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TBT-induced Apoptosis in Tunicate Haemocytes

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Early events in apoptosis include chromatin condensation followed by DNA fragmentation as well as translocation of phosphatidylserine (PS) in the outer plasma membrane. Organotin compounds increase intracellular Ca^{2+} levels and cause apoptosis in mammalian cells. In investigating whether TBT may also induce apoptosis in haemocytes of the ascidian *Botryllus schlosseri*, we exposed haemocytes to this xenobiotic at the sublethal dose of 10 μM, causing cell shrinkage and inhibition of phagocytosis and respiratory burst. Apoptosis was revealed as (i) chromatin condensation, with Acridine Orange nuclear staining; (ii) DNA fragmentation, with the TUNEL reaction; (iii) PS translocation, with the annexin-V assay; and (iv) loss of membrane permeability with the Trypan Blue diffusion assay. After 1 h of exposure, nuclear changes, i.e. significant collapse and cleavage of chromatin, were observed and cytoplasm blebbing occurred, together with surface alterations triggered by PS exposure. Haemocyte mortality increased significantly only after 2 h. All these apoptotic events may be closely related to a TBT-induced cytosolic calcium increase resulting in activation of endonucleases.

Keywords: tributyltin (TBT); immunotoxicity; apoptosis; haemocytes; ascidians; *Botryllus*

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INTRODUCTION

Many xenobiotics become lethally cytotoxic according to their concentration. These toxins induce programmed cell death with completely different mechanisms of action. Induction of apoptosis has recently been reported in various mammalian cell lines in the presence of *p*-benzoquinone[1] and isothiocyanates[2] in a time- and dose-dependent manner. Environmental contaminants, such as arsenite and cadmium, cause cytoplasm protrusions, indicative of cells entering apoptosis in primary cultures of hepatocytes of the teleostean *Salmo salar*.[3]

A number of xenobiotics have immunotoxic effects in mammals, but relatively little information is available regarding their immunotoxicity in fish and aquatic invertebrates. Organochlorines may induce direct or indirect toxicity, altered functionality and cell death in the fish thymus, an organ important for immunocompetence and survival.[4] Organotin compounds are a widespread class of synthetic substances which persist in aquatic environments, with dramatic long-term impact on life, mainly related to their immunosuppressive activity. In mammals and teleosts, decreased resistance to infections has been found after persistent organotin exposure,[5] and depletions of lymphocytes and leukocytes accompanied by atrophy of spleen and thymus have also been described.[6,7] Trisubstituted organotins are the most diffuse compounds known to cause apoptosis in various mammalian cell lines. Recently, triphenyltin chloride has been reported to cause apoptosis in mammalian HL-60 promyelocytic cells, by means of a cascade of events which include (i) increase of intracellular Ca^{2+}, (ii) alteration of actin polymerization and (iii) induction of DNA degradation.[8] In previous reports we demonstrated the immunotoxicity of tributyltin (TBT) in the colonial ascidian *Botryllus schlosseri*, a filter-feeding marine invertebrate, inhibiting phagocytosis and the respiratory burst.[9] The effect is mediated by an extensive cytosolic calcium increase, resulting in volume loss and morphological changes related to cytoskeletal alterations.[10,11]

We stress that, in contrast to the situation for vertebrates, studies on apoptosis generally induced in invertebrates by xenobiotic exposure are still

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greatly lacking. As tunicates are closely related to vertebrates, they can be chosen as a comparative model for the immunotoxicity. In a first approach, widely used techniques for detecting general events leading to apoptosis in vertebrates were extended to ascidian haemocytes. The morphological changes induced by TBT exposure are the main subject of this study to clarify the sequence of events linked to apoptosis.

MATERIALS AND METHODS

Animals

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

Haemocyte collection and cultures

Haemocytes 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 centrifuged at 780 *g* for 10 min, and pellets were resuspended in FSW at a final concentration of (8–10) $\times$ 10^6 cells ml^{-1}

Culture chambers were made according to a previously reported method[12] and loaded with 50 μl of haemocyte suspension.

