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Effects of Metal-Based Environmental Pollutants on Tunicate Hemocytes

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Tunicates are filter feeding marine invertebrates that are susceptible to environmental contamination by toxic metals and polyaromatic hydrocarbons. Recently, we have shown that tunicate immune reactions are profoundly affected by exposure to tributyltin (TBT) and copper, both of which are components of marine antifouling paints. This study tests the effects of those pollutants on the hemocytes of tunicates. Immunofluorescence labeling with an anti-hemocyte monoclonal antibody demonstrated that the antigenic structure of the circulating hemocyte population was substantially affected by TBT and copper. Antigen-positive hemocytes were also found to accumulate in the pharyngeal papillae of TBT-exposed tunicates. Histological analyses indicated that this cellular accumulation in pharyngeal papillae involved refractile vacuolated hemocytes. Refractile vacuolated cells from TBT-exposed tunicates also occurred at greater frequencies in the circulating hemolymph, and had altered morphologies, compared to cells from non-treated controls. These data confirm that exogenous metals can have profound effects on the hemocytes of tunicates.

Key Words: **tunicate; ascidian; *Styela plicata;* environmental pollutants; tributyltin; TBT; copper; hemocyte.**

INTRODUCTION

Many purpose-specific chemicals have been used to control the growth of marine organisms. Tributyltin (TBT) and copper, both active components of antifouling paints, are common examples of such toxicants. Even though the use of these metals as antifoulers has now been restricted in Australia and elsewhere, residual contamination persists and both are still registered for use on larger vessels (>25 m) (Batley *et al.*, 1996; Belfroid *et al.*, 2000; Evans, 1999).

Given their role in preventing the growth of sessile invertebrates on boats and ships, the effects of TBT and copper on marine and freshwater organisms have been extensively studied (Batley *et al.*, 1996; Cima *et al.*, 1995, 1998a,b, 1999; Langston, 1990; Lawson *et al.*, 1995; Stebbing, 1985; Waldock and Thain, 1983). Test organisms have included carp, trout, shrimp, crabs, mollusks, and tunicates. In these animals, organotin complexes (mono-, di-, and tri-butyltin) have been shown to affect development and differentiation. They decrease the viability of tunicate embryos, with the most susceptible species being those with embryonic gestation times longer than 24 h. Organotins also reduce gastrula cell adhesion, impair cytoskeletal function, and block mitosis leading to anomalous embryos (Cima *et al.*, 1998a,b; Mansueto *et al.*, 1993).

Both copper and TBT are also known to modulate immune reactions. TBT reduces the level of phagocytosis in the tunicates *Styela plicata* (Raftos and Hutchinson, 1997), *Ciona intestinalis* (Cooper *et al.*, 1995), and *Botryllus schlosseri* (Cima *et al.*, 1995). Sublethal doses of copper decrease cell proliferation and viability in *S. plicata* and reduce the cytotoxic activity of hemocytes (Raftos and Hutchinson, 1997). Raftos and Hutchinson (1997) have suggested that these detrimental effects on tunicate immune function arise from the ability of TBT and copper to modulate cellular activity. The current study tests that hypothesis by investigating the effects of TBT and copper on the hemocytes of *S. plicata*. A monoclonal antibody (MAb) developed against hemocytes of *S. plicata* is used here in conjunction with histological analysis to gauge cellular responses to these metals.

MATERIALS AND METHODS

Collection of tunicates. *S. plicata* were collected from two sites on Sydney Harbor, Birkenhead Point Marina and Quakers Hat Bay. Animals were held in 40-liter aerated aquaria at 14°C with a minimum of 10 liters of seawater per animal. Tunicates were equili-

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brated in aquaria for at least 24 h before being exposed to toxicants.

