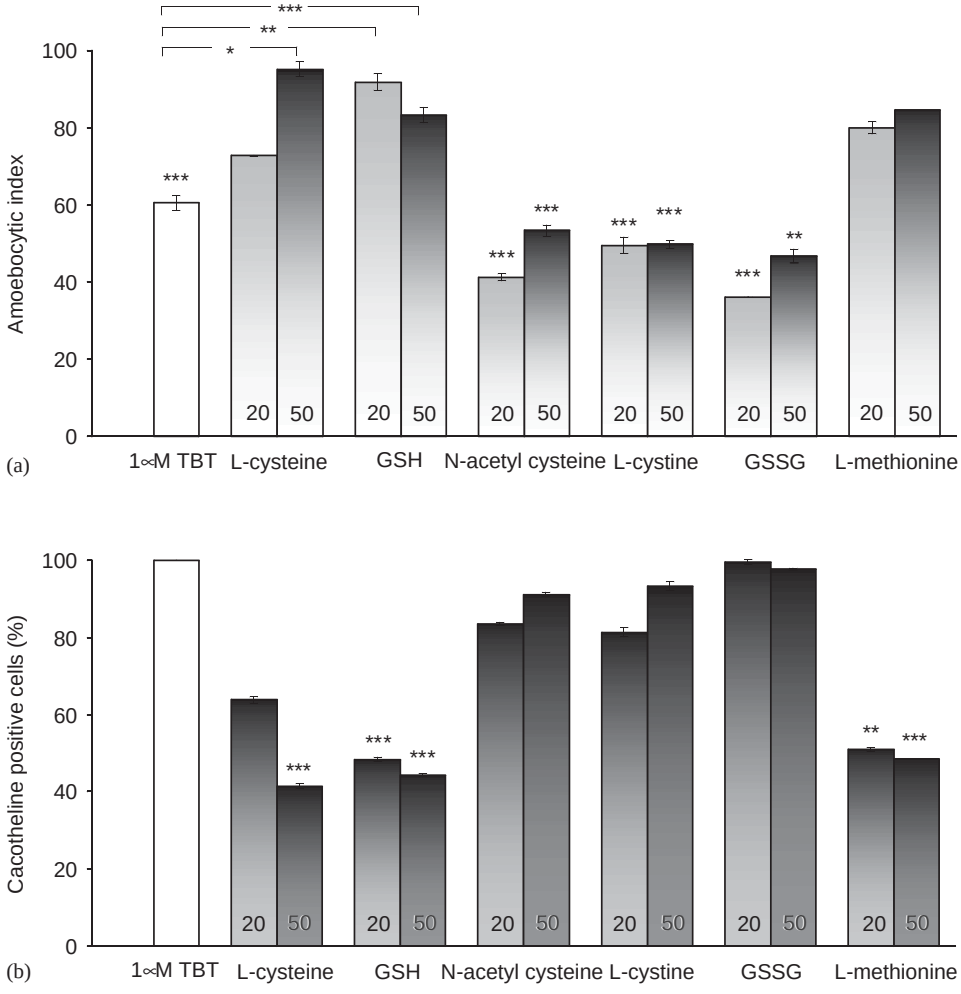

Fig. 2. Effects of coexposure of *B. schlosseri* hemocytes to 1 μM TBT and increasing concentrations (20, 50 mM) of various natural, exogenous sulfhydryl and sulfide substances on cell morphology. (a) Amebocytic index. (b) Tin content, as percentage of cells positive to cacotheline. Control values are reported as 100. Unless otherwise indicated, asterisks show levels of significance with respect to controls referred to unexposed hemocytes (for amebocytic index) and to hemocytes with tin content after TBT exposure (for percentage of cacotheline-positive cells). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

and the percentage of cell with intracellular tin content still remained very high. Unlike both the other sulfhydryl compounds and L-methionine, *N*-acetylcysteine behaved like the disulfides.

