

Proliferative Responses of Harbor Seal (*Phoca vitulina*) T Lymphocytes to Model Marine Pollutants

JENNIFER C.C. NEALE^{a,*}, JUDITH A. VAN DE WATER^b, JAMES T. HARVEY^c, RONALD S. TJEERDEMA^a and M. ERIC GERSHWIN^b

^aDepartment of Environmental Toxicology, University of California, Davis, One Shields Avenue, Davis, CA, 95616, USA; ^bDivision of Rheumatology, Allergy and Clinical Immunology, TB 192, One Shields Avenue, Davis, CA, 95616, USA; ^cMoss Landing Marine Laboratories, California State University, 8272 Moss Landing Road, Moss Landing, CA, 95039, USA

In recent years, population declines related to viral outbreaks in marine mammals have been associated with polluted coastal waters and high tissue concentrations of certain persistent, lipophilic contaminants. Such observations suggest a contributing role of contaminant-induced suppression of cell-mediated immunity leading to decreased host resistance. Here, we assessed the effects of the prototypic polycyclic aromatic hydrocarbon (PAH), benzo[a]pyrene (B[a]P), and two polychlorinated biphenyls (PCBs), CB-156 and CB-80, on the T-cell proliferative response to mitogen in harbor seal peripheral lymphocytes. Despite the variability associated with our samples from free-ranging harbor seals, we observed a clear suppressive effect of B[a]P (10 uM) exposure on T cell mitogenesis. Exposures to 10 uM CB-156 and CB-80, and 1.0 and 0.1 uM B[a]P, did not produce significant depression in lymphoproliferation. Exposure to the model PAH at 10 uM resulted in a 61% (range 34–97%) average reduction in lymphoproliferation. We were able to rule out a direct cytotoxic effect of B[a]P, indicating that observed effects were due to altered T cell function. Based on our *in vitro* results, we hypothesize that extensive accumulation of PAH by top-trophic-level marine mammals could alter T cell activation *in vivo* and impaired cell-mediated immunity against viral pathogens.

Keywords: Benzo[a]pyrene; Harbor seal; Immunotoxicity; Lymphoproliferation; PAH; PCB

INTRODUCTION

Understanding immune alterations related to environmental contamination has become an important priority in marine mammal research because of the high incidence in recent years of mass mortality events, unexplained population declines, and strandings among marine mammal populations (Dietz *et al.*, 1989; Marine Mammal Commission, 1999; Kennedy *et al.*, 2000). These events have been largely attributed to disease outbreaks caused by established or newly identified members of the genus *Morbillivirus* (Geraci *et al.*, 1982; Osterhaus and Vedder, 1988; Duignan *et al.*, 1995). Common features of these massive die-offs include highly polluted habitat and high tissue concentrations in affected individuals of persistent, lipophilic contaminants, including those known to have deleterious effects on the immune systems of rodents and humans (Hall *et al.*, 1992; Visser *et al.*, 1993; Reijnders, 1994). Whereas contaminant-induced immune suppression has been suggested as a major contributing factor in marine mammal disease epizootics, causal relationships and potential mechanisms of immunotoxicity have not been

established (Olsson *et al.*, 1994; Marine Mammal Commission, 1999; van Loveren *et al.*, 2000).

The harbor seal (*Phoca vitulina*) is an ideal model organism for investigating contaminant-induced immune alterations in marine mammals. Fish-eating seals are long-lived, maintain large adipose deposits, and occupy high trophic levels in the marine food chain, thus accumulating relatively high levels of lipophilic contaminants (Boon *et al.*, 1987; Young *et al.*, 1998; Neale, unpublished data). Among marine mammals, harbor seals experience relatively high exposures to such chemicals, as they are non-migratory and frequent coastal habitats characterized by industrial centers, heavy marine traffic, and urban and agricultural runoff. Accordingly, Pacific harbor seals have been identified as one of four key indicators for coastal marine habitats by the California Department of Fish and Game. As an integrator of contamination of nearshore marine environments, the harbor seal also serves as a model for humans in communities dependent on consumption of seafood from polluted local waters.

