

# IMMUNOMODULATORY EFFECTS UPON IN VITRO EXPOSURE OF CALIFORNIA SEA LION AND SOUTHERN SEA OTTER PERIPHERAL BLOOD LEUKOCYTES TO DOMOIC ACID

Milton Levin,<sup>1,5</sup> Dhanashree Joshi,<sup>1</sup> Andrew Draghi II,<sup>1,4</sup> Frances M. Gulland,<sup>2</sup> David Jessup,<sup>3</sup> and Sylvain De Guise<sup>1</sup>

<sup>1</sup> Department of Pathobiology and Veterinary Science, University of Connecticut, 61 North, Eagleville Road, U-3089, Storrs, Connecticut 06269, USA

<sup>2</sup> The Marine Mammal Center, 1065 Fort Cronkite, Sausalito, California 94965, USA

<sup>3</sup> California Department of Fish and Game, Marine Wildlife Veterinary Care and Research Center, 1451 Shaffer Road, Santa Cruz, California 95060, USA

<sup>4</sup> Current address: Division of Gastroenterology and Nutrition, Connecticut Children's Medical Center, 282 Washington Street, Hartford, Connecticut 06106, USA

<sup>5</sup> Corresponding author (email: milton.levin@uconn.edu)

**ABSTRACT:** During red tide bloom events, the marine diatom *Pseudo-nitzschia* produces the toxin domoic acid (DA), which has been associated with stranding and mortality events involving California sea lions (*Zalophus californianus*) and southern sea otters (*Enhydra lutris*). In addition to these well-documented DA-induced neurotoxic events, there is increasing concern that DA may exert chronic effects, such as immunomodulation, which may potentially increase an individual's susceptibility to a number of opportunistic infections following nonlethal exposure. We investigated the effects of DA on innate (phagocytosis and respiratory burst) and adaptive (mitogen-induced lymphocyte proliferation) immune functions with the use of peripheral blood leukocytes collected from healthy California sea lions and southern sea otters upon in vitro exposure to 0 (unexposed control), 0.0001, 0.001, 0.01, 0.1, 1.0, 10, and 100  $\mu\text{M}$  DA. Domoic acid did not significantly modulate phagocytosis or respiratory burst in either species. For California sea lions, DA significantly increased ConA-induced T-lymphocyte proliferation upon exposure to DA concentrations ranging from 0.0001 to 10  $\mu\text{M}$ , resulting in a nonlinear dose-response curve. There was no effect on lymphocyte proliferation at the highest concentration of DA tested. No effects on lymphocyte proliferation were observed in southern sea otters. Importantly, the in vitro DA concentrations affecting T-cell proliferation were within or below the range of DA in serum measured in free-ranging California sea lions following natural exposure, suggesting a risk for immunomodulation in free-ranging animals. Understanding the risk for immunomodulation upon DA exposure will contribute in the health assessment and management of California sea lions and southern sea otters, as well as guide veterinarians and wildlife rehabilitators in caring for and treating afflicted animals.

**Key words:** California sea lion, domoic acid, immunomodulation, southern sea otter.

## INTRODUCTION

Over the last several decades, the increased frequency and global distribution of harmful algal blooms (HAB; commonly referred to as red tides) has been suggested to be linked to human activity, such as increased pollution runoff into aquatic ecosystems or global warming (Van Dolah, 2000). Certain species of the marine diatom *Pseudo-nitzschia* produce the neurotoxin domoic acid (DA), which biomagnifies in the food web and can adversely affect marine mammal species (Scholin et al., 2000; Gulland et al., 2002; Lefebvre et al., 2002; Kreuder et al., 2003)

Exposure to DA has been associated with stranding and mortality events involving California sea lions (*Zalophus californianus*) and southern sea otters (*Enhydra lutris*). Along the central California coast during 1998 and 2000, over 400 California sea lions died and many others showed signs of neurotoxicity (Lefebvre et al., 1999; Scholin et al., 2000; Gulland et al., 2002). The event was associated with the ingestion of prey, including northern anchovy, which fed on the DA toxin-producing *Pseudo-nitzschia australis*, which bloomed in the Monterey Bay region during the same period (Scholin et al., 2000). About half of

the stranded sea lions died despite supportive treatment. Because these acute mortality events associated with DA exposure were first recognized in marine mammals, more subtle long-term neurological and behavioral effects have been identified, presumably resulting from low-dose, sublethal exposure (Goldstein et al., 2008). Changes in hematologic parameters also have been documented in sea lions exposed to DA, but the pathogenesis and significance to health and survival are unclear (Gulland et al., 2002).

Southern sea otters were listed as threatened by the US Fish and Wildlife Service (USFWS) in 1977. Despite this protection, population growth has been lower than for other sea otter populations. DA was implicated in the deaths of sea otters during an unusual mortality event (UME) in California in 2003 (Jessup et al., 2007). The temporal and spatial pattern of southern sea otter exposure to DA is similar to that of California sea lion strandings due to DA toxicosis. DA intoxication was a primary cause of death in 4 out of 105 otters examined from February 1998 to July 2001 (Kreuder et al., 2003). Additional adverse health effects associated with DA exposure in southern sea otters include myocarditis and dilated cardiomyopathy (Kreuder et al., 2005). Domoic acid-exposed sea otters in the southern aspect of their range were 55 times more likely to have died with myocarditis than unexposed sea otters.