Monoclonal antibody production. Monoclonal antibodies to hemocyte surface antigens were produced by the method of Radford *et al.* (1998). Mice were immunized with hemocytes by a modification of the method of Gilbertson *et al.* (1986). The hemocytes were harvested from five tunicates into sterile-filtered seawater (FSW, 0.45-μm filtration) before being washed once and resuspended in FSW. Three Balb/c female mice were injected intraperitoneally with 200 μL of the cell suspensions (5×10^7 cells per mL) on three separate occasions at 2-week intervals. Mice were sacrificed 3 days after the final immunization and the spleens were removed.

Splenocytes were fused with NS-1 mouse myeloma cells using polyethylene glycol according to a modified method of Kohler and Milstein (1975). Antibody secretion was monitored by immunofluorescence staining of tunicate hemocytes with culture supernatants followed by flow cytometric analysis. Hybridomas were cloned by limiting dilution if they exhibited anti-hemocyte surface reactivity in flow cytometric analyses. Supernatants were collected and stored at 4°C in the presence of 0.02% w/v sodium azide as a preservative.

The specificities of MAbs for hemocytes are described in detail by Radford *et al.* (1997). MAb A62 was selected for use in this study because the expression of its ligand is known to fluctuate in response to environmental stressors such as bacterial immunization (D. Raftos, unpublished data). A62 stains approximately 20% of hemocytes from normal tunicates, but is not specific to any individual hemocyte type (Radford *et al.*, 1997).

Exposure of tunicates to TBT and copper. TBT (ICN Chemicals, NSW Australia) and $CuSO_4$ (Jaegar Chemicals, NSW, Australia) were added to aquaria at least 48 h before tunicates. Tunicates were incubated with metals for periods of 12–72 h before being subjected to cellular analysis. The concentrations of TBT, dibutyltin, monobutyltin, and copper in solution at the beginning of exposure periods, and the concentrations of metals in whole-body tissues (pharnyx, hepatopancreas, body wall, and gonads) dissected from groups of four tunicates that had been exposed to TBT or copper for 48 h, were determined by the CSIRO Centre for Advanced Analytical Chemistry (Lucas Heights, NSW).

Hemocyte harvesting. Circulating hemocytes were harvested from incisions in the incurrent siphons of tunicates. The cells were collected into prechilled (on ice) marine anticoagulant (MAC), which contained 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 10 mM ethylenediaminetetra acetic acid (EDTA), and 0.45 M sodium chloride (Smith and Peddie, 1992). Hemocyte suspensions were then adjusted to 1×10^6 hemocytes/mL in MAC.

Cytocentrifugation. Cytocentrifuge sample chambers (Shandon, NSW) were prepared using poly-L-lysine (Sigma Chemicals, St Louis, MO) coated slides mounted in a Cytospin cytocentrifuge (Shandon, NSW, Australia). Hemocytes (30 μL, 1×10^6 cells/mL) were loaded into each sample chamber and centrifuged for 10 min (130*g;* room temperature). Cytocentrifuge preparations were then removed from the sample chambers and allowed to air dry before being fixed and stained.

Tissue processing. Pharyngeal tissue was excised from tunicates that had been longitudinally bisected. Tissue samples (approximately 3×5 mm) were fixed in formaldehyde (4% in FSW) for 24 h at 4°C. Fixed pharyngeal tissue was dehydrated through a graded series of alcohols (30% v/v, 50% v/v, 70% v/v for 1 h each with constant rotation). The tissue was held in 70% ethanol overnight and then loaded into a Citadel 2000 automated tissue processor (Shandon) in plastic tissue cassettes. Sections (5 μm) were cut and mounted on poly-L-lysine-coated glass slides. Sections were deparaffinized before either histological or immunohistochemical staining.

