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The chemiluminescence response of bivalve haemocytes: utility in screening for immunomodulators and as a biomarker

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Resistance to infectious diseases in bivalves depends primarily on the vigour and efficacy of haemocyte-dependent antimicrobial defence mechanisms. Like other phagocytes, haemocytes seem to rely on oxygen-independent (lysosomal hydrolases, lysozyme) and oxygen-dependent (reactive oxygen species) mechanisms to destroy ingested microorganisms. The generation of cytotoxic oxyradicals by haemocytes can be precisely quantified by means of a simple chemiluminescence (CL) assay using luminol or other CL probes. Tributyltin (TBT), and other environmental contaminants, at sublethal levels, will produce dose-dependent suppression of CL activity in haemocytes exposed in short-term, *in vitro* assays. Presumably, this suppression would find expression as impaired host defence capability. In fact, TBT has been shown to exacerbate progression and lethality of *Perkinsus marinus* infections in the oyster, *Crassostrea virginica*. This suggests that CL assays on haemocytes exposed *in vitro* to single agents or complex mixtures might be useful in screening for aquatic immunomodulators. Statistically significant alterations in CL responses of haemocytes withdrawn from bivalves exposed to xenobiotics in the laboratory or field are more difficult to identify because of high inter-animal variation; however, the use of haemocyte CL as a biomarker of effect merits further investigation.

Keywords: molluscan immunity, immunotoxicant screening, biomarker of effect, oysters, dermo disease.

Introduction

Immunotoxicological phenomena may in part explain the apparent linkage between chronic exposure of aquatic organisms to environmental contaminants and enhanced susceptibility and/or progression of infectious diseases. Some measures of immunocompetency in bivalves can be precisely quantified and are modulated by xenobiotic exposure. This, in turn, suggests that immunoassays may hold promise as potential biomarkers or as screening assays to identify immunotoxicants.

Reactive oxygen species (ROS) are thought to participate in phagocyte-mediated antimicrobial responses of vertebrate monocyte/macrophages and neutrophils (Klebanoff and Hamon 1972, Klebanoff 1985). A similar role for ROS in bivalve haemocytes seems likely, although direct evidence for this is limited. The level of ROS production by haemocytes can be markedly enhanced by administration of typical activators of mammalian blood phagocytes (Anderson 1994); however, the level of this ROS production is significantly lower in haemocytes than that seen in mammalian cells (Bramble and Anderson 1998). Production of ROS by bivalve haemocytes can be easily measured by the incorporation of chemiluminogenic probes in the medium during cell stimulation. The most commonly used probe is luminol (5´-amino,2,3-dihydro-1,4-phtalazinedione), which is thought to measure predominantly activity of the myeloperoxidase–hydrogen peroxide–halide anti-microbial pathway (Albrecht and Jungi 1993). Bivalve haemocytes are known to

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produce hydrogen peroxide (H_2O_2) upon stimulation (Nakamura *et al*. 1985) and to contain myeloperoxidase (MPO) (Schlenk *et al*. 1991); furthermore, luminol-dependent chemiluminescence (CL) is reduced in haemocytes treated with inhibitors of MPO (Austin and Paynter 1995). There is little doubt that appropriate membrane perturbations produced by phagocytosis or receptor-ligand interactions can stimulate the generation of superoxide anions (O_2^-) by haemocytes, as measured by lucigenin-augmented CL (Bramble and Anderson 1997) or superoxide dismutase (SOD) sensitive reduction of nitroblue tetrazolium (Anderson *et al.* 1992a). Also, inhibition of the enzyme that catalyses O_2^- generation (NADPH oxidase) reduces lucigenin-augmented CL (Bramble and Anderson 1999). Haemocytic H_2O_2 is produced from O_2^- via SOD; H_2O_2 can be converted to even more cytotoxic products, such as HOCl, in the presence of chloride ions and MPO. The production of photons (CL) arising from the interaction of HOCl and other ROS with luminol is directly correlated with cellular antimicrobial activity in mammalian phagocytes (Horan *et al*. 1982). Cellular CL has been recommended for screening for macrophage immunodulators in vertebrate animals (Tam and Hinsdill 1990).