Domoic acid-contaminated prey, especially bivalves, can also put humans at risk. Domoic acid is the causative agent in amnesic shellfish poisoning (ASP) of humans, with symptoms including vomiting, diarrhea, dizziness, seizures, and permanent loss of short-term memory (Jeffery et al., 2004). In 1987, >100 people became ill and 4 died after eating mussels contaminated with DA from waters off Prince Edward Island, Canada (Perl et al., 1990).

Although the acute manifestations of

DA neurotoxicity are relatively well understood (Berman et al., 2002; Jakobsen et al., 2002; Qiu and Curras-Collazo, 2006), there is increasing concern that DA may exert sublethal, chronic effects, such as immunomodulation, following individual and repeated DA bloom events. The immunomodulatory potential of DA in mice was recently demonstrated for the first time in our laboratory (Levin et al., 2008). Sublethal DA-induced immunomodulation may be a contributing factor in an animal's susceptibility to opportunistic infections. The two leading causes of death in southern sea otters are acanthocephalan and *Toxoplasma gondii* infections (Kreuder et al., 2003). Compromised immune functions following DA exposure would have the potential of contributing to the sea otter's susceptibility to these or other pathogens, as has been reported in mice undergoing chemical-induced immunomodulation (Luster et al., 1993). Contaminant-induced immunosuppression by organochlorines, including polychlorinated biphenyls (PCBs), has been suggested as one cofactor in the morbilliviral-attributable deaths of tens of thousands of marine mammals (Ross et al., 1996; Van Loveren et al., 2000).

We explored the *in vitro* effects of DA on peripheral blood leukocyte immune functions in two marine mammals to elucidate the sublethal effects of DA. Assays to measure immunotoxicity included leukocyte phagocytosis and respiratory burst, as well as mitogen-induced lymphocyte proliferation upon *in vitro* DA exposure. These functional immune assays were validated by and are part of the National Toxicology Program to predict the immunotoxicity of chemicals (Luster et al., 1992, 1993). This study provides the foundation to determine the immunomodulatory potential of DA (the hazard identification and dose-assessment steps in risk assessment) and elucidate the mechanisms and pathways involved in the response to toxic levels of DA in marine mammals.

## MATERIALS AND METHODS

### Animals and blood sampling

Blood samples from captive southern sea otters were collected from the California Department of Fish and Game, Marine Wildlife Veterinary Care and Research Center, Santa Cruz, California, USA, and the Monterey Bay Aquarium, Monterey, California, USA. Captive sea otters (all males) were previously wild animals that had been declared nonreleasable by the USFWS and held under appropriate permits. All animals were clinically healthy based on a veterinary examination and complete blood count and serum chemistry panel values were within normal ranges. Blood samples from California sea lions were collected from wild-stranded animals housed at The Marine Mammal Center, Sausalito, California for rehabilitation. All animals were subadult or adult, of both sexes, and clinically healthy based on clinical examination, hematology, and serum chemistry. Blood samples for our studies were collected into sodium heparin tubes (Vacutainer, Becton Dickinson, Franklin Lakes, New Jersey, USA), kept on ice packs in the dark, shipped overnight, and processed within 24 hr of collection.

### Isolation of leukocytes

Isolation of leukocytes was performed prior to the evaluation of immune functions. For phagocytosis and respiratory burst, erythrocytes were lysed with the use of  $\text{NH}_4\text{Cl}$  and the leukocytes were resuspended in Hanks balanced salt solution (HBSS, Gibco BRL, Grand Island, New York, USA). Cells were washed twice with HBSS, and their viability was assessed using the exclusion dye trypan blue. Viability was typically greater than 90%.

For lymphocyte proliferation, whole blood was centrifuged for 20 min at  $220 \times G$ , and the buffy coat was collected and resuspended into Dulbecco's modified eagle medium (DMEM, Gibco BRL) supplemented with 1 mM sodium pyruvate, 100  $\mu\text{M}$  nonessential amino acids, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin (all from Gibco BRL), along with 10% fetal bovine serum (Hyclone, Logan, Utah, USA), hereafter referred to as complete DMEM. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Paque plus (Amersham Biosciences, Uppsala, Sweden) for 35 min at  $990 \times G$ . The PBMCs were collected, washed once, and enumerated, with their viability assessed with the use of the

exclusion dye trypan blue. Viability was typically greater than 90%.

### Domoic acid

Domoic acid (Sigma, St Louis, Missouri, USA) was resuspended in complete DMEM for phagocytosis and proliferation assays or phosphate-buffered saline with glucose (1 g/l; Sigma; referred to as PBS-G) for the respiratory burst. The final concentrations of DA were 0 (unexposed control), 0.0001, 0.001, 0.01, 0.1, 1.0, 10, and 100  $\mu\text{M}$ . These concentrations represent environmentally relevant exposure, as they are within the range measured in serum of naturally exposed California sea lions (Scholin et al., 2000; Goldstein et al., 2008).