Effects of TBT

Tributyltin chloride (Sigma) was first dissolved in 95% ethanol at 10 mM Concentration and then diluted at final concentrations of 0.1, 1 and 10 μM in the FSW used for culture incubation.

After 30 min of cell adhesion to the chamber coverslip, FSW-containing debris was discarded and replaced with equal volumes of the TBT solution. FSW containing 0.1% of 95% ethanol was used in controls. Treated haemocyte monolayers were incubated for 1, 2, 3 and 4 h at 25 °C and then washed several times in FSW.

Morphological assays for apoptosis

a) Acridine Orange and Pfitzner's Safranin

After TBT exposure, haemocytes were stained *in vivo* with Acridine Orange (Sigma) (0.1 mg ml^{-1} in FSW) for 90 s and then immediately observed under a Leitz Dialux 22 light and fluorescent microscope with an I2/3 filter block at a magnification of 1250. The phenotype of apoptotic cells was identified as follows:

(i) *Viable*: bright green nucleus with intact structure.
(ii) *Early apoptotic*: bright yellow nucleus showing condensation of chromatin as dense yellow areas.
(iii) *Late apoptotic*: orange nucleus showing condensation and cleavage of chromatin as dense orange areas.

The apoptotic index was expressed as a percentage of cultured haemocytes showing chromatin condensation — yellow and orange nuclei — revealed by Acridine Orange nuclear staining.

The haemocytes were first fixed for 30 min at 25 °C in Sanfelice solution (16 ml of 1% chromic trioxide, 8 ml of formaldehyde, and 1 ml of 100% acetic acid). Condensed chromatin was then revealed as dense red areas in nuclei after staining for 2 h in Pfitzner's Safranin. The working solution was first prepared by adding 50 ml of distilled water to a stock solution of Safranin (Merck, Germany; 1 g/100 ml in absolute ethanol). Haemocytes were then stained with Mayer's haematoxylin to detect euchromatin as blue strands in nuclei of non-apoptotic cells.

b) Trypan Blue diffusion

Inability to exclude the Trypan Blue vital dye is a useful procedure for estimation of cell death. Haemocyte monolayers were exposed to 0.25% Trypan Blue in FSW for 5 min at 25 °C and then observed *in vivo* under a light microscope. In this case, the apoptotic index was expressed as the percentage of haemocytes positive to Trypan Blue dye due to loss of membrane permeability.

c) TUNEL reaction

Nuclear collapse, associated with extensive damage to chromatin and DNA cleavage into oligonucleo-

somal-length DNA fragments, was investigated by means of analysis and quantification of enzymic *in situ* labelling of apoptosis-induced DNA strand breaks under light microscopy, i.e. the TUNEL reaction (In Situ Cell Death Detection Kit, Boehringer Mannheim GmbH, Germany). In this assay haemocyte monolayers were fixed in paraformaldehyde (Serva) solution (4% in ISO buffer, i.e. 20 mM Tris, 0.5 M NaCl, pH 7.5) for 30 min at 25 °C, rinsed with PBS and incubated with blocking solution (0.3% H_2O_2 in methanol) for 30 min at 25 °C. After washing in PBS, they were incubated in a permeabilization solution (0.1% Triton X-100, Merck, in 0.1% sodium citrate) for 2 min at 4 °C. Haemocytes were then rinsed twice with PBS and incubated in the TUNEL reaction mixture for 60 min at 37 °C. In this reaction, the enzyme deoxynucleotidyl transferase (TdT) catalyses the addition of fluorescein isothiocyanate (FITC)-labelled dUTP to the free 3′-OH DNA ends: samples may be analysed under a fluorescence microscope in this state. However, incorporated FITC was detected by incubating haemocytes in anti-FITC antibody Fab fragments from sheep, conjugated with horseradish peroxidase (signal conversion) for 30 min at 37 °C. After the haemocyte monolayers had been rinsed in PBS, they were incubated in 3,3′-diaminobenzidine (Sigma) solution for 10 min at 25 °C. Lastly, they were washed in PBS and mounted in Acquovitrex (Carlo Erba). They were observed and quantified by light microscopy. Haemocytes incubated without TdT were used as negative control.