Methods

There are numerous variants of the luminol-augmented CL assay, the following method is used in this laboratory and is representative of this technique. Haemolymph from individual oysters was incubated for 15 min at room temperature (~20 °C) in a 35 mm plastic Petri dish, during which time the majority of the cells became firmly attached. Filter-sterilized ambient estuarine water (FA) from the oyster holding tanks was used to wash the cells, removing the fluid phase of the haemolymph and any unadhered haemocytes. The resultant cell monolayer was then overlaid with FA and incubated for 2.5 h at room temperature. During this period of time, the cells became progressively more loosely attached and could be finally washed off the glass and resuspended in FA. These cells retained phagocytic activity, but showed little of the clumping typically encountered during centrifugation and resuspension of freshly collected haemocytes. The cells were removed from suspension by centrifugation (200 *g*, 15 min, 20 °C), resuspended in cell support medium (CSM), and counted in a haemacytometer. The composition of CSM was 0.5% antibiotic solution (10 000 U penicillin, 10 mg streptomycin, and 25 µg amphotericin B per ml), 5% foetal calf serum, and 1 mg ml^{-1} glucose in FA. Defined media such as Hanks balanced salt solution may be substituted for FA, and the foetal calf serum can be omitted. These changes may be beneficial when carrying out immunotoxicological studies using metals or other xenobiotics in the medium. The haemocyte concentration was determined and 10^6 cells in 2.275 ml CSM were placed in each of a series of small plastic liquid scintillation vials. Luminol was prepared by the method of Scott and Klesius (1981), 25 µl of this solution was mixed with the cell suspensions (10 µM final concentration), and the vials were placed in a scintillation counter programmed for single photon monitoring. The vials were counted at room temperature (~20 °C) at ~30 min to establish the level of unstimulated, background CL for each sample. During this time, the apparent background CL activity generally decreased and reached a stable level that was taken to be the 'true' level of spontaneous activity. The initial high level of activity sometimes seen probably resulted from the haemocytes being physically agitated during their introduction into the vials, and from ROS activity associated with adhesion and spreading on the plastic substrate. Finally, a suspension of heat-killed, washed zymosan particles (0.2 ml of 10.0 mg zymosan per ml CSM) was added to each vial. The haemocytes rapidly phagocytosed large numbers of these particles, which were present in the medium in numbers exceeding the phagocytic potential of the haemocytes. The vials were counted at room temperature (~20 °C) at ~0.3 min intervals for ~2 h after the addition of zymosan. The background CL level was defined as the counts per minute (cpm) recorded immediately before the addition of the phagocytic stimulus. The peak CL was the maximal CL response elicited by the phagocytic event, corrected for background CL activity. The total CL response was expressed as the area under the phagocytically induced portion of the CL curve, corrected for background activity, and estimated by a polygon summation method.

Results and discussion

The toxic effects of TBT on locomotion, spreading, and CL of oyster haemocytes was first studied by Fisher *et al*. (1990). Clearly, *in vitro* exposure of

Table 1. Inhibition of zymosan-stimulated total CL activity by *Crassostrea virginica* haemocuytes after *in vitro* exposure to tributyltin.

Nominal TBT concentration (ppb)	Measured TBT concentration (ppb)	Percent inhibition of total CL expressed as x ± SD(n)	
		1 h exposure	20 h exposure
50	13	–2.5 ± 14.2 (4)	1.0 ± 13.0 (6)
100	38	7.0 ± 12.3 (5)	7.0 ± 12.2 (8)
250	84	36.6 ± 20.4 (5)**	61.9 ± 24.7 (7)***
500	170	67.3 ± 16.3 (5)***	82.5 ± 14.5 (9)***
1000	420	91.4 ± 8.5 (5)***	

Significance between controls and treatment groups was analysed by the Bonferroni multiple comparison test, if ANOVA $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

C. virginica haemocytes to TBT produces a dose-dependent suppression of phagocytosis-stimulated, luminol-augmented CL (table 1). The TBT concentrations used were generally not cytolethal after 1 h or 20 h exposure (85–90% of the haemocytes remained viable, based on trypan blue exclusion assays), only 1000 ppb for 20 h produced significant cell mortality, accounting for the omission of data for this group in table 1. Concentrations of ≥ 250 ppb TBT were shown to significantly inhibit CL. The concentrations required to produce 50% CL inhibition were calculated by log probability analysis to be: 1 h–IC_{50} ≅ 340 ppb, and 20 h–IC_{50} ≅ 240 ppb TBT (nominal), actual (measured by gas chromatography) IC_{50} estimates were about 116 and 82 ppb TBT, respectively (Anderson *et al.* 1997). If TBT-exposed haemocytes were carefully washed and placed in TBT-free medium, they partially recovered their ability to respond to CL stimulators. Other studies have also shown that exposure of molluscan haemocytes to sublethal concentrations of environmental contaminants such as metals, organometals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated phenols, etc. often result in decreased ability to produce ROS, as measured by reduced CL responses (Larson *et al.* 1989, Anderson *et al.* 1992b, 1994, Roszell and Anderson 1992, Baier-Anderson and Anderson 1977). These data suggest that xenobiotic exposure can cause impairment of oxygen-dependent cellular defence mechanisms, this may result in reduced resistance to infection.