### Phagocytosis

Phagocytosis was evaluated *in vitro* as previously described (Levin et al., 2005). Briefly, the leukocyte concentration was adjusted to  $2 \times 10^6/\text{ml}$  in HBSS, followed by incubation at 37 C with 5%  $\text{CO}_2$  with DA for 3 hr in round-bottomed 96-well plates (Falcon, Becton Dickinson) in triplicate. Fluorescent latex beads measuring 1  $\mu\text{m}$  diameter (Molecular Probes, Eugene, Oregon, USA) were added to the cell suspension to obtain a ratio of approximately 100 beads/cell, and cells were incubated for 1 additional hour at 37 C, under agitation at 300 rpm, with the use of a Thermomixer R (Eppendorf, Hamburg, Germany). The cell suspension from each well was then layered on a cushion of ice cold 3% bovine serum albumin (Sigma) and centrifuged at  $150 \times G$  for 8 min at 4 C. The supernatant containing the free beads was discarded and the cells were resuspended in 200  $\mu\text{l}$  of phosphate-buffered saline (PBS, Gibco) containing 1% neutral buffered formalin (Decal Corp, Tallman, New York, USA). Cells were stored at 4 C until analysis (within 24 hr).

### Respiratory burst

The respiratory burst was evaluated *in vitro* as previously described (Levin et al., 2007a). Briefly, the leukocyte concentration was adjusted to  $2 \times 10^6/\text{ml}$  in HBSS. Cell suspensions were incubated for 30 min at 37 C, in the dark, with 5  $\mu\text{M}$  of 2,7-dichlorofluorescein diacetate (DCFDA, Molecular Probe, Eugene, Oregon, USA), a probe used to quantify the production of  $\text{H}_2\text{O}_2$ . The cell suspension was centrifuged for 10 min at  $220 \times G$  and resuspended in PBS-G. For each individual animal, leukocytes were plated in triplicate with DA in each of

two round-bottomed 96-well plates (Falcon, Becton Dickinson). Phorbol myristate acetate (PMA, Molecular Probe), a cell activator, was added at  $10^{-9}$  M to the first plate and PBS-G (control) was added to the second plate. Both plates were incubated for 1 hr at 37 C. After incubation, cells were fixed with 1% neutral buffered formalin and stored at 4 C until analysis (within 24 hr).

#### Flow cytometry

The fluorescence of approximately 5,000 (respiratory burst) and 10,000 (phagocytosis) cells was read with a FACScan (Becton Dickinson) flow cytometer with the use of the CellQuest software (Becton Dickinson Immunocytometry System, San Jose, California, USA). Neutrophils were gated electronically according to their relative size (forward scatter [FSC]) and complexity (side scatter [SCC]). For phagocytosis, the fluorescence of the cells was read at 530 nm (FL-1) on a logarithmic scale, with the fluorescence of free beads used as reference. Cells acquired fluorescence equal to that of the number of beads they ingested. Phagocytosis was reported as the percentage of cells that had phagocytized one or more beads (1+, the proportion of all cells that participate in phagocytosis), the endpoint of phagocytosis routinely reported (Burleson et al., 1995; Brousseau et al., 1999, 2000). The respiratory burst was evaluated as the mean fluorescence at 530 nm (FL-1) of the PMA stimulated cells and reported as the ratio (stimulation index) of the mean fluorescence of PMA stimulated cells divided by the mean fluorescence of the unstimulated cells.

#### Lymphocyte proliferation

Mitogen-induced lymphocyte proliferation was evaluated in vitro as previously described (Mori et al., 2006). PBMCs in complete DMEM were plated ( $2 \times 10^5$  cells/well) in 96-well flat-bottomed tissue culture plates (Falcon, Becton Dickinson), along with DA, in triplicate. Cells were incubated at 37 C with 5% CO<sub>2</sub> for 66 hr with the T-cell mitogen, concanavalin A (Con A, Sigma). Con A was used at the optimal and suboptimal concentration of 1.0 µg/ml and 0.10 µg/ml, respectively. Suboptimal concentrations were used, as they previously proved more sensitive in detecting immunotoxicity (Mori et al., 2006). Lymphocyte proliferation was evaluated as the incorporation of 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue, added for the last 18 hr of incubation, and further detected with a monoclonal antibody and a colorimetric

enzymatic reaction (Cell Proliferation ELISA BrdU [colorimetric]), Roche Diagnostics GmbH, Mannheim, Germany) as per manufacturer's instructions with the use of an ELISA plate reader (Multiskan EX v.1.0) at 450 nm with a reference wavelength of 690 nm. Proliferation was expressed as a percentage of the unexposed control (0 µM).

#### Statistics

A repeated-measures one-way analysis of variance (RM ANOVA) was performed to compare the exposed groups to the unexposed group using  $P < 0.05$  for statistical significance. Differences among animals were handled through the use of RM ANOVA tests, which allow for the detection of consistent effects of a series of experimental interventions (in vitro exposure to various concentrations of DA) in different individuals despite differences in baseline values between animals. Examining the changes rather than the values observed before and after interventions removes the differences due to individual responses, producing a more sensitive (or more powerful) test. All analyses were performed using the SigmaStat 3.5.1 (Systat, San Jose, California, USA) software. Data were expressed as a percentage (%) of the unexposed control (0 µMDA).

## RESULTS

#### Effects of DA on phagocytosis and respiratory burst

Domoic acid did not significantly modulate California sea lion and southern sea otter neutrophil phagocytosis or neutrophils respiratory burst (data not shown).

#### Effects of DA on lymphocyte proliferation

Domoic acid did not significantly modulate southern sea otter ConA-induced T-lymphocyte proliferation. However, DA significantly increased California sea lion ConA-induced T lymphocyte proliferation at 1 and 10 µM DA with suboptimal and from 0.0001 to 1 µM DA with optimal concentrations of ConA (Fig. 1). The maximum change was greater at a lower concentration with the optimal concentration of ConA (0.01 µM) compared to the suboptimal concentration (1 µM). The magnitude of the change was up to 120% of control with suboptimal concen-

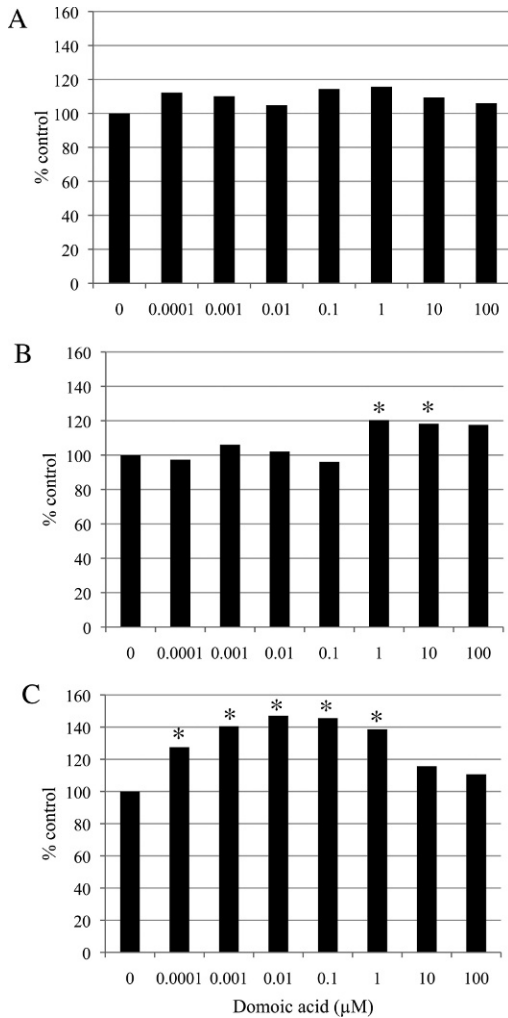


FIGURE 1. California sea lion T-lymphocyte proliferation upon increasing concentrations of domoic acid with no mitogen (A;  $n=9$ ), suboptimal ConA (B;  $n=7$ ; Sub ConA), and optimal ConA (C;  $n=8$ ; Opt ConA). Data are expressed as a percent of the unexposed ( $0 \mu\text{M}$ ). \* $P<0.05$  compared to unexposed ( $0 \mu\text{M}$ ) for the same mitogen.

trations of ConA, and up to 147% of control with optimal concentrations of ConA. The relationship between concentration and effect was quantified for the window of concentrations that had significant effects using optimal concentrations of ConA (Fig. 2). The relationship was best resolved with a quadratic equation with  $R^2=0.99$ .

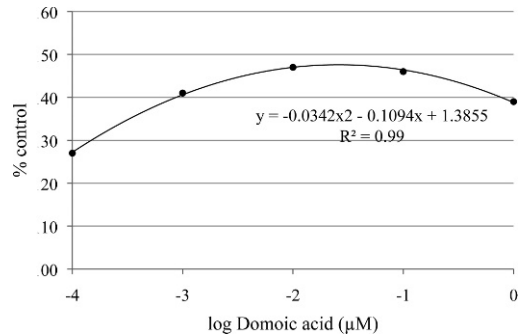


FIGURE 2. California sea lion dose-response curve for optimal ConA-induced T-lymphocyte proliferation with range of domoic acid concentrations that were significantly different ( $P<0.05$ ) from the unexposed control ( $0 \mu\text{M}$ ; see Fig. 1). Data are expressed as a percent of the unexposed ( $0 \mu\text{M}$ ).

## DISCUSSION

This is the first report to document the immunomodulatory effects upon in vitro exposure to DA in isolated California sea lion PBMCs. No effects on either innate or adaptive immune functions were documented in southern sea otter cells. However, DA may modulate other immune functions not evaluated in the present study, such as natural killer (NK) cell activity or B cell proliferation. The lack of published accounts of DA concentration in the blood of southern sea otters would complicate future risk assessment for immunomodulation should other effects be documented upon in vitro exposure.

For California sea lions, DA did not modulate the two innate immune functions tested. However, T-lymphocyte proliferation, an adaptive immune function, was significantly modulated. Unstimulated T lymphocyte proliferation was unaffected upon exposure to DA. However, T lymphocytes that were stimulated with the optimal mitogen concentration were sensitive to the effects of DA, even at lower concentrations of DA. In other words, lymphocytes in a maximum state of stimulation were most at risk for the toxic effects of DA. Nevertheless, T lymphocytes that were weakly stimulated need

higher concentrations of DA to induce detectable modulatory effects in our study. In either situation, a sea lion's immune response to a "real" antigen may be influenced by its DA exposure, with chronic, low-level DA exposure a potential threat to the health of an individual.

The dose-response curve (Fig. 2) demonstrated an inverted U-shape response when stimulated with ConA at an optimal concentration. Such nonlinear dose-response curves have been demonstrated for a variety of environmental chemicals when lymphocyte proliferation is the immune endpoint measured (Calabrese, 2005). Although the lowest concentration to modulate T-cell proliferation was not determined in this study, it appears that 10  $\mu$  M represents the highest concentration at which significant effects on proliferation would occur. The high  $R^2$  value for the portion of the dose-response curve (Fig. 2) for which there were significant effects suggest interpolation may be appropriate within the range of concentrations. Over the entire range, the highest concentration was not necessarily immunomodulatory.

Further work is needed to elucidate the mechanism(s) and pathway(s) involved in DA-induced immunotoxicity, which may help elucidate the nature of the dose-response curve observed in Figure 2. Domoic acid-induced neurotoxicity is mediated through glutamate cell surface receptors, including kainate, alpha amino-5-methyl-3-hydroxyisoxazolone-4-propionate (AMPA), and N-methyl-D-aspartate (NMDA) receptors (Jakobsen et al., 2002). Glutamate receptors on California sea lion lymphocytes may help explain DA-induced immunotoxicity, as similar receptors have been shown to be present on human and rodent immune cells (Ganor et al., 2003; Boldyrev et al., 2004, 2005; Miglio et al., 2005). Differential receptor expression and signaling through these receptors may help explain the initial increase in the magnitude of proliferation at low concentrations of DA followed by a

reduction from the highest magnitude of proliferation at higher DA concentrations. For example, differential expression of metabotropic glutamate receptor subtypes mediated the opposite effects of glutamate, for which DA is an analog, on human lymphocyte proliferation (Pacheco et al., 2004).

The DA concentrations tested in vitro that induced significant changes in T lymphocyte proliferation (Fig. 1) were within and below those measured in the serum of 13 naturally exposed California sea lions (ranging from 0.01 to 0.642  $\mu$ M). The concentrations of DA in the serum of two sea lions during a toxic diatom bloom along the central California coast in 1998 (Scholin et al., 2000) were approximately 0.540 and 0.640  $\mu$ M. Serum concentrations of DA were measured in animals suffering acute DA toxicosis and animals suffering from chronic consequences of previous sublethal exposure (Goldstein et al., 2008). Ten animals suffering acute toxicity had serum concentrations ranging approximately 0.013–0.642  $\mu$ M, whereas one animal suffering chronic effects had a concentration of approximately 0.01  $\mu$ M. Immunomodulation for California sea lion cells upon in vitro exposure was found at concentrations 5,000 times lower than those found in the blood of naturally exposed animals upon acute exposure resulting in clinical signs, and 100 times lower than for animals suffering from chronic exposure (Goldstein et al., 2008). However, measurement of DA levels in wild sea lions is limited by detection limits of the tests used for routine screening of animal fluids (Brodie et al., 2006), and by the time between sampling animals and ingestion of DA, as DA is rapidly cleared from the blood stream (99% cleared within 4 hr of administration to primates; Truelove and Iverson, 1994).

During red tide events, morbidity and mortality have resulted from the well-documented DA-induced neurotoxic effects. However, DA-induced immunomodulation may adversely affect those

individuals who survive the initial toxic insult. The data in this report suggest that naturally exposed California sea lions may be at risk for the sublethal immunomodulatory effects upon DA exposure. Modulation of immune functions may increase the risk for opportunistic infections, autoimmunity, or neoplasia (Kuby, 1997; Germolec, 2004; Vas and Monestier, 2008). In testing over 50 chemical compounds, a good correlation was documented in mice between chemical-induced changes in the immune tests and altered host resistance in that there were no instances where host resistance was altered without affecting immune test results (Luster et al., 1993). More specifically, the statistically significant relationship between changes in T-cell proliferation and increased sensitivity to two infectious diseases and one tumor challenge model was linear, with susceptibility increasing with more profound effects on T cells (Luster et al., 1993).

The increase in T-lymphocyte proliferation upon DA exposure may decrease host resistance in the following manner. T-cell activation occurs by contact with antigen displayed by an antigen-presenting cell (APC). However, this interaction, by itself, is not sufficient to activate naive T lymphocytes fully. Naive T cells require two distinct signals for activation, proliferation, and subsequent differentiation into effector and memory cells. Signal one is generated by interaction of an antigenic peptide with the T-cell receptor-CD3 complex, and signal two is delivered by an antigen-nonspecific costimulatory molecule provided primarily by interactions between CD28 on the T cell and members of the B7 family on the APC (Greenfield et al., 1998). The presence of signal one without signal two leads to a tolerance mechanism in which the lymphocyte enters an active state of unresponsiveness or anergy following an antigen encounter, a situation in which the cells remain alive for an extended period of time but remain unable to mount a

functional immune response (Schwartz, 2003). A continuous low-grade stimulation by DA could mimic signal one, in the absence of signal two, and lead to anergy, which would prevent an appropriate response to pathogens.

Chemical-induced increase in T-cell proliferation may also increase the risk for autoimmunity. For example, animal models have demonstrated that environmental agents, such as mercury, can lead to immune activation (specifically T cells) and loss of self-tolerance, resulting in autoimmunity (Vas and Monestier, 2008).

Nonspecific and unregulated increases in lymphocyte proliferation may be the initiating step in the transformation of a lymphocyte into a cancer cell (Kuby, 1997). Interestingly, California sea lions have a high prevalence of spontaneous neoplasia (Gulland et al., 1996). Glutamate receptor antagonists are important in the suppression of some tumors (Rzeski et al., 2002; Stepulak et al., 2007), and DA increases chromosomal abnormalities in the Caco-2 cell line (Carvalho et al., 2006), suggesting a potential role of DA in tumor formation. Future work evaluating DA-induced immunomodulation should consider these complex immune processes, especially in view of potential continuous and low-level DA exposure.

The first demonstration of DA-induced immunomodulation in any mammal species was recently described (Levin et al., 2008). Institute for Cancer Research (ICR) mouse innate and adaptive immune functions were significantly modulated upon *in vitro* DA exposure, with the use of assays similar to those used in our study. In an effort to validate the use of the mouse model to predict the immunomodulatory effects of DA in other mammalian species, the immunomodulatory effects upon *in vitro* exposure were compared among mice, California sea lions, and southern sea otters for similar assays (phagocytosis and T-cell proliferation) and DA concentrations (0, 1, 10, and 100  $\mu\text{M}$ ) used in both studies (Table 1).

TABLE 1. The immunomodulatory effects on phagocytosis by neutrophils and T-lymphocyte proliferation upon in vitro domoic acid exposure ( $\mu\text{M}$ ) in mice, California sea lions, and southern sea otters. Results are reported as the concentrations that had statistically significant immunomodulatory effects upon in vitro exposure compared to the unexposed control (0  $\mu\text{M}$ ).

	Phagocytosis neutrophils	Proliferation spontaneous	Proliferation suboptimal ConA	Proliferation optimal ConA
Mouse <sup>a</sup>	1 $\mu\text{M}$	1, 100 $\mu\text{M}$	1, 10, 100 $\mu\text{M}$	No effect
California sea lion	No effect	No effect	1, 10 $\mu\text{M}$	1 $\mu\text{M}$
Southern sea otter	No effect	No effect	Not done	No effect

<sup>a</sup> Levin et al., 2005

For phagocytosis and spontaneous proliferation, effects were observed only in the mouse model. For these data, the mouse model overestimated the risk for immunomodulation in the two marine mammals. For proliferation with suboptimal ConA, the mouse correctly predicted the effects in California sea lions at the two lower concentrations, but not at the highest concentration, where the effects would have overestimated the risk to sea lions (in which no effects were detected). Proliferation with the use of suboptimal ConA was not done for southern sea otters. For proliferation with optimal ConA, no effects were detected in mice; however, significant effects were observed in sea lions at 1  $\mu\text{M}$ . For these data, the mouse model would have underestimated the risk in California sea lions, but would have correctly predicted the effects in sea otters. Overall, the commonly used mouse model did not always correctly predict the immunomodulatory effects of DA in other mammalian species. These results were not surprising, as the mouse model also failed to predict consistently and correctly the immunotoxicity upon in vitro exposure to PCBs in humans, and several marine mammal species (Levin et al., 2005; Mori et al., 2006; Levin et al., 2007a, 2007b; Mori et al., 2008).

In conclusion, future DA blooms are likely to occur, putting marine mammals at risk for both acute sublethal and lethal exposure. Understanding the risk for

immunomodulation upon DA exposure will contribute in the health assessment and management of southern sea otters and California sea lions, as well as guide veterinarians and wildlife rehabilitators in caring for and treating afflicted animals.

#### ACKNOWLEDGMENTS

This work was supported by a grant from the Morris Animal Foundation. We are grateful to the Marine Mammal Center, California Department of Fish and Game, and the Monterey Aquarium for providing the marine mammal blood samples used as a source of immune cells in those experiments.

#### LITERATURE CITED

- BERMAN, F. W., K. T. LEPAGE, AND T. F. MURRAY. 2002. Domoic acid neurotoxicity in cultured cerebellar granule neurons is controlled preferentially by the NMDA receptor  $\text{Ca}^{2+}$  influx pathway. *Brain Research* 924: 20–29.
- BOLDYREV, A. A., V. I. KAZEY, T. A. LEINSOO, A. P. MASHKINA, O. V. TYULINA, P. JOHNSON, J. O. TUNEVA, S. CHITTUR, AND D. O. CARPENTER. 2004. Rodent lymphocytes express functionally active glutamate receptors. *Biochemical and Biophysical Research Communications* 324: 133–139.
- , D. O. CARPENTER, AND P. JOHNSON. 2005. Emerging evidence for a similar role of glutamate receptors in the nervous and immune systems. *Journal of Neurochemistry* 95: 913–918.
- BRODIE, E. C., F. M. D. GULLAND, D. J. GREIG, M. HUNTER, J. JAAKOLA, J. ST. LEGER, T. A. LEIGHFIELD, AND F. M. VAN DOLAH. 2006. Domoic acid causes reproductive failure in California sea lions (*Zalophus californianus*). *Marine Mammal Science* 22: 700–707.
- BROUSSEAU, P., Y. PAYETTE, H. TRYPHONAS, B. BLAKLEY, H. BOERMANS, D. FLIPO, AND M. FOURNIER. 1999. *Manual of immunological*