Immunofluorescence staining. Hemocytes (1×10^6 cells in 1 mL MAC) were centrifuged (14,000*g,* 8 s), the supernatants removed, and the cell pellets resuspended in 500 μL MAC–immunostaining buffer (MAC-IS; 199 mL MAC, 0.2 g bovine serum albumin, 1 mL 20% sodium azide) for 20 min on ice. Supernatants were removed and replaced with 100 μL of anti-hemocyte monoclonal antibody, A62, (1/100 in MAC-IS) and incubated with constant agitation at 4°C for 1 h. The cell suspensions were then washed twice by centrifugation through MAC-IS and the hemocytes resuspended in 100 μL of fluorescein isothiocyanate (FITC)-labeled sheep anti-mouse immunoglobulin (Sileneus, NSW, Australia) (1/200, 4°C, 1 h). Cells were then washed with MAC-IS three times before being resuspended in 400 μL of paraformaldehyde (1% w/v in FSW) for flow cytometric analysis.

Flow cytometry. A single-laser FACScan flow cytometer (Becton–Dickinson; San Jose, CA) was used to analyze immunofluorescent-stained hemocytes. FITC was excited by an argon ion laser tuned to 488 nm. Fluorescence emissions of 10,000 cells per sample were analyzed using a 535 ± 15-nm band-pass filter. Gates were set to eliminate debris. Within any one experiment, parameters (photomultiplier tube voltage, scatter, fluorescence gain) were kept constant.

"Positive" hemocytes were deemed to be those that yielded fluorescence intensities that were greater than 95% of cells stained with an irrelevant control antibody (anti-hagfish cell surface immunoglobulin kindly sup-

plied by Mr. J. Hooke, University of Technology, Sydney).

Immunohistochemistry. Sections of pharyngeal tissue were cleared in xylene and rehydrated through a graded series of alcohols to water. A Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) was used for immunostaining according to the manufacturer's instructions. Sections were incubated for 20 min in a humidified chamber with phosphate-buffered saline (PBS; 150 mM sodium chloride, 20 mM phosphates, pH 7.2) containing 1.5% v/v heat-inactivated normal horse serum (PBS–NHS). PBS–NHS was removed without washing before A62 (1:1000 in PBS–NHS) was added and incubated for 30 min. Slides were then washed in PBS for 5 min and drained thoroughly before incubation with biotinylated secondary antibody. Slides were washed after 30 min and further incubated with 0.3% hydrogen peroxide (in methanol; 30 min) to quench endogenous peroxidase activity. Quenched slides were washed in PBS and then incubated with Vectastain Elite ABC reagent for 30 min. After a final wash in PBS, the slides were incubated with 3,3′-diaminobenzidine (DAB) until suitable staining intensity developed (210 to 360 s). Staining was stopped by rinsing in tap water.

Harris' hematoxylin (Gurr, 1973) was used to counterstain cells for 3 min followed by rinsing in tap water for 5 min. After rinsing, slides were placed in Scott's Bluing solution (saturated lithium carbonate) for 3 min and washed in tap water for 3 min before being dehydrated and mounted in Fastmount (Frontline, NSW, Australia). Negative controls were used in all experiments.

Cells were considered to be positive for antigen if they displayed a brown coloration either around their cell membrane or internally. Those cells that were negative for the antigen remained blue, reflecting the hematoxylin counterstaining.

Histology. The relative frequencies of morphologically distinct cell types were determined after TBT exposure using routine hematological stains. May Grunwald Giemsa stain (Gurr, Poole, England) was used for cytocentrifuge preparations of circulating hemocytes, and hematoxylin and eosin were used for paraffin sections of pharyngeal tissue. Staining was performed by the methods of Gurr (1973). Differential cell counts were made of eight previously identified hemocyte types (Radford *et al.*, 1998). All counts were carried out using double-blind techniques to preclude operator bias.

Statistical analyses. The statistical significance of differences between treatments was determined using Student's two-tailed *t* test for the difference between means (Sokal and Rohlf, 1981).