To clarify the nature of the TBT interaction with GSH, the amebocytic index was assessed in the presence of 1 μM TBT and GSH fragments, Cys-Gly and γ-Glu-Cys. In these experiments, whereas the Cys-Gly dipeptide had no effect (Fig. 3a), the γ-Glu-Cys dipeptide was able to restore the amebocytic index (Fig. 3b) over a concentration range similar to that of GSH, that is, beginning from 20 mM (Fig. 2a).

3.4. *N-ethylmaleimide, carmustine, thimerosal, and tributyltin*

The mode of interaction of TBT with possible molecular targets inside ascidian phagocytes was evaluated through a series of experiments of combined action with substances with well-known effects, particularly involving mammals, and extensively reported in the literature.

With regard to the percentage of hemocytes with intracellular GSH content, assayed with chlorobimane dye, isodynamic mixtures of ED_{50} of TBT (20 μM) and NEM (0.8 μM) showed an isobologram indicative of antagonism (Fig. 4a), whereas those of TBT (20 μM) and carmustine (100 μM) showed no interactions (Fig. 4b).

After exposure to 1 and 10 μM thimerosal, a considerable increase was observed in the percentage of cells with a higher Ca^{2+} concentration in the cytosol, in comparison with a range of 1.15 ± 0.25–$1.45 \pm 0.23\%$ in controls. This effect is similar to that observed after exposure to 1 and 10 μM TBT (Fig. 5a). Experiments with isodynamic mixtures of ED_{50} of TBT (10 μM) and thimerosal (2 μM) showed an isobologram indicative of combined action of partial additivity (Fig. 5b).

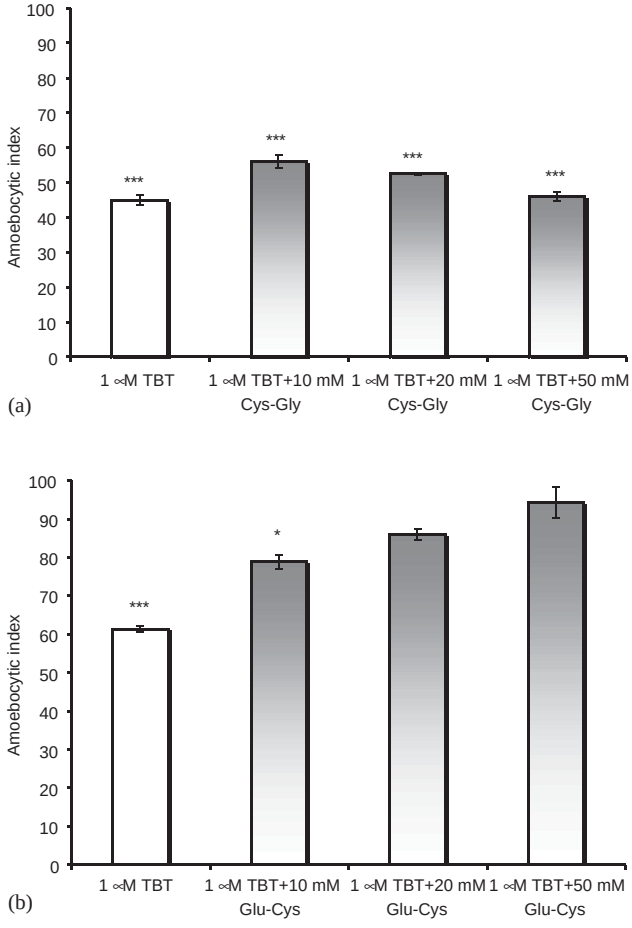

Fig. 3. Variations in amebocytic index of *B. schlosseri* hemocytes exposed to 1 μM TBT and coexposed to increasing concentrations (10, 20, 50 mM) of GSH fragments. (a) Cysteinyl-glycine (Cys-Gly). (b) L-cysteinyl-glutamic acid (γ-Glu-Cys). Asterisks: levels of significance with respect to control values referred to unexposed hemocytes and reported as 100. $^{*}P<0.05$; $^{***}P<0.001$.

