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Cytoskeleton Alterations by Tributyltin (TBT) in Tunicate Phagocytes

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The effects of tributyltin chloride (TBT) on cytoskeletal components, as possible cell targets of toxicity, were examined on cultured hemocytes of the colonial ascidian *Botryllus schlosseri* by means of indirect immunofluorescence. The immunotoxic effect of 10 μM TBT (sublethal concentration) consists of (1) inhibition of yeast phagocytosis, Ca^{2+}ATPase activity, and respiratory burst; (2) increase in intracellular Ca^{2+} concentration; and (3) alterations in cell morphology. After 60 min, TBT-exposed amebocytes become spherical, withdrawing their long pseudopodia, and lose motility. Their microfilaments assemble in clusters around the peripheric cytoplasm, indicating massive disassembly, with the exception of unaltered adhesion plaques. Analogously, their microtubules reveal extensive disaggregation, being scattered in the cytoplasm and not recognizable as single filaments, whereas the microtubule organizing center (MTOC) is still visible. Treatment together with 20 μg/ml calmodulin (CaM) can partially restore the cytoskeleton architecture. These results suggest a relationship between TBT and Ca^{2+} homeostasis in ascidian hemocytes. By interfering with Ca^{2+}ATPase activity through CaM inhibition, either directly or indirectly, TBT induces an excess of intracellular Ca^{2+} accumulation, which first causes internal disorganization of cytoskeletal proteins and consequently inhibition of phagocytosis, beginning from chemotaxis and particle adhesion. © 1998 Academic Press

INTRODUCTION

The cytoskeleton is involved in determining the distinctive shape of cells and in regulating cell motility, chromosome movements during mitosis and meiosis, and intracellular transport of vesicles and organelles, including the processes of exocytosis and endocytosis. The main components of the cytoskeleton, such as straight filaments (microfilaments) and hollow tubules (microtubules) running throughout the cytoplasm, are the targets of some poisons. For certain poisons, the molecular components of the cytoskeleton (i.e., actin, tubulin, intermediate filaments, and associated proteins) are the primary targets. Some toxic compounds interact selectively with cytoskeleton components and bind directly to actin and tubulin, causing either breakdown of the polymer into its constitutive elements or stabilization of the fibers. Both structural rearrangements affect cell shape and motility, but some changes in the cytoskeleton may also be secondary. Various families of natural and derived compounds are well-known poisons with specific mechanisms of action on the cytoskeleton, but only in recent years have several environmental substances or pollutants been revealed to cause disorders of cytoskeletal components (Syversen *et al*., 1984; Scapigliati *et al*., 1988).

In the past decade, the severe and continual contamination of coastal sea waters by organotin compounds used as biocides in antifouling paints has led to worldwide interest in the toxic effects induced by tributyltin (TBT) and its degradation products on various marine organisms, especially on filter-feeding bentonic invertebrates [see WHO (1980, 1990) and Bryan and Gibbs (1991) for reviews]. In mammals, organotin compounds cause *in vitro* disaggregation of actin filaments and inhibition of tubulin polymerization, to which cytoskeletal modifications have been ascribed (Tan *et al*., 1978; Bondy and Hall, 1986). These environmental toxicants also have detrimental effects on the mammalian immune system, impairing phagocytosis, decreasing the activity and number of polymorphonuclear leukocytes (Elferink *et al*., 1986), and inhibiting F-actin polymerization in resting human neutrophils (Marinovich *et al*., 1990; Galli *et al*., 1993).

The *in vitro* immunotoxicity of organotin compounds at low concentrations in the blood cells of the colonial ascidian *Botryllus schlosseri* has been demonstrated. In this species, their effects are not associated with any cytolysis or mortality of the hemocytes or both, but irreversible and significant decreases in yeast phagocytosis, respiratory burst, and calmodulin-Ca^{2+}ATPase activity do occur (Cima *et al*., 1995, 1997). Hyaline amebocytes with long pseudopodia and uni- or multivacuolated macrophage-like cells are the two hemocytic types involved in phagocytosis that represent two functional stages of a single cell type

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(Ballarin *et al*., 1993, 1994). After their exposure to organotin compounds, a considerable change in the shape of the hemocyte was always observed which became spherical. Actively phagocytizing amebocytes withdrew their characteristic pseudopodia, suggesting structural damage to cytoskeleton components.

The aim of the present study was an immunocytochemical study of the toxic effects on microfilaments and microtubules of *B. schlosseri* phagocytes exposed to TBT concentrations sufficient to impair phagocytosis.