TABLE 1

Concentrations of TBT, Dibutyltin (DBT), Monobutyltin (MBT), and Copper in Whole-Body Tissues from Tunicates Exposed to either 50 μg/L TBT or 5 mg/L Copper for 48 h

	MBT (ng/g)	DBT (ng/g)	TBT (ng/g)	Copper (μg/g)
Control (no metal exposure)	40 ± 3	12 ± 2	5.8 ± 0.2	<0.5
50 μg/L TBT	94 ± 66	16 ± 3	20 ± 2	
5 mg/L copper				2.2 ± 0.2

RESULTS

Metal concentrations. The maximum doses of metals added to aquaria (50 μg/L TBT; 5 mg/L copper) yielded soluble concentrations of 1.88 μg/L TBT and 0.57 mg/L copper after 48 h. Monobutyltin (56 ng/L) and dibutyltin (41 ng/L) were also detected 48 h after the addition of 50 μg/L TBT. Normal seawater used in the aquaria had copper and TBT concentrations of 10 μg/L and 1.5 ng/L, respectively.

The concentrations of metals in whole-body tissues from tunicates that had been exposed to TBT (50 μg/L) and copper (5 mg/L) for 48 h are shown in Table 1. Tunicates exposed to TBT contained 20 ± 2 ng TBT/g tissue, which was approximately four times the level of TBT detected in nonexposed controls. However, the majority of butyltin appeared as monobutyltin, which again was far more abundant in TBT-exposed tunicates than in nonexposed controls. Tunicates exposed to 5 mg/L copper retained 2.2 ± 0.2 mg copper/g tissue, whereas <0.5 mg copper/g tissue was evident in nonexposed controls.

Fixed time and dose experiments. In initial experiments, tunicates were exposed to single doses of metals (TBT 50 μg/L; copper 5 mg/L) that were known to significantly modulate immune function (Raftos and Hutchinson, 1997). Circulating hemocytes were then immunofluorescently stained using A62 and analyzed by flow cytometry. Significant differences ($P < 0.05$) in the percentages of cells stained by A62 were observed between control tunicates and those exposed to copper or TBT (Fig. 1). Up to twice as many hemocytes from TBT- or copper-exposed tunicates were stained by A62 compared to nonexposed controls.

Dose response analyses. The fixed dose analyses described above were extended using A62 to test the effect of a range of TBT (0.5–50 μg/L) and copper (0.2–5 mg/L) concentrations. Significant differences ($P < 0.05$) in A62 staining were observed between controls and tunicates exposed to 5 and 50 μg/L TBT (Fig. 2A) or to 0.2 and 5 mg/L copper (Fig. 2B). Up to twice as many hemocytes were stained with A62 at the highest dose of copper and TBT compared to controls.

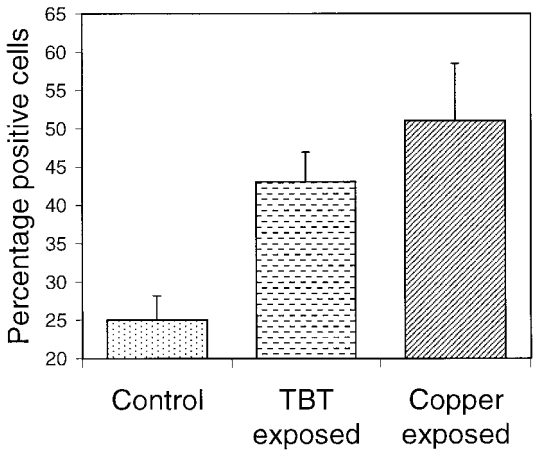

FIG. 1. Fixed time and dose analyses. Percentage (mean ± SE, $n > 7$) of circulating hemocytes stained with MAb A62 after exposure of tunicates to copper (5 mg/L) or TBT (50 μg/L) for 48 h.