Among the lipophilic chemicals that accumulate in marine mammal tissues are polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls

*Corresponding author. Tel.: +1-530-752-2534/3286. Fax: +1-530-752-3394. E-mail: jcneale@ucdavis.edu

(PCBs). PAHs constitute a diverse class of ubiquitous industrial and environmental pollutants. Sources include forest fires and fossil fuel combustions, decay of organic materials, volcanic eruptions, and crude oil spills. PCBs are widespread anthropogenic pollutants that were manufactured in the U.S. until 1977 for use in electrical equipment. Decades after the ban on manufacture of these compounds, PCBs remain priority contaminants due to their great environmental persistence. Because PCBs and PAHs are bioconcentrated through the food chain, food sources represent the greatest exposure route for these top trophic level predators.

In this pilot study, we used a model PAH and two PCBs in an *in vitro* system to address the link between exposure to marine pollutants and lymphocyte function relevant to host resistance in the harbor seal. The cell-mediated arm of acquired immunity is most relevant to the mammalian immune response to viral pathogens. The proliferation of T cells in response to mitogens is one measure of T-cell function that is used in Tier I immunotoxicity tests *in vitro* and is frequently evaluated because of its correlation with *in vivo* host resistance (Luster *et al.*, 1988). This assay is considered a robust measure of the T-cell-mediated immune response to viral pathogens and of immunocompetence in general. We assessed the effects of the prototypic PAH, benzo[a]pyrene (B[a]P), a compound having both widespread occurrence in the environment and known immunotoxic effects in laboratory animals, on the T-cell proliferative response to mitogen of harbor seal peripheral lymphocytes (Ladics *et al.*, 1992; White *et al.*, 1994). For comparative purposes, two PCB congeners also were evaluated in this assay—CB-156 (2,3,3',4,4',5-hexachlorobiphenyl), a moderately chlorinated, mono-ortho PCB congener often present in biological samples, and CB-80 (3,3',5,5'-tetrachlorobiphenyl), a less chlorinated, non-ortho congener.

MATERIALS AND METHODS

Samples

Seals were captured via land-grab and beach seine net techniques at haul-out sites in north-central California between May 2001 and February 2002 (Yochem *et al.*, 1987; Jeffries *et al.*, 1993; NMFS Scientific Research Permit No. 555–1565). Individuals were physically restrained and sex, mass, standard length (SL), and estimated age were recorded. A condition index (ratio of mass to SL) was calculated for each individual (Kopec and Harvey, 1995). Blood was drawn from the extradural venous sinus into sterile evacuated blood collection tubes containing acid citrate dextrose (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA).

Cell Preparation and Exposures

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of blood for 20 min at 300g. Buffy coats

were diluted 1:2 in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA). Mononuclear cells were further purified by density gradient centrifugation using 5 ml Histopaque-1077 (Sigma-Aldrich, St Louis, MO, USA) per 8.5 ml whole blood. Gradients were centrifuged for 30 min at 700g. The resulting white blood cell layer was removed and washed twice in Hank's Balanced Salt Solution (JRH Biosciences, Lenexa, KS, USA) with 10 min centrifugations at 200g. Cell viability was assessed via Trypan Blue solution (Sigma-Aldrich, St Louis, MO, USA) exclusion and viable cells were counted microscopically using a hemacytometer. Cell pellets were suspended in RPMI 1640 medium supplemented with L-glutamine (2 mM), gentamicin (0.08%), and fetal bovine serum (10%; hereafter referred to as "media").

B[a]P (Sigma-Aldrich, St Louis, MO, USA), and CB-156 and CB-80 (AccuStandard, New Haven, CT, USA) were suspended in anhydrous dimethylsulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA) which also served as the vehicle control. Treatment solutions were diluted with media immediately before addition to wells. Final exposure concentrations in wells were 0.1, 1.0, and 10 μ M for B[a]P and 10 μ M for the vehicle and PCBs. A media control also was included.

Mitogenesis

The proliferative response to mitogen by T lymphocytes was assessed using a standard blastogenesis assay (DiMolfetto-Landon *et al.*, 1995; Davila *et al.*, 1996). PBMC were cultured in 96-well microtiter plates (Corning, Incorporated, Corning, NY, USA) containing approximately 2×10^5 cells/well in a total volume of 0.2 ml. Cell cultures were incubated without mitogen or in the presence of phytohemagglutinin (Sigma-Aldrich, St Louis, MO, USA; 20 μ g/ml, determined optimal concentration), a T-cell-specific mitogