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Immunomodulatory effects of brevetoxin (PbTx-3) upon *in vitro* exposure in bottlenose dolphins (*Tursiops truncatus*)

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ABSTRACT

Harmful algal blooms (HAB), commonly referred to as 'red tides', involving the dinoflagellate *Karenia brevis* produce a series of neurotoxins known as brevetoxins (PbTx). Brevetoxins have long been associated with extensive fish kills, adverse human health effects such as neurotoxic shellfish poisoning, and have been associated with mortality events in aquatic mammals such as bottlenose dolphins (*Tursiops truncatus*). The immunotoxicological effects of these brevetoxins have been studied in manatees, humans, and cell lines, however, the effects in bottlenose dolphins remain unclear. There are increasing concerns that dolphins may be exposed to repeated/chronic, sub-lethal concentrations, which may impact their overall health. The objectives of this study were to measure the changes in innate (phagocytosis, respiratory burst, NK cell activity) and adaptive (mitogen-induced B and T lymphocyte proliferation) immune functions upon *in vitro* exposure to increasing concentrations of brevetoxin (PbTx-3) (0, 0.01, 0.1, 1, 10, 100, 500, and 1000 nM) using bottlenose dolphin peripheral blood immune cells. Brevetoxin significantly increased spontaneous lymphocyte proliferation at 0.1–1000 nM compared to the unexposed control. Brevetoxin significantly increased T lymphocyte proliferation upon suboptimal (0.1 μg/ml) and optimal (1.0 μg/ml) Con-A stimulation at 0.01–100 nM and 0.1 nM of PbTx-3 respectively, as well as suboptimal (0.05 μg/ml) and optimal (5.0 μg/ml) LPS-induced B lymphocyte proliferation at 0.01–100 nM and 0.01–500 nM of PbTx-3, respectively. Both neutrophil and monocyte respiratory burst were significantly increased at 500 and 1000 nM. There were no significant effects on neutrophil or monocyte phagocytosis or NK cell activity. Importantly, concentrations that modulated immune functions *in vitro* were within the range measured in the blood of dolphins during two unusual mortality events, suggesting that naturally exposed dolphins may be at risk for immunomodulation. Brevetoxin-induced immunomodulation may increase an animal's susceptibility to bacterial, viral, or fungal infections. Understanding the risk for immunomodulation upon HAB toxin exposure can contribute in the health assessment and management of marine mammals, as well as guide veterinarians and wildlife rehabilitators in caring for and treating afflicted animals.

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1. Introduction

Harmful algal blooms (HAB), commonly referred to as 'red tides,' produce biotoxins with deleterious health effects in marine organisms. Over the last several decades, the frequency and global distribution of HAB have increased (Van Dolah, 2000). Brevetoxins (PbTx) produced by the dinoflagellate *Karenia brevis* represent a significant health threat to humans, marine mammals, sea turtles, fish, and sea birds (Fleming et al., 2005; Van Dolah et al., 2003; Kreuder et al., 2002; Naar et al., 2007; Landsberg, 2002). They have been implicated in the death of large numbers of fish (Ingersoll, 1881; Davis, 1948; Steidinger et al., 1998), and associated with morbidity and mortality in marine mammals (Layne, 1965; Geraci et al., 1989; Bossart et al., 1998; Flewelling et al., 2005). Bottlenose dolphins (*Tursiops truncatus*) can be exposed to PbTx either by ingestion of contaminated food or by inhalation of aerosolized toxin (Woofter et al., 2005; Landsberg and Steindinger, 1998). Furthermore, exposure to PbTx is not always acutely lethal in exposed animals and the sub-lethal, chronic effects of PbTx are of increasing concern (Van Dolah, 2000; Flewelling et al., 2005). Due

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to the persistence of PbTxs in the environment (Naar et al., 2007; Landsberg et al., 2009), dolphins may be exposed to repeated/chronic, sub-lethal concentrations, which may be impacting their overall health.

Brevetoxins are a suite of lipophilic, polyether neurotoxins that are active *in vivo* in the nanomolar to picomolar concentrations (Baden, 1989). They specifically bind to cell membranes and activate voltage gated sodium channels (VGSC), leading to an influx of sodium into the cell (Fleming et al., 2005; Baden, 1989). These VGSCs have been described on immune cells such as lymphocytes, neutrophils, and natural killer (NK) cells, suggesting that brevetoxin may interact with immune cells and modulate their functions (Roselli et al., 2006).

Brevetoxin exposure was also associated with several unusual mortality events (UMEs) along the coast of Florida where hundreds of bottlenose dolphins have died. Three bottlenose dolphin unusual mortality events (UMEs) have been recorded in the Florida panhandle region between 1999 and 2006 (Twiner et al., 2012). From August 1999 to May 2000, 152 dolphins died following extensive *Karenia brevis* blooms (NOAA, 2004). In the spring of 2004, there were 105 bottlenose dolphin mortalities in the absence of a detectable *K. brevis* bloom, but stomach contents from stranded dolphins, as well as their prey items showed high levels of PbTx (Flewelling et al., 2005; NOAA, 2004). From September 2005 though April 2006, 90 bottlenose dolphins died, with high levels of *K. brevis* (ranging between 1.3×10^6 cells/L and 3.5×10^6 cells/L) detected in the water during the first few months, which then dissipated (Twiner et al., 2012). Although by the spring of 2006 *K. brevis* was not detected in the water (Naar et al., 2007), the number of strandings remained elevated with moderate levels of PbTx detected in dolphin tissues (Fire et al., 2007). Investigations of these UMEs indicated finfish were not necessarily killed by *K. brevis*, and they have the potential to accumulate and serve as a vector of PbTxs to higher trophic levels, such as bottlenose dolphins (Flewelling et al., 2005; Landsberg et al., 2009). Therefore, it is possible that these dolphins may be exposed to chronic, relatively low dose concentrations of PbTx from unrecognized events.

This study measured the effects of PbTx-3 on innate and adaptive immune functions of bottlenose dolphin leukocytes following *in vitro* exposure. Innate immune functions including phagocytosis, respiratory burst and NK cell activity, that help remove and destroy microorganisms, whereas adaptive immune functions, including B and T cell proliferation leading to effector and memory B and T lymphocytes, to specifically remove pathogens and prevent re-infection. Brevetoxin induced immunomodulation in bottlenose dolphins may increase their susceptibility to bacterial, viral, or fungal infections. Understanding this risk will contribute in the health assessment and potential treatment of afflicted animals.

2. Materials and methods

2.1. Brevetoxin

Brevetoxin-3 (PbTx-3) in solid form was purchased from EMD Biosciences, Inc. (San Diego, CA), reconstituted in sterile water, aliquoted and frozen in glass vials at −20 °C as per manufacturer's instructions. Prior to each assay, PbTx-3 was re-suspended in complete DMEM for phagocytosis, NK cell activity, and proliferation assays, or phosphate buffered saline with glucose (1 g/L; Sigma; referred to as PBS-G) for respiratory burst.

Final concentrations of PbTx-3 were tested at 0 nM (unexposed control), 0.01, 0.1, 1.0, 10, 100, 500, and 1000 nM, in all experiments. These concentrations have been documented to induce changes in immune cells compared to that of an unexposed control in manatees and Jurkat cells (Walsh et al., 2008; Sayer et al., 2005; Murrell and Gibson, 2009). Brevetoxin-3 was chosen for these studies because it is a major component of the brevetoxin mixture produced by *Karenia brevis* (Van Dolah, 2000; Landsberg, 2002; Baden, 1989).

2.2. Animals and blood collection

Whole blood from bottlenose dolphins (*Tursiops truncatus*) was opportunistically collected from healthy captive adult animals housed by the US Navy Marine Mammal Program as assessed by their attending veterinarian. All blood samples were collected into sodium heparin tubes, kept cool, and shipped to our laboratory for processing within 24 h. Both male and female dolphins were sampled in order to represent a whole population rather than a subpopulation within the species.

2.3. Isolation of leukocytes

Blood leukocytes were isolated from whole blood prior to evaluation of phagocytosis and respiratory burst as previously reported (De Guise et al., 1995; Levin et al., 2004, 2007). Erythrocytes were lysed with NH_4Cl, and re-suspended in Hanks balanced salt solution (HBSS, Gibco BRL, Grand Island, NY). Cells were washed twice in HBSS and viability was assessed using the exclusion dye trypan blue.

2.4. Isolation of peripheral blood mononuclear cells (PBMCs)

Whole blood was mixed 1:1 with PBS/2 mM sodium (Tetra) ethylenediamine tetraacetate (EDTA; Fisher Scientific, Pittsburg, PA), layered on top of equal volume of Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ) and centrifuged at $600 \times g$ for 30 min. PBMCs were re-suspended in Dulbecco's modified eagle medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with (all from Gibco BRL, Grand Island, NY) 1 mM sodium pyruvate, 100 μM non-essential amino acids, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, along with 10% fetal bovine serum (Hyclone, Logan, UT), thereafter referred to as complete DMEM. Cells were washed twice in complete DMEM, and viability assessed using the exclusion dye trypan blue.

2.5. Phagocytosis

Phagocytosis was evaluated by methods previously described (Levin et al., 2004). Briefly, leukocytes were adjusted to 2×10^6 cells/ml in HBSS, followed by incubation at 37 °C with 5% CO_2 with PbTx-3 for 3 h in 96-well round bottom plates (Falcon, Becton Dickinson, Lincoln Park, NJ) in triplicate. One micrometer-diameter fluorescent latex beads (Molecular Probes, Eugene, OR) were added to the cell suspension at a ratio of approximately 100:1 and incubated for an additional hour at 37 °C under agitation at 300 rpm using a Thermomixer R (Eppendorf, Hamburg, Germany).

Cell-associated fluorescence was evaluated using a FACScan (Becton Dickinson, Rutherford, NJ) flow cytometer with the CellQuest software (Becton Dickinson Immunocytometry System, San Jose, CA). Neutrophils and monocytes were gated electronically according to their relative size (forward scatter, FSC) and complexity (side scatter, SCC). The fluorescence of the cells was read at 530 nm (FL-1) on a logarithmic scale using the fluorescence of free beads as reference. Cells acquire a fluorescence equal to the number of beads they ingested. Phagocytosis was evaluated as the proportion of neutrophils or monocytes that have phagocytized one or more beads.

2.6. Respiratory burst

Respiratory burst was evaluated as previously described (De Guise et al., 1995; Levin et al., 2007). Briefly, isolated leukocytes

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