Time course studies. Responses to TBT (50 μg/L) and copper (5 mg/L) were also tested over various periods of exposure using A62. Significant ($P < 0.05$) increases in the relative frequency of circulatory hemocytes stained with A62 were observed between TBT-exposed tunicates and nonexposed controls after 8 h (Fig. 3A). At this time, there were 1.5 times as many A62-positive hemocytes in TBT-exposed animals relative to controls. However, responses decreased over longer periods so that, after 72 h of TBT exposure, there was a significant decrease in the percentage of A62-stained cells relative to the 48-h time point ($P < 0.05$; Fig. 3A).

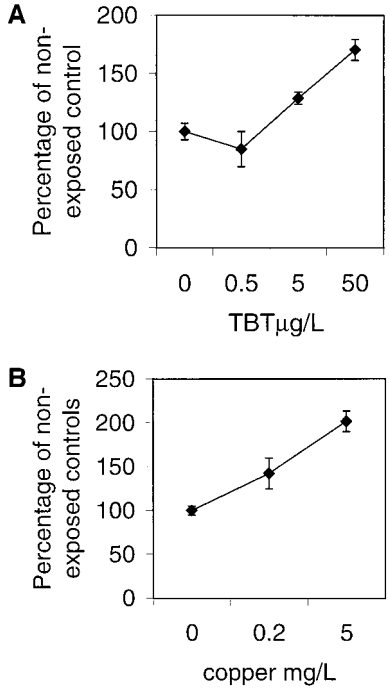

FIG. 2. Dose response analysis for A62. Circulating hemocytes were stained with MAb A62 after exposure of tunicates to various doses of (A) TBT (0.5–50 μg/L) or (B) copper (0.2–5 mg/L) for 48 h. Relative frequencies of positive cells are shown as percentages (mean ± SE, $n > 7$) of mean levels in nontreated controls.

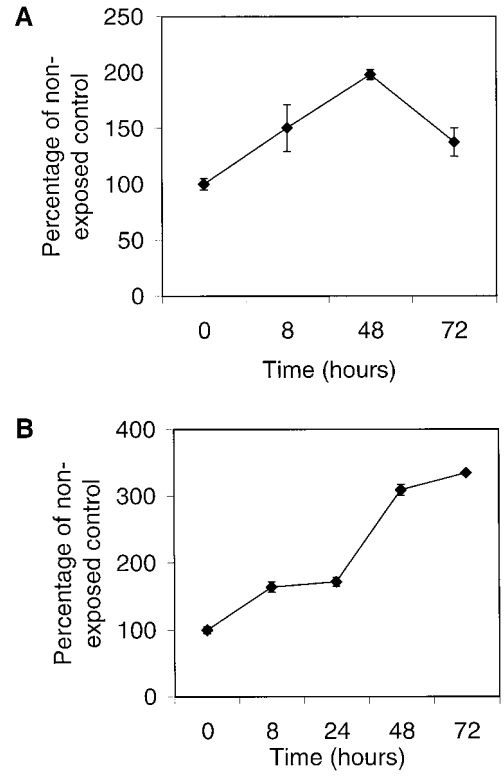

FIG. 3. Time course analyses using A62. Circulating hemocytes were stained with MAb A62 after exposure of tunicates to (A) TBT (50 μg/L) or (B) copper (5 mg/L) for up to 72 h. Relative frequencies of positive cells are shown as percentages (mean ± SE, $n = 4$) of mean levels in nontreated controls.

The time course of copper exposure also revealed a significant ($P < 0.05$ versus nontreated controls) increase in the percentage of circulatory hemocytes stained positively with A62 after 8 h (Fig. 3B). The percentage of A62-positive hemocytes continued to increase over the remainder of the experimental period such that, after 72 h, there were approximately 3.5 times more A62-positive hemocytes in copper-exposed tunicates than in nontreated controls.

Immunohistochemistry of pharyngeal tissue. Immunohistochemistry revealed differences in the tissue distribution of A62-positive hemocytes in TBT-exposed and control tunicates. In TBT-exposed animals, large numbers of A62-stained cells were found in close contact with the thinnest epithelial layer of pharyngeal papillae (Fig. 4A). This is the same area where histology revealed an accumulation of the refractile vacuolated cells (see below). Similar accumulations of A62-positive cells were not observed in pharyngeal tissue from nonexposed controls (Fig. 4B).