The apoptotic index was expressed as the percentage of haemocytes with positivity to the TUNEL reaction, showing brown nuclei.

d) Annexin-V

The specific FITC-coupled annexin-V probe (Annexin-V-FLUOS Staining Kit, Boehringer Mannheim GmbH, Germany) was used to monitor changes in the distribution of phosphatidylserine in the plasma membrane during apoptosis. In this assay, haemocyte monolayers were incubated in a staining solution obtained by diluting 20 μl annexin-V-FITC labelling reagent in 1000 μl ISO buffer and adding 20 μl propidium iodide. The latter reagent was used to distinguish necrotic from apoptotic cells.

Haemocytes were observed *in vivo* after 15 min at a magnification of 1250 under a Leitz Dialux 22 light and fluorescent microscope equipped with an I2/3 filter block for FITC excitation.

The apoptotic index was expressed as the percentage of haemocytes positive to PS translocation with the annexin-V assay.

Statistical analysis

All experiments were repeated in triplicate. The number of haemocytes positive to various assays, at least 200 cells per monolayer in 10 fields, were counted, expressed as a percentage $\pm$ SD of the total number and analysed by the χ^2 test with the FREQ procedure of the SAS statistical package (SAS Institute Inc., Cary, NC, USA).

RESULTS

In order to find the threshold value of the apoptosis stages, the dose-dependent course was first evaluated after exposure of *B. schlosseri* cultured haemocytes to various TBT concentrations, from 0.1 to 10 μM. In experiments with a TBT concentration of 1 μM, after 1 h haemocytes showed many morphological alterations related to apoptosis, i.e. cell shrinkage, chromatin condensation and PS translocation, but these results did not appear repeatable. This TBT concentration probably represents the threshold value of exposure around which a remarkable variability occurs following an 'all or nothing' behaviour. Therefore, 10 μM TBT was chosen for exposure of haemocytes for 1–4 h, since this concentration constantly and significantly changes cytoskeletal structure and does not cause mortality within 1 h.[9,10]

In controls, both viability and apoptotic indexes, detected with Trypan Blue and Acridine Orange respectively, remained unchanged up to 4 h. For this reason, in Fig. 1 the control data are conventionally reported as time = 0.

In the presence of 10 μM TBT, haemocytes rapidly exhibited several morphological changes, resulting in loss of spherical shape and volume.

Nuclear changes

A significant ($P < 0.001$) increase in nuclear condensation, corresponding to early apoptosis, as revealed with Acridine Orange staining of haemocytes, could already be detected after 1 h (Fig. 1). Chromatin formed dense, crescent-shaped aggregates which lined the nuclear membrane. Interestingly, post-treatment with either Acridine Orange or Pfitzner's Safranin revealed that most of the

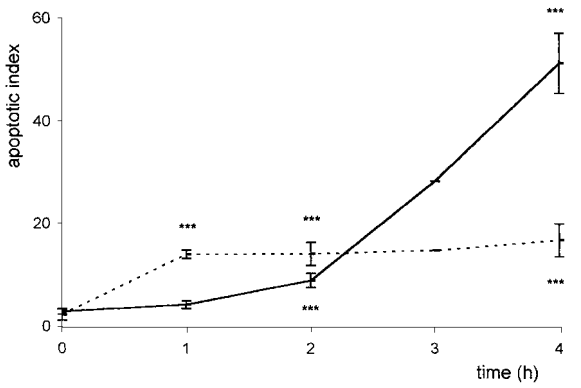

Figure 1 Apoptotic index expressed as percentage of *B. schlosseri* cultured haemocytes positive to Trypan Blue dye (thick line) and as a percentage of blood cells showing chromatin condensation revealed by Acridine Orange staining (dotted line), after 1, 2, 3 and 4 h of 10 μM TBT exposure. Asterisks indicate the levels of significance with respect to controls (time = 0): *** $P < 0.001$.

periphery of the nucleus was occupied by condensed chromatin (Fig. 2). At the same time, the cytoplasm of haemocytes exposed to TBT and then to Acridine Orange also showed an orange fluorescence, unlike the typical green fluorescence of controls.