MATERIALS AND METHODS

Animals

Colonies of *Botryllus schlosseri* from the Lagoon of Venice were reared in this laboratory, attached to glass slides immersed in aquaria and fed Liquifry Marine (Liquifry Co., Dorking, UK) and algae.

Hemocyte 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 (Sigma), adjusted to pH 7.5 to prevent clotting. Cells were then centrifuged at 780*g* for 15 min, and pellets were resuspended in FSW at a final concentration of $8–10 \times 10^6$ cells/ml.

Culture Chambers

Culture chambers were made by gluing Teflon rings (15-mm internal diameter, 1-mm thick) to siliconized glass slides, according to a previously reported method (Ballarin *et al*., 1994). Fifty microliters of the hemocyte suspension was placed in the center of each well, and washed coverslips were laid over the Teflon rings, previously smeared with Vaseline, and gently pressed down to touch the drop of cell suspension. The culture slides were kept upside down for 30 min to allow hemocytes to settle and adhere to the coverslips.

Effects of Tributyltin Chloride

Tributyltin chloride (Sigma) was first dissolved in 95% ethanol at 10 mM and then diluted at final concentrations of 0.1, 1.0, and 10.0 μM in the FSW used for culture incubation.

After the hemocytes had adhered to the culture chambers, FSW containing debris was discarded and replaced with equal volumes of TBT solution or FSW containing 0.1% of 95% ethanol for the toxicity assay and as controls, respectively. Treated cultures were kept upside down for 60 min at 25°C and then washed several times in FSW. Cell viability was assessed by the Trypan blue dye exclusion test, the dye being dissolved in FSW. Parallel experiments were performed by adding 20 μg/ml calmodulin (CaM) (Sigma) both in the presence of TBT and for 30 min after TBT treatment.

Fluorescent Microscopy

To stain microfilaments and microtubules for indirect immunofluorescence, blood cells adhering to coverslips were fixed in a solution of 4% paraformaldehyde (Serva) and 1% sucrose in an isotonic salt solution (ISO: 20 mM Tris, 0.5 M NaCl, pH 7.5) at 4°C for 30 min, rehydrated in PBS (0.15 M NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 8 mM Na_2HPO_4, pH 7.4), permeabilized for 5 min with 0.1% Triton X-100 (Merck) in PBS, and preincubated in PBS containing 1% powdered milk and 0.5% newborn calf serum (GIBCO) for 30 min at room temperature. Washed hemocytes were then incubated with monoclonal mouse anti-actin (Sigma) (dilution 1/100 in PBS) or monoclonal anti-α-tubulin (Sigma) (dilution 1/2,000 in PBS) for 60 min at room temperature. After having been rinsed twice in PBS, hemocytes were incubated for 30 min at room temperature with 5 μg/ml FITC-labeled goat anti-mouse IgG (Sigma) in PBS. After staining, the coverslips were rinsed in 0.1 M carbonate buffer, pH 9.5, and mounted with Acquovitrex (Carlo Erba), a nonfluorescent aqueous mounting solution. In controls, PBS was used instead of the primary antibody. Hemocytes were observed under a Leitz Dialux 22 light and fluorescent microscope equipped with a I2/3 filter block for FITC excitation.

Statistical Analysis

All experiments were repeated in triplicate. The number of hemocytes with an ameboid shape, counted at a magnification of ×1250—at least 200 cells per coverslip in ten fields—was expressed as the percentage of the total number of hemocytes ± SD and analyzed by using the χ^2 test with the FREQ procedure of the SAS statistical package (SAS Institute Inc., Cary, NC).

RESULTS

Cell Morphology and Viability

It was previously observed that TBT had inhibitory effects on the *in vitro* phagocytosis of yeast cells and Ca^{2+}ATPase activity of *B. schlosseri* hemocytes in a concentration-dependent manner, its lower effective concentration being 1 μM for phagocytosis and 10 μM for Ca^{2+}ATPase activity (Cima *et al*., 1995). At the end of treatment with TBT, the viability of blood cells, as monitored by Trypan blue, exceeded 99.5%. A similar value was observed in controls not containing TBT. Therefore, all exposure concentrations were to be considered sublethal.

A significant decrease ($P < 0.05$) in hemocyte viability was observed only after 60-min exposure at concentrations higher than 100 μM.