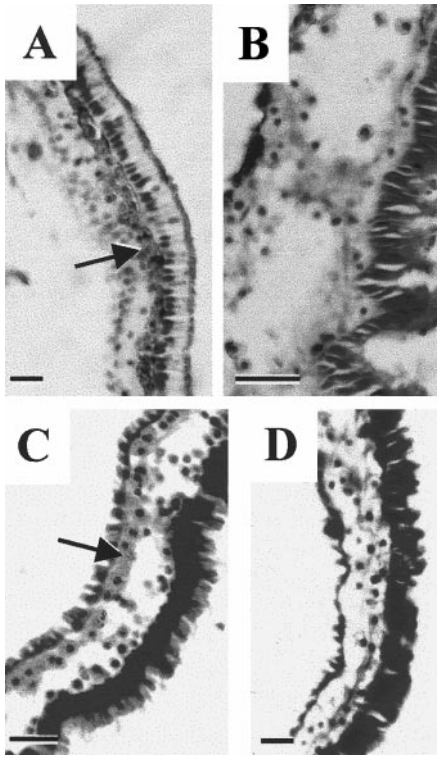

FIG. 4. Localization of (A and B) A62-positive or (C and D) refractile vacuolated cells in the pharyngeal papillae of (B and D) nonexposed controls or (A and C) tunicates exposed to TBT (50 μg/L) for 48 h. Note that the A62-positive and refractile vacuolated cells (arrows) have accumulated in the pharynx of TBT-exposed tunicates adjacent to the thinnest epithelial layer of the pharyngeal papilla. Bars, 20 μm.

Histological analyses. Differential cell counts of circulatory hemocyte preparations revealed that the frequencies of refractile vacuolated cells and nonrefractile vacuolated cells differed significantly ($P < 0.05$) in nonexposed controls and TBT-exposed tunicates after 48 h (Fig. 5). The frequency of refractile vacuolated cells was approximately 1.5 times greater in TBT-exposed animals than in nonexposed controls, whereas the frequency of nonrefractile cells was halved in TBT-exposed tunicates (Fig. 5). None of the other hemocyte types differed significantly in frequency in nonexposed controls and TBT-treated tunicates when tested after 48 h. However, significant variation was evident in the frequencies of fine granular cells and hemoblasts from nontreated controls assays after 0 and 48 h ($P < 0.05$).

Histological analysis of pharyngeal tissue showed that refractile vacuolated cells accumulated in the pharynx of TBT-exposed tunicates, most often in close proximity to the thinnest epithelial layer of pharyngeal papillae (Fig. 4C). Such accumulations were not evident in nontreated controls (Fig. 4D).

Subcellular alterations. A change in vacuole shape was evident in TBT-exposed tunicates compared to controls. The acidophilic refractile vacuoles that were

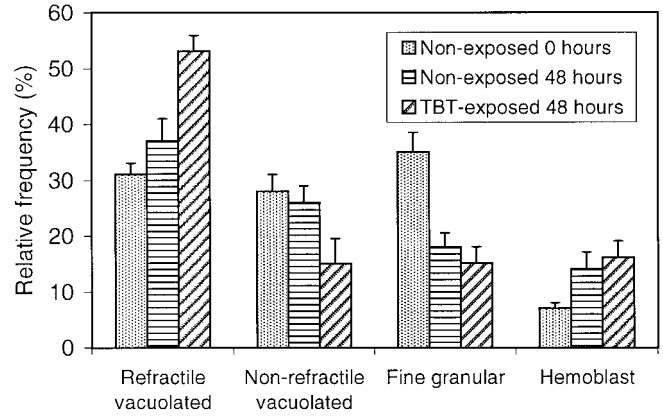

FIG. 5. Relative frequencies of different circulating hemocyte types in TBT-treated (50 μg/L) and control tunicates after 0 and 48 h of exposure. The frequencies of individual cell types are expressed as percentages (mean ± SE, $n = 5$) of the total hemocyte population. This histogram shows only the most common hemocyte types (refractile vacuolated cells, nonrefractile vacuolated cells, fine granular cells, and hemoblasts).