Haemocytes positive to the TUNEL reaction (Figs 3a, 3b) significantly increased ($P < 0.001$) after 1 h (Fig. 4) of TBT treatment and their number regularly augmented after 2 h. These results are comparable with those above reported using Acridine Orange, and both point up the DNA changes. The TUNEL reaction still appears to be more sensitive than the Acridine Orange assay for detecting DNA cleavage as TBT exposure continues after 1 h.

Plasma membrane changes

Correlating the chromatin condensation with the loss of membrane integrity by means of Acridine Orange and Trypan Blue respectively, the apoptotic index was not significantly different after 2 h, whereas after 4 h haemocytes positive to Trypan Blue increased significantly and exceeded those containing a picnotic nucleus (Fig. 1).

With the annexin-V test, haemocytes exposed to TBT showed cytoplasm blebbing together with surface alterations triggered by translocation of plasma-membrane phosphatidylserine to the outer side of the phospholipidic bilayer (Figs 3c, 3d). As a result of the excessive invagination of the plasma membrane, apoptotic cells dissociated into a number of membrane-bound particles. These apoptotic bodies were recognized and ingested by phagocytes. After 1 h of TBT exposure, the percentage of haemocytes positive to the annexin-V assay was significantly ($P < 0.001$) higher than in untreated cells and significantly ($P < 0.001$) increased after 2 h (Fig. 4).

DISCUSSION

Generally, cells undergoing death by apoptosis share a number of strikingly similar features, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation. These events were observed in *B. schlosseri* haemocytes exposed to TBT up to 4 h. Time-lapse studies on mammalian cells have shown that the changes occurred rapidly, with the induction of apoptosis taking about 1–3 h, and apoptotic structures persisting for about 1 h.[13]

The first common event, i.e. cell shrinkage, is due to the release of fluid from the cell. In mammals, it has been suggested that dilatation of the endoplasmic reticulum may be involved in transport of water to the cell exterior.[14] Moreover, previous observations on disassembly of microfilaments and microtubules in *B. schlosseri* haemocytes suggest the interaction of TBT with cytoskeletal components directly regulated by calmodulin, leading to cell shape alteration.[10]

Accompanying the decrease in cell volume, the plasma membrane of *B. schlosseri* haemocytes becomes extensively convoluted and acquires a characteristic blebbed appearance, together with phosphatidylserine externalization. The latter is a common event, well described during apoptosis of murine and human cells.[15–17] In particular, tunicate haemocytes show an increasing pattern of apoptosis even after 2 h of TBT exposure: nuclei already appear in late apoptosis after 1 h, as observed with Acridine Orange and the TUNEL reaction, both giving more information than Pfitzner's Safranin; instead, after 2 h, DNA fragmentation is more extensive and alterations of plasma membrane continue, as observed with Trypan Blue and annexin-V. Colour changes in Acridine Orange observed in the cytoplasm of haemocytes exposed to TBT may be related to pH lowering due to increased halide/hydroxyl exchange across plasma membranes.[18]