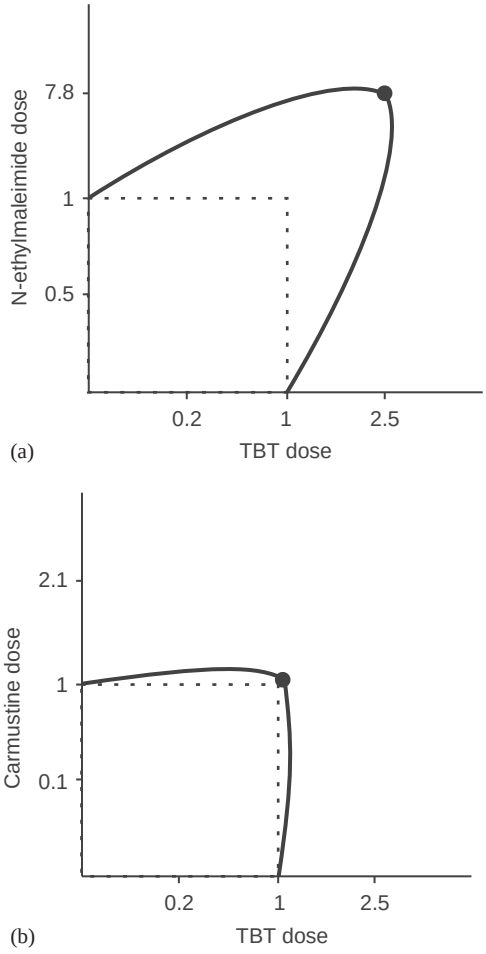

Fig. 4. Isoboles of isodynamic mixtures of (a) TBT and NEM and (b) TBT and carmustine, obtained by evaluation of effects of both compounds on GSH cell content revealed through chlorobimane staining in cross-experiments using (1) maximum inactive dose of TBT plus minimum active nonlethal dose of other compound; (2) minimum active nonlethal dose of TBT plus maximum inactive dose of other compound; (3) ED_{50} doses of both compounds.

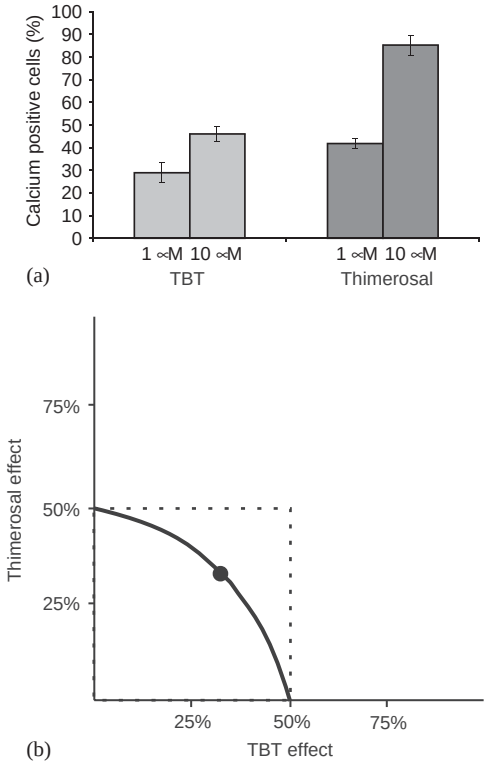

Fig. 5. (a) Percentage of *B. schlosseri* hemocytes showing internal Ca^{2+} increase revealed through Von Kossa's silver nitrate method after incubation in filtered seawater containing 1 and 10 μM of TBT or thimerosal. (b) Isobole of isodynamic mixtures of TBT and thimerosal obtained by evaluation of effects of both compounds on cytosolic Ca^{2+} increase.

4. Discussion

Bioassays conducted on short-term hemocyte cultures of the ascidian *B. schlosseri* to investigate the effects of TBT on cell physiology and its possible interactions with sulfhydryl substances revealed a complex picture with some constant characteristics, allowing us to propose a mechanism of action of this toxicant that seems to be integrated with the Ca^{2+}-dependent mechanism (Cima et al., 2002a).

A substantial decrease in both total thiol and particularly GSH contents occurred after exposure to low concentrations of TBT, indicating the possible interaction of this xenobiotic with the synthesis and/or metabolism of sulfhydryl peptides in cells, compromising their functionality. This confirms the ability of TBT to cross cell membranes, as a result of its high lipophilicity, and to enter the cell, as revealed with cacotheline dye, interacting with various intracellular molecular targets. Indeed, the co-presence of TBT and natural sulfhydryl compounds in the incubation medium can restore cell morphology, evaluated as the amebocytic index.

Among the exogenous, natural sulfhydryl compounds assayed, GSH is certainly the most active substance, because it can restore the amebocytic index even at the concentration of 20 mM. Moreover, cell tin contents, evaluated with cacotheline, show a symmetrical decrement, suggesting the scavenging activity of GSH toward TBT. In our opinion, as earlier proposed for the interaction between TBT and calmodulin (Cima and Ballarin, 2000; Cima et al., 2002a), exogenous GSH may bind directly to TBT present in the culture medium, preventing much of the contaminant from entering the cells. Indeed, the same mechanism of action was also demonstrated in coincubation experiments with mercapto- chelating compounds such as 2,3-dimercaptopropanol (BAL), dithiothreitol (DTT), and meso-2,3-dimercaptosuccinic acid (DMSA) added to TBT-treated erythrocytes, with reduction of hemolysis rate attributable to the removal of TBT from cells and the formation of membrane-associated aggregates (Byington et al., 1974; Gray et al., 1986). It is possible that, in cultured cells exposed to sublethal TBT concentrations, similar scavenger interaction occurs with endogenous GSH after the TBT entry, protecting the thiol redox potential by keeping sulfhydryl groups of proteins in the reduced form (Cotgreave and Gerdes, 1998).

Generally, comparison of our results concerning the restorative ability of natural thiol compounds on cell morphology indicates that their scavenging activity toward TBT is due to the presence not only of at least one thiol group, but also of both one free amino and one free carboxyl group. This proposal is supported by the restorative ability observed for L-methionine, the sulfur atom of which does not form a thiol group but is closely bound inside the molecule, though with available valence electrons. Moreover, with reference to the different behavior of the GSH fragments, the lower restorative activity of Cys-Gly compared with that of γ-Glu-Cys probably depends on the profound change that takes place in the molecular conformation of Cys-Gly in aqueous solution, resulting in a 2,5-diketopiperazine derivative.

The series of experiments studying the combined actions between TBT and NEM, and TBT and carmustine on GSH contents—which aimed at determining whether the interaction of TBT occurs in the cell directly with thiol groups, or indirectly through inhibition of enzymes that control GSH metabolism, respectively—supports the proposal of direct interaction with GSH. TBT acts as an antagonist of NEM on the same molecular target, which is represented by thiol group-containing compounds. Instead, interaction with glutathione reductase, the enzyme necessary to restore GSH from GSSG specifically inhibited by carmustine, is probably negligible or indirect, because indifferent behavior was observed when cells were coexposed to the ED_{50} of TBT and carmustine.

Therefore, the significant variation in the amebocytic index observed after exposure to TBT, explained earlier

as a consequence of direct interaction with intracellular Ca^{2+} homeostasis (Cima and Ballarin, 2000), may also be supported by Ca^{2+}-independent mechanisms based on direct interaction of this xenobiotic with both GSH and other thiol group-containing proteins. Although Jensen et al. (1991) believe that inhibition of microtubule assembly by organotin compounds is caused by direct interaction with tubulin without the involvement of sulfhydryl groups of proteins, other authors have shown that, in cell cultures of human and other mammalian leukocytes, dithiols such as BAL and DTT can counteract some immunotoxic effects of organotins at high concentrations (Elferink et al., 1986; Mirabelli et al., 1988; Galli et al., 1993; Marinovich et al., 1990, 1996). Moreover, organotin-induced depolymerization of F-actin observed in mammalian thymocytes (Chow and Orrenius, 1994; Nebbia et al., 1999) involves oxidation of thiol groups, because the presence of thiol-reducing substances such as DTT protects against the disruptive action of organotins.