- methods. CRC Press, Boca Raton, Florida, 141 pp.
- , J. PELLERIN, Y. MORIN, D. CYR, B. BLAKLEY, H. BOERMANS, AND M. FOURNIER. 2000. Flow cytometry as a tool to monitor the disturbance of phagocytosis in the clam *Mya arenaria* hemocytes following in vitro exposure to heavy metals. *Toxicology* 142: 145–156.
- BURLESON, G. R., J. H. DEAN, AND A. E. MUNSON (EDS.). 1995. *Methods in immunotoxicology*. Wiley-Liss, Inc., New York, New York, 456 pp.
- CALABRESE, E. 2005. Hormetic dose-response relationships in immunology: Occurrence, quantitative features of the dose response, mechanistic foundations, and clinical implications. *Critical Reviews in Toxicology* 35: 89–295.
- CARVALHO, P. S., R. CATIAN, S. MOUKHA, W. G. MATIAS, AND E. E. CREPPY. 2006. Comparative study of domoic acid and okadaic acid induced-chromosomal abnormalities in the Caco-2 cell line. *International Journal of Environmental Research and Public Health* 3: 4–10.
- GANOR, Y., M. BESSER, N. BEN-ZAKAY, T. UNGER, AND M. LEVITE. 2003. Human T cells express a functional ionotropic glutamate receptor GluR3, and glutamate by itself triggers integrin-mediated adhesion to laminin and fibronectin and chemotactic migration. *Journal of Immunology* 170: 4362–4372.
- GERMOLEC, D. R. 2004. Sensitivity and predictivity in immunotoxicity testing: Immune endpoints and disease resistance. *Toxicology Letters* 149: 109–114.
- GOLDSTEIN, T., J. A. MAZET, T. S. ZABKA, G. LANGLOIS, K. M. COLEGROVE, M. SILVER, S. BARGU, F. VAN DOLAH, T. LEIGHFIELD, P. A. CONRAD, J. BARAKOS, D. C. WILLIAMS, S. DENNISON, M. HAULENA, AND F. M. GULLAND. 2008. Novel symptomatology and changing epidemiology of domoic acid toxicosis in California sea lions (*Zalophus californianus*): An increasing risk to marine mammal health. *Proceedings of the Royal Society B* 275: 267–276.
- GREENFIELD, E. A., K. A. NGUYEN, AND V. K. KUCHROO. 1998. CD28/B7 costimulation: A review. *Critical Reviews in Immunology* 18: 389–418.
- GULLAND, F. M., J. G. TRUPKIEWICZ, T. R. SPRAKER, AND L. J. LOWENSTINE. 1996. Metastatic carcinoma of probable transitional cell origin in 66 free-living California sea lions (*Zalophus californianus*), 1979 to 1994. *Journal of Wildlife Diseases* 32: 250–258.
- , M. HAULENA, D. FAUQUIER, G. LANGLOIS, M. E. LANDER, T. ZABKA, AND R. DUERR. 2002. Domoic acid toxicity in Californian sea lions (*Zalophus californianus*): Clinical signs, treatment and survival. *Veterinary Record* 150: 475–480.
- JAKOBSEN, B., A. TASKER, AND J. ZIMMER. 2002. Domoic acid neurotoxicity in hippocampal slice cultures. *Amino Acids* 23: 37–44.
- JEFFERY, B., T. BARLOW, K. MOIZER, S. PAUL, AND C. BOYLE. 2004. Amnesic shellfish poison. *Food and Chemical Toxicology* 42: 545–557.
- JESSUP, D. A., M. A. MILLER, C. KREUDER-JOHNSON, P. A. CONRAD, M. T. TINKER, J. ESTES, AND J. A. MAZET. 2007. Sea otters in a dirty ocean. *Journal of the American Veterinary Medical Association* 231: 1648–1652.
- KREUDER, C., M. A. MILLER, D. A. JESSUP, L. J. LOWENSTINE, M. D. HARRIS, J. A. AMES, T. E. CARPENTER, P. A. CONRAD, AND J. A. MAZET. 2003. Patterns of mortality in southern sea otters (*Enhydra lutris nereis*) from 1998–2001. *Journal of Wildlife Diseases* 39: 495–509.
- , ———, L. J. LOWENSTINE, P. A. CONRAD, T. E. CARPENTER, D. A. JESSUP, AND J. A. MAZET. 2005. Evaluation of cardiac lesions and risk factors associated with myocarditis and dilated cardiomyopathy in southern sea otters (*Enhydra lutris nereis*). *American Journal of Veterinary Research* 66: 289–299.
- KUBY, J. 1997. *Immunology*. 3rd edition. W. H. Freeman and Company, New York, New York, 313 pp.
- LEFEBVRE, K. A., C. L. POWELL, M. BUSMAN, G. J. DOUCETTE, P. D. MOELLER, J. B. SILVER, P. E. MILLER, M. P. HUGHES, S. SINGARAM, M. W. SILVER, AND R. S. TJEERDEMA. 1999. Detection of domoic acid in northern anchovies and California sea lions associated with an unusual mortality event. *Natural Toxins* 7: 85–92.
- , S. BARGU, T. KIECKHEFER, AND M. W. SILVER. 2002. From sanddabs to blue whales: The pervasiveness of domoic acid. *Toxicon* 40: 971–977.
- LEVIN, M., B. MORSEY, C. MORI, P. R. NAMBIAR, AND S. DE GUISE. 2005. PCBs and TCDD, alone and in mixtures, modulate marine mammal but not B6C3F1 mouse leukocyte phagocytosis. *Journal of Toxicology and Environmental Health A* 68: 635–656.
- , ———, AND S. DE GUISE. 2007a. Modulation of the respiratory burst by organochlorine mixtures in marine mammals, humans, and mice. *Journal of Toxicology and Environmental Health A* 70: 73–83.
- , ———, AND ———. 2007b. Non-coplanar PCBs induce calcium mobilization in bottlenose dolphin and beluga whale, but not in mouse leukocytes. *Journal of Toxicology and Environmental Health A* 70: 1220–1231.
- , H. LEIBRECHT, J. RYAN, F. VAN DOLAH, AND S. DE GUISE. 2008. Immunomodulatory effects of domoic acid differ between in vivo and in vitro exposure in mice. *Marine Drugs* 6: 636–659.
- LUSTER, M. I., C. PORTIER, D. G. PAIT, K. L. WHITE, JR., C. GENNINGS, A. E. MUNSON, AND G. J. ROSENTHAL. 1992. Risk assessment in immuno-