Cultured hemocytes, appearing as a polymorphic population in normal conditions, changed to a common spherical morphology in the presence of TBT (Figs. 1a and 1b). Only a few morphological changes were observed in hemocytes treated with 1 μM TBT, whereas considerable alterations in cell shape occurred after incubation with 10 μM TBT. In the latter case, phagocytizing amebocytes completely withdrew their pseudopodia, became rounded, and stopped moving.

The number of cultured hemocytes never decreased, indicating that probable alteration of cell components by TBT did not interfere with the ability of the cells to attach to the substrate through their adhesion plaques.

Effects of TBT on Microfilaments

The relationships between the aforedescribed morphological changes and induced cytoskeletal modifications were tested by indirect immunofluorescence microscopy, by using antibodies against actin and tubulin to reveal structural alterations in microfilaments and microtubules, respectively.

In controls (Fig. 1c), the immunofluorescence distribution of the antibody revealed that F-actin consisted of an

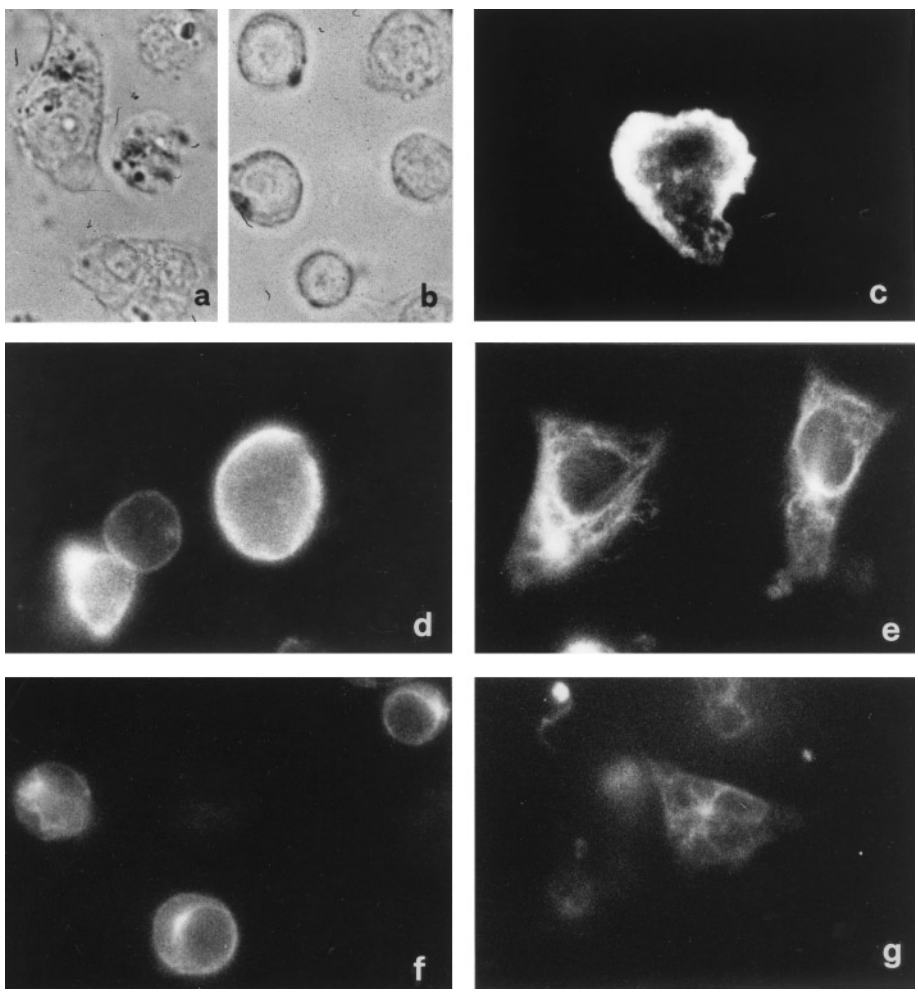

FIG. 1. *Botryllus schlosseri* cultured amebocytes; ×1400. (a, b) Light microscope images of control hemocytes (a) and rounded hemocytes after treatment with 10 μM TBT for 60 min (b). (c, d) Immunofluorescent images of microfilaments visualized by anti-actin antibodies: (c) control hemocyte, with F-actin distributed in cortical layer, pseudopodia, and adhesion plaques; (d) TBT-treated hemocytes, with F-actin reorganized in clusters around peripheral cytoplasm. (e–g) Immunofluorescent images of microtubules visualized by anti-α-tubulin antibodies: (e) control hemocytes, with thick network of thin microtubules rising from MTOC near nucleus; (f) TBT-treated hemocytes, still with MTOC but with microtubules scattered, diffuse, and arranged in a ring around nucleus; (g) TBT + CaM-treated hemocytes in which microtubules form an aster and pseudopodia are rebuilt.