Jewell et al. (1982) suggest that modification of sulfhydryl groups of cytoskeletal proteins during oxidative stress provokes the destruction of the whole cytoskeleton. However, TBT does not seem to increase either generation of ROS, which is generally an index of oxidative stress, or consequent lipid peroxidation, as reported for mouse hepatocytes (Ueno et al., 1994). It has recently been observed that TBT, though causing a decrease in cell levels of GSH that plays a role in catabolizing H_2O_2, does not behave as a catalyst in the generation of O_2 from H_2O_2 in cultured rat thymocytes (Sakai et al., 2001). Therefore, TBT is a powerful xenobiotic capable of oxidizing thiol groups directly without producing ROS and of reacting with antioxidant enzymes GST and GPX, affecting both the defense ability of organisms against oxidative stress caused by environmental xenobiotics and the resistance to disease (Cima et al., 2002b).

It is well known that an important event in TBT cytotoxicity in both vertebrates and invertebrates is a deleterious increase in cytosolic Ca^{2+} contents (Oyama et al., 1994; Cima et al., 1995), which is maintained through inhibition of Ca^{2+}-ATPase by the interaction of TBT with calmodulin (Yallapragada et al., 1991; Cima et al., 2002a). However, the mechanism by which TBT is able to open intracellular Ca^{2+} channels is still unknown. Our results indicate that increased cytosolic Ca^{2+} after exposure to TBT is similar to that observed after exposure to thimerosal, which is known to deplete intracellular Ca^{2+} stores by blocking the inositol 1,4,5-trisphosphate ($InsP_3$) receptor on the membrane of internal compartments (Swann, 1991). Actually, our experiments of coexposure to isodynamic mixtures of ED_{50} of TBT and thimerosal show combined behavior of partial additivity, suggesting a different type of interaction with the $InsP_3$ receptor. Also in human platelets (van Gorp et al., 1997), both thimerosal and carmustine provoke a significant increase in intracellular Ca^{2+} contents, due to stimulation of the $InsP_3$ receptor. Thimerosal reacts directly with the sulfhydryl groups of the $InsP_3$ receptor, whereas carmustine, acting as an inhibitor of glutathione reductase, causes an irreversible decrease in the cytosolic GSH concentration (Chen et al., 2000). Indeed, in permeabilized hepatocytes, $InsP_3$-dependent Ca^{2+} channels are not stimulated by GSH but by GSSG, which modifies the oxidation state of the sulfhydryl groups of the receptor, fundamental for the regulation of Ca^{2+} permeability (Renard et al., 1992). Moreover, in endothelial cells, oxidation of GSH is accompanied by a stimulation of Ca^{2+} mobilization (Elliott et al., 1995). Consequently, the redox state of GSH is a control system of the function of the $InsP_3$ receptor, and represents the preferential molecular target of TBT inside cells.

5. Conclusions

The immunotoxicity induced by TBT on tunicate phagocytes seems to involve a series of molecular perturbations that recall many effects also described in mammalian leukocytes, suggesting general mechanisms of action that may be summarized as follows: (1) extensive oxidation of thiol groups, a process known to affect cytoskeletal proteins, which causes alteration in cell morphology; (2) a decrease in the intracellular concentration of glutathione, due to direct interaction of TBT with GSH; (3) an increase in intracellular Ca^{2+} concentration, due not only to direct, hydrophobic interaction of TBT with calmodulin and consequently to inhibition of Ca^{2+}-ATPase activity, but also to interaction with GSH, the redox state of which is a control system for the activity of the $InsP_3$ receptor. Indeed, although TBT does not interact directly with glutathione reductase, the activity of this enzyme is insufficient to restore the reduced state of glutathione: the presence of high amounts of GSSG causes stimulation of the Ca^{2+}-releasing property of the $InsP_3$ receptor, and both the resulting calcium mobilization and inhibition of calmodulin-dependent Ca^{2+}-ATPase increase intracellular Ca^{2+} contents.

As a consequence, the two proposed Ca^{2+}-dependent and Ca^{2+}-independent mechanisms of action of TBT are linked and synergistic in triggering the cascade of secondary events that lead to the immunosuppressive activity and cell death.

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