Several investigators have measured changes in *P. marinus* infection levels and dermo-related mortality produced by exposure to chemicals such as diethylnitrosamine (Winstead and Couch 1988), PAH- and metal-containing sediments (Chu and Hale 1994), and TBT (Fisher *et al*, 1995, Anderson *et al.* 1996, 1998). The synergy between xenobiotic exposure and reduced resistance to *P. marinus* infection was seen in oysters with naturally-occurring infections, as well as those infected experimentally. Apparently other stressors, such as hypoxia, will serve to exacerbate the effects of chemicals on resistance to infectious disease. For example, Anderson *et al.* (1998) followed the progression of dermo disease in four groups of naturally-infected oysters. Group one was exposed to ~ 100 ng l^{-1} TBT, group two was exposed to ~100 ng l^{-1} TBT and hypoxic conditions (~3 ng dissolved oxygen l^{-1}), group three was exposed to similar hypoxic conditions, and a control group received no experimental treatments. All groups comprised 50 oysters and were maintained at 15 ppt and 25 °C for the 6-week course of the study. The level of infection increased for all groups, based on numbers of *P. marinus* in the

Table 2. Effects of hypoxia and/or tributyltin exposure on the percent cumulative mortality of *Crassostrea virginica* infected in the field with *Perkinsus marinus*.

	Percent cumulative mortality					
	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
TBT	7	7	12	22	25	29
TBT/hypoxia	16	29	38	41	55	58
Hypoxia	8	19	27	31	41	46
Control	2	15	16	17	26	33

haemolymph; the percent cumulative mortality also increased with time (table 2). Statistical analysis of these data was carried out using Fisher's exact test. Significant mortality differences between the controls and TBT-treated oysters were not seen; differences between these groups were probably obscured because both control and experimental oysters started the study with moderate levels of infection. Mortality was higher in the hypoxic group than in the controls after 4 weeks ($P < 0.05$). However, oysters in the TBT-exposed plus hypoxia group consistently had a higher mortality that the controls ($P < 0.05$ on week 1, $P < 0.001$ on subsequent sampling dates).

Therefore, in the case of TBT, it appears that a compound which can be shown *in vitro* to suppress a putative defence mechanism (the production of ROS, as shown by reduced CL), will also enhance infections and lethality of a parasitic infection in the oyster. However, we have found it difficult to demonstrate significant TBT-associated ROS modulation in haemocytes withdrawn from oysters receiving TBT exposures shown to enhance dermo disease; this has been attempted using several methods including CL (Anderson *et al*. 1996) and nitroblue tetrazolium reduction (Anderson *et al*. 1998). This suggests that the course of *P. marinus* infections may be influenced by TBT-sensitive defence mechanisms unrelated to oxygen-dependent defence mechanisms. In fact, it is known that phagocytosis of *P. marinus* cells by oyster haemocytes sensitizes the cells to ROS production when stimulated by other particles (Anderson *et al*. 1995), but fails to produce any net CL (LaPeyre *et al*. 1995, Volety and Chu 1995). However, the lack of CL modulation in haemocytes from oysters exposed to TBT levels adequate to enhance *P. marinus* infections does not imply that CL suppression cannot be seen in cells from bivalves chronically exposed to other environmental contaminants. For example, CL and other defence-related mechanisms are suppressed in haemocytes of oysters exposed to sediments amended with selected PAHs commonly present in contaminated areas of the Chesapeake Bay (F.-L. Chu, personal communication).

In conclusion, it is likely that measuring ROS production by bivalve haemocytes via CL activity is a potentially useful *in vitro* method to screen for immunomodulatory environmental chemicals. Inhibition of ROS activity is an immunological lesion which probably causes decreased host resistance to infectious disease via reduced antimicrobial potential. It seems unlikely that this response will be useful in indicating exposure to specific pollutants or classes of pollutants; it probably functions as a more general indicator of anthropogenic stress. It is likely that the CL response is affected by salinity, DO, temperature, and other natural environmental variables, these variables must be adequately addressed when

designing experimental controls. Preliminary evidence indicates that CL activity may find application as part of a suite of bivalve biomarkers of effect.

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