In the colonial ascidian *B. schlosseri*, percen-

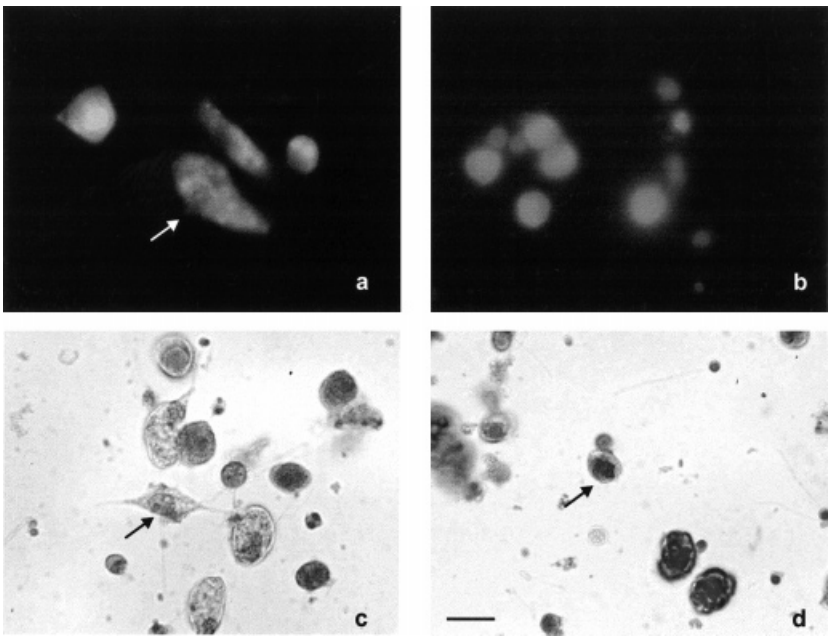

Figure 2 *B. schlosseri* haemocytes stained *in vivo* with Acridine Orange (a, b), and with Mayer's haematoxylin and Pfitzner's Safranin after fixation in Sanfelice solution (c, d). Normal conditions: (a) bright green nuclei (arrow) with intact structure and (c) blue strands of euchromatin (arrow) and an amoeboid shape. After 1 h of 10 μM TBT exposure: (b) orange nucleus with condensation and cleavage of chromatin and (d) dense red areas in nucleus (arrow) and a spherical shape. Bar length: 10 μm.

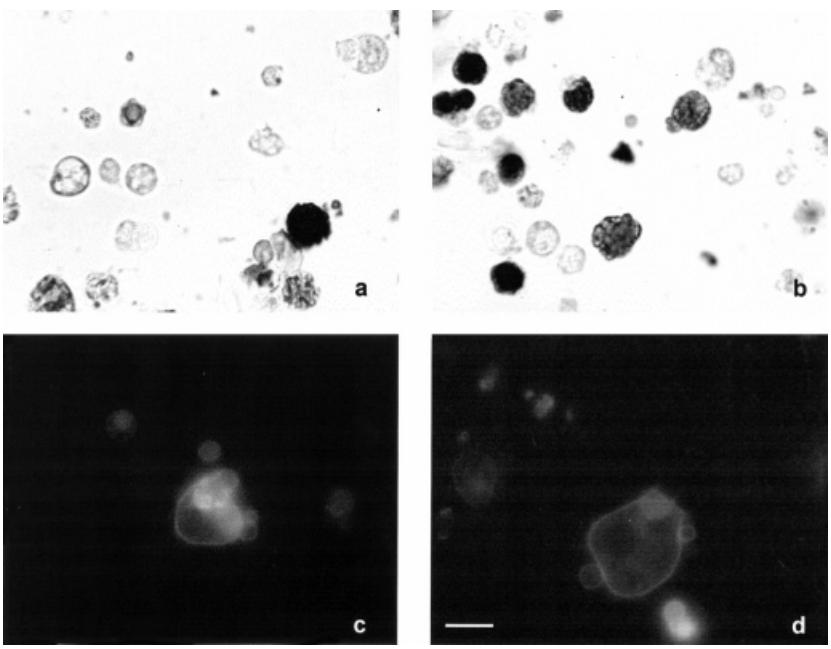

Figure 3 *B. schlosseri* haemocytes positive to enzymic labelling *in situ* of apoptosis-induced DNA strand breaks under light microscopy (TUNEL reaction) showing brown nuclei (a, b) and positive to the specific fluorescein isothiocyanate-coupled annexin-V probe showing cytoplasm blebbing and phagocytized apoptotic bodies (c, d). (a) Normal conditions and (b, c, d) after 1 h of 10 μM TBT exposure. Bar length: 10 μm.