lobulated in control animals (Fig. 6A) became more frequent and developed a crescent shape, rather than being lobulated, in tunicates exposed to TBT (Fig. 6B). Electron microscopy has shown that the vacuoles take up a significantly increased volume within the refractile vacuolated cells after exposure to TBT (data not shown).

DISCUSSION

We have shown that toxic metals can alter the antigen expression, relative frequencies, morphology, and tissue distribution of hemocytes from the tunicate *S. plicata*. Differences between normal (control) tunicates and those exposed to metals were detected by immunofluorescence and immunohistochemistry using MAb A62 and by classical histology.

Significant increases in the relative frequencies of A62-positive hemocytes were evident in the circulating hemolymph of tunicates exposed to both metals. Changes in A62 staining were dose and time depen-

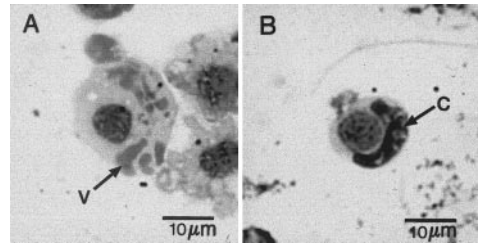

FIG. 6. May-Grunwald Giemsa-stained cytocentrifuge preparations of circulating refractile vacuolated cells from (A) a nonexposed control and (B) a tunicate exposed to TBT (5 μg/L) for 48 h. Note that vacuoles (v) are lobulated or separate in the nonexposed control, but are crescent shaped (c) and curve around the nucleus in the TBT-treated tunicate.

dent, confirming that they were correlated with metal exposure. Significant differences in the frequencies of A62-positive cells could be detected at doses of 5 μg/L TBT and 200 μg/L copper. Whereas these concentrations are extremely high relative to known environmental ranges, atomic absorption spectroscopy confirmed that both copper and TBT rapidly partitioned in aquaria, so that the levels of metal remaining in solution at the beginning of exposures were far lower than the initial doses added to aquaria. Hence, the doses of metals that elicited detectable responses in this study fall within the concentration ranges reported for heavily contaminated estuarine environments. TBT concentrations >2 μg/L were reported before its use was restricted in the late 1980's, and levels >100 ng/L still occur in some locations (Waldcock and Miller, 1983; Michel and Averty, 1999). Similarly, copper concentrations of up to 25 μg/L have been identified in areas suffering from severe industrial pollution (Stauber *et al.*, 2000).

Tissue burden data for TBT and copper accumulation also suggest that the dose regime used here yields tissue concentrations of metals that are comparable to those commonly reported in animals sampled from the wild. Whereas tissue burden data for tunicates is not available in the literature, tissue burdens of up to 5 mg copper/g tissue and 500 ngTBT/g tissue have been reported among other marine filter feeders such as oysters (Elgethun *et al.*, 2000; Han *et al.*, 1994; Harino *et al.*, 2000; Shim *et al.*, 2000; Lin and Hsieh, 1999). These tissue burdens are far in excess of the levels reported here for tunicates exposed to TBT (20 ng/g) and copper (2.2 μg/g) in aquaria.

Histological analysis showed that the increased A62 reactivity evident during TBT treatment corresponded with an increased frequency of refractile vacuolated cells in the circulating hemolymph. The relative frequency of refractile vacuolated cells increased by 43% in TBT-exposed tunicates, apparently at the expense of nonrefractile vacuolated cells, which decreased in frequency by 45% relative to nontreated controls.