- toxicology. I. Sensitivity and predictability of immune tests. *Fundamental and Applied Toxicology* 18: 200–210.
- , ———, ———, G. J. ROSENTHAL, D. R. GERMOLEC, E. CORSINI, B. L. BLAYLOCK, P. POLLOCK, Y. KOUCHI, W. CRAIG, K. L. WHITE, A. E. MUNSON, AND C. E. COMMENT. 1993. Risk assessment in immunotoxicology. II. Relationships between immune and host resistance tests. *Fundamental and Applied Toxicology* 21: 71–82.
- MIGLIO, G., F. VARSALDI, AND G. LOMBARDI. 2005. Human T lymphocytes express N-methyl-D-aspartate receptors functionally active in controlling T cell activation. *Biochemical and Biophysical Research Communications* 338: 1875–1883.
- MORI, C., B. MORSEY, M. LEVIN, P. R. NAMBIAR, AND S. DE GUISE. 2006. Immunomodulatory effects of in vitro exposure to organochlorines on T-cell proliferation in marine mammals and mice. *Journal of Toxicology and Environmental Health A* 69: 283–302.
- , ———, ———, T. S. GORTON, AND S. DE GUISE. 2008. Effects of organochlorines, individually and in mixtures, on B-cell proliferation in marine mammals and mice. *Journal of Toxicology and Environmental Health A* 71: 266–275.
- PACHECO, R., F. CIRUELA, V. CASADO, J. MALLOL, T. GALLART, C. LLUIS, AND R. FRANCO. 2004. Group I metabotropic glutamate receptors mediate a dual role of glutamate in T cell activation. *Journal of Biological Chemistry* 279: 33352–33358.
- PERL, T. M., L. BEDARD, T. KOSATSKY, J. C. HOCKIN, E. C. TODD, AND R. S. REMIS. 1990. An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *New England Journal of Medicine* 322: 1775–1780.
- QIU, S., AND M. C. CURRAS-COLLAZO. 2006. Histopathological and molecular changes produced by hippocampal microinjection of domoic acid. *Neurotoxicology and Teratology* 28: 354–362.
- ROSS, P. S., R. L. DE SWART, H. VAN LOVEREN, AND A. D. M. E. OSTERHAUS. 1996. The immunotoxicity of environmental contaminants to marine wildlife: A review. *Annual Review of Fish Diseases* 6: 151–165.
- RZESKI, W., C. IKONOMIDOU, AND L. TURSKEI. 2002. Glutamate antagonists limit tumor growth. *Biochemical Pharmacology* 64: 1195–1200.
- SCHOLIN, C. A., F. GULLAND, G. J. DOUCETTE, S. BENSON, M. BUSMAN, F. P. CHAVEZ, J. CORDARO, R. DELONG, A. DE VOGELAERE, J. HARVEY, M. HAULENA, K. LEFEBVRE, T. LIPSCOMB, S. LOSCUTOFF, L. J. LOWENSTINE, R. MARIN, 3RD, P. E. MILLER, W. A. McLELLAN, P. D. MOELLER, C. L. POWELL, T. ROWLES, P. SILVAGNI, M. SILVER, T. SPRAKER, V. TRAINER, AND F. M. VAN DOLAH. 2000. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature* 403: 80–84.
- SCHWARTZ, R. H. 2003. T cell anergy. *Annual Review of Immunology* 21: 305–334.
- STEPULAK, A., M. SIFRINGER, W. RZESKI, K. BROCKE, A. GRATOPP, E. E. POHL, L. TURSKEI, AND C. IKONOMIDOU. 2007. AMPA antagonists inhibit the extracellular signal regulated kinase pathway and suppress lung cancer growth. *Cancer Biology and Therapy* 6: 1908–1915.
- TRUELOVE, J., AND F. IVERSON. 1994. Serum domoic acid clearance and clinical observations in the cynomolgus monkey and Sprague-Dawley rat following a single i.v. dose. *Bulletin of Environmental Contamination and Toxicology* 52: 479–486.
- VAN DOLAH, F. M. 2000. Marine algal toxins: Origins, health effects, and their increased occurrence. *Environmental Health Perspectives* 108 (Suppl 1): 133–141.
- VAN LOVEREN, H., P. S. ROSS, A. D. OSTERHAUS, AND J. G. VOS. 2000. Contaminant-induced immunosuppression and mass mortalities among harbor seals. *Toxicology Letters* 112–113: 319–324.
- VAS, J., AND M. MONESTIER. 2008. Immunology of mercury. *Annals of the New York Academy of Sciences* 1143: 240–267.

*Submitted for publication 15 June 2009.*