extensive system of cross-linked bundles with higher density and organization, traversing the cell through the entire cytoplasm both in the cortical layer immediately underlying the cell membrane and in the superficial dynamic extensions of the advancing pseudopodia. Small, more intense fluorescent points scattered on the surface represented the adhesion plaques, in which localized cables of F-actin were bound to the cell membrane and exerted traction on the substrate. These observations are in agreement with the pattern of microfilament organization described by Boyles and Bainton (1979) in mammalian polymorphonuclear leukocytes.

In 10 μM TBT-treated hemocytes (Fig. 1d), F-actin distribution was dramatically reorganized from a diffuse cytoplasmic location to clusters distributed around the peripheral cytoplasm, indicating massive breakdown of the microfilaments. However, the adhesion plaques were still evident as scattered fluorescent points.

Effects of TBT on Microtubules

In controls (Fig. 1e), microtubules appeared to spread out from a major microtubule organizing center near the nucleus (i.e., the centrosome), to which the microtubule (−) end was anchored. Microtubules radiated with the (+) end toward the cell periphery along various directions, prevalently toward the leading edge of pseudopodia, making up a thick network of thin filaments in the cytoplasm.

In 10 μM TBT-treated hemocytes (Fig. 1f), the microtubules were scattered in the cytoplasm and lacked orientation. The MTOC was still present, but the microtubules were not recognizable as single filaments and appeared as a poorly defined mass in a ring around the nucleus. Their diffuse fluorescence indicated extensive microtubule disassembly that did not affect the MTOC.

Effects of CaM on TBT-Altered Cell Morphology

The observed 10 μM TBT-induced microtubule and microfilament disaggregation was a rapid and irreversible process. To evaluate the recovery ability of the hemocytes, samples were incubated in FSW for 60 min after TBT treatment, but altered cell morphology remained so and cytoskeletal protein depolymerization was still evident. However, 20 μg/ml CaM did seem to significantly restore the morphology of some amebocytes, both after TBT treatment and in the presence of TBT. Table 1 presents the number of hemocytes with ameboid morphology expressed as a percentage of total hemocytes. When CaM was added after TBT treatment, it was less efficient in restoring cell morphology.

Cytoskeletal architecture was partially restored by CaM, and this restoration was particularly evident in the microtubules. In samples treated simultaneously with 20 μg/ml CaM and 10 μM TBT, immunofluorescence revealed that a few microtubules had reassembled, extending from the aster chiefly toward the rebuilt pseudopodia (Fig. 1g).

TABLE 1
Effects of CaM on TBT-Altered Cell Morphology

Treatment	Percentage of ameboid hemocytes
TBT 60 min/W/FSW 60 min	1.70 ± 0.14
TBT 60 min/W/CaM 30 min	3.45 ± 0.35*
TBT + CaM 60 min	7.25 ± 0.07***
Untreated (FSW) 60 min	14.27 ± 1.18***

Note. Experiments involved addition of 20 μg/ml CaM both in the presence of 10 μM TBT and after TBT treatment (W, washing). Number of hemocytes with ameboid morphology expressed as a percentage of total hemocytes. Significant differences with respect to TBT/FSW treatment: $*P < 0.05$; $***P < 0.001$.

DISCUSSION

Previous observations verified that ascidian hemocytes exposed to organotin compounds undergo rapid changes in shape (Cima *et al*., 1995, 1997). The present study reveals that these changes in shape are closely related to severe interference with cytoskeletal proteins, an important event associated with the manifestation of cellular toxicity (Marinovich *et al*., 1990). In embryos of the ascidian *Styela plicata*, it had already been observed that TBT causes cleavage block, ascribed to inhibition of microtubule polymerization and deep changes in blastomere morphology probably due to inhibition of microfilament polymerization (Cima *et al*., 1996). In the present study, the remarkable and irreversible breakdown and dispersion of both microfilaments and microtubules in ascidian hemocytes exposed to TBT is demonstrated, although the adhesion plaques and MTOC of the hemocytes appear to be unaltered. The immunotoxic effect of TBT, at the same concentration that affects cell morphology in *B. schlosseri* phagocytes, is closely associated with inhibition of Ca^{2+}ATPase activity and high accumulation of intracellular Ca^{2+} ions (Cima *et al*., 1995). Both events are responsible for impaired phagocytosis, but their role in the mechanism of action of TBT was unclear until now.

In rat brain synaptic membranes, it has been demonstrated that, although TBT inhibits CaM-stimulated Ca^{2+}ATPase in a concentration-dependent manner, this effect may be partially reversed by excess addition of CaM (Yallapragada *et al*., 1990, 1991). The same response was obtained by exposing *B. schlosseri* phagocytes to 20 μg/ml CaM in the presence of triphenyltin (TPT) compounds (Cima *et al*., 1997).

In mouse thymocytes, TBT has also been found to increase the membrane Ca^{2+} permeability of cellular calcium stores and to decrease Ca^{2+}ATPase activity, resulting in a sustained increase in intracellular Ca^{2+} concentrations (Oyama *et al*., 1994). The latter effect appears to be a multifactorial process, involving release of Ca^{2+} from intracellular stores, inhibition of the Ca^{2+} extrusion system, and activation of Ca^{2+} influx (Orrenius *et al*., 1992). In the same cells, the immunotoxicant effect of TBT, resulting in depletion of thymocytes by apoptosis, has been related to rapid cytoskeleton modifications such as Ca^{2+}-dependent depolymerization of F-actin (Chow and Orrenius, 1994).

From the results of the present study, it appears probable that the immunotoxicity of TBT on *B. schlosseri* hemocytes is mediated by its direct mechanism of action on the cytoskeleton through alteration of calcium homeostasis. Therefore direct interaction of TBT with the Ca^{2+} pump or calmodulin is suggested. In the first case, the lypophilic butyl groups of TBT may interact with membrane-bound Ca^{2+}ATPase, causing inhibition of Ca^{2+} extrusion. In the second case, the tin ion of TBT may interact directly with CaM, altering its conformation, as observed with certain heavy metals (Desaiah, 1989). Consequently, CaM-dependent Ca^{2+}ATPase activity is inhibited.

Both these hypotheses involve an increase in intracellular Ca^{2+} concentration to activate a series of cytotoxic mechanisms, the first of which is a change in cell morphology through the disassembly of cytoskeletal proteins and extensive activation of fluidifying proteins such as gelsolin. An excess of CaM added to the incubation medium may act as a TBT competitor for the same active site on Ca^{2+}ATPase or as a substitute for TBT-inhibited CaM. In any case, added CaM partially restores cytoskeletal architecture by restoring Ca^{2+} pump activity and intracellular Ca^{2+} homeostasis. It has been proposed that the rapid depolymerization of F-actin content observed in mammalian thymocytes may also be a Ca^{2+}-independent process, involving the oxidation of thiol groups of microfilaments by TBT (Chow and Orrenius, 1994).

In conclusion, the severe immunotoxic effects of TBT in ascidian hemocytes may result in the inhibition of yeast phagocytosis at a very early stage, probably beginning at the stage of particle adhesion or, as described in mammal neutrophils, from chemotaxis process (Arakawa and Wada, 1984). The toxic effects on the oxidative phosphorylation of mitochondria (Aldridge *et al*., 1977) take place later. They may inhibit the respiratory burst in ascidian phagocytes (Cima *et al*., 1997), but it is unlikely that inhibition of oxidative phosphorylation and ATP depletion are directly involved in cytoskeletal alterations, because ATP and GTP are not strictly necessary for organizing microfilaments and microtubules (Alberts *et al*., 1983).

Although more data are needed to verify the foregoing hypotheses of the mechanisms of action of TBT through Ca^{2+}-dependent and Ca^{2+}-independent processes, previous and present studies strongly support the observation that the potent immunotoxic effects of TBT on ascidian phagocytes involve cytoskeletal modifications and perturbation of intracellular Ca^{2+} homeostasis.

CONCLUSION

In tunicates, the immunotoxic effect of TBT at sublethal concentrations is principally evident in inhibition of phagocytosis and related respiratory burst. The mechanism of action is still unknown, but observations on alterations in cell morphology and inhibition of Ca^{2+}ATPase activity strongly suggest that TBT interacts with various cell components, causing consequent effects; that is, (1) increase in intracellular Ca^{2+} concentration, (2) internal disorganization of cytoskeletal proteins, (3) hindering of chemotaxis and particle adhesion, and (4) inhibition of the oxidative phosphorylation of mitochondria.

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