The origin of the refractile vacuolated cells that appeared in the circulatory hemolymph during exposure to TBT has not been determined. Either they may have been recruited from another compartment of the hemolymph or they may reflect an altered subcellular structure of existing circulatory hemocytes. It is already known that many tunicate hemocytes, rather than circulating freely, are sequestered in parietal endocarps of the body wall (Goodbody, 1974). The selective release of refractile vacuolated cells from these endocarps could explain their appearance in the circulating hemolymph. However, if existing refractile vacuolated cells were recruited from parietal endocarps during TBT treatment, a proportional decrease in the frequencies of all other hemocyte types would have been expected in the circulating hemolymph. Instead, the increased frequency of refractile vacuolated cells was accompanied only by a decrease in the frequency of nonrefractile vacuolated cells. This indicates that nonrefractile vacuolated cells may have given rise to refractile vacuolated cells, or that hematopoiesis produced more refractile vacuolated cells at the expense of the nonrefractile cell type. Such explanations fit well with previous studies, which have concluded that most tunicate hemocyte types represent different morphological states of a single, plastic cell lineage (Wright, 1981).

This plasticity of cellular structure is also reflected by the morphological changes observed in refractile vacuolated cells after exposure to TBT. The increased frequency of refractile vacuolated cells in response to TBT exposure was accompanied by changes in the shape of vacuoles within these cells. A large proportion of refractile vacuolated cells from TBT-treated tunicates had vacuoles with a distinctive crescent shape that occupied a volume of the cell similar to that of the nucleus. This contrasts the normal, lobulated shape of the vacuoles in hemocytes from nonexposed controls.

Such changes in vacuole morphology may be due to an altered differentiation state of the cells, or they might have resulted from direct toxic effects of TBT on lipid membranes or cytoskeletal structure. Alternatively, the altered size and shape of vacuoles may reflect the ability of refractile vacuolated cells to sequester TBT or its metabolites. Refractile vacuolated cells are known to accumulate vanadium or iron for normal physiological purposes, possibly related to tunic formation or defense (Wright, 1981). Such cellular mechanisms for metal sequestration might also allow the bioaccumulation of TBT absorbed from the environment. Indeed, Cima *et al.* (1996) have shown that embryonic cells from *S. plicata* can sequester TBT in electron-dense granules comparable to those identified in the current study.

If TBT or its metabolites are sequestered in the vacuoles of refractile vacuolated cells, the altered tissue distribution of refractile vacuolated cells during TBT exposure could reflect an inducible mechanism for expelling or sequestering metals. Refractile vacuolated and A62-positive cells amassed along the epithelium comprising the thinnest edge of pharyngeal papillae during TBT exposure. Similar accumulations were not evident in nontreated controls. Papillae are thin projections of the pharyngeal wall, which are used for gas, water, and nutrient exchange (Goodbody, 1974). The anterior surface of papillae is one cell thick and is directly exposed to water being filtered through the pharynx. Hence, it represents an obvious surface for the sequestration or expulsion of toxic metals passing into the tissue.

Alternatively, the refractile vacuolated cells that accumulate in the pharynx during TBT exposure might be involved in repair or inflammatory responses to epithelial damage. Ballarin *et al.* (1995) and Arizza *et*

al. (1995) have shown that refractile vacuolated cells (morula cells) are involved in defense processes. These cells have been observed in necrotic regions of surgically fused incompatible colonies, and cell separation techniques have shown that they contain large amounts of the defensive enzyme phenoloxidase (Smith and Peddie, 1992). Ballarin *et al.* (1995) also found that refractile vacuolated cells are chemotactically attracted to inflammatory sites where they release granules containing phenoloxidase.

We are currently testing whether refractile vacuolated cells are involved in toxic metal sequestration and expulsion by determining the metallic content of vacuoles and by tracing the transmigration of vacuoles through the pharyngeal epithelium.

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