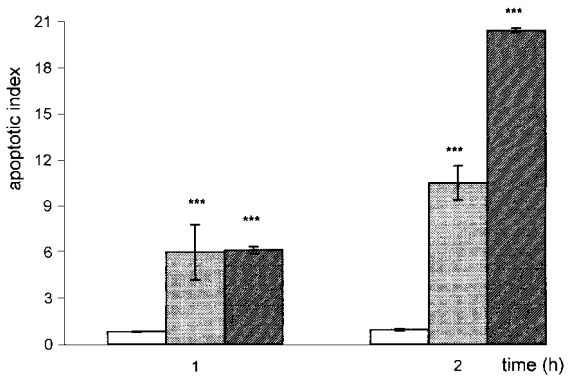

Figure 4 Apoptotic index of *B. schlosseri* haemocytes exposed to 10 μM TBT for 1 and 2 h as detected with the annexin-V assay (grey bars) and the TUNEL reaction (black bars). Asterisks indicate significant differences with respect to controls (unexposed haemocytes: open bars): *** $P < 0.001$.

tages of apoptotic haemocytes revealed with Acridine Orange and Trypan Blue after 1 h of TBT exposure differ significantly, and this result may be related to a more rapid loss of nuclear functions than of membrane integrity. Haemocyte mortality, detected with Trypan Blue, takes place only after 2 h, since cells in the early stages of apoptosis may retain their membrane integrity for several hours and therefore do not stain with Trypan Blue, leading an underestimate of the extent of death. On the other hand, a plasma-membrane alteration already occurs within 1 h, before uptake of vital dyes, as observed with the annexin assay. Hence, this method appears more sensitive than Trypan Blue for assessing early apoptosis.

Elevations in the cytosolic Ca^{2+} level are associated with apoptotic cell death in a number of model systems.[8] Transient Ca^{2+} rises are more typically associated with cell proliferation and inhibition of apoptosis, whereas sustained Ca^{2+} increases are most often linked to growth inhibition and cell death, since they are sufficient to promote activation of endogenous endonucleases which are responsible for cleaving chromatin in the internucleosomal linker regions.[19,20] In this framework, the dramatic changes observed within the nucleus of *B. schlosseri* haemocytes, i.e. chromatin condensation and nuclear fragmentation, may be due to activation of endogenous endonucleases, which cleave the DNA into oligonucleosomal fragments of about 180–200 bp, as described in mammals.[21–23]

Chemical toxins can trigger apoptosis by promoting sustained Ca^{2+} increases.[24] In particular, organotin compounds have been reported to increase intracellular Ca^{2+} levels and to cause apoptosis in mammalian cells.[8,25] Analogously, they are known to increase cytosolic Ca^{2+} in our species.[9] Noteworthy is the ATP- and calmodulin-dependent nuclear Ca^{2+}-uptake system, which appears to facilitate endonuclease activation directly during apoptosis.[26] Moreover, calmodulin plays an important role in regulating the response.[27] Therefore, we suggest that, in *Botryllus* haemocytes, most of the observed apoptotic events are Ca^{2+}-dependent because they are closely related to TBT-triggered cytosolic calcium increase, leading to cell death, mediated by the inhibition of calmodulin and calmodulin-dependent enzymes (Ca^{2+}-ATPase), disassembly of cytoskeletal components (microfilaments and microtubules), and activation of Ca^{2+}-dependent DNA lytic enzymes (endonucleases). The above-mentioned cell shrinkage may be closely related to an alteration in the Ca^{2+}-transport system associated to the cisternae of smooth endoplasmic reticulum.

Other apoptosis mechanisms can be involved differently from or correlated with Ca^{2+} changes in haemocytes exposed to TBT and will be the subject of further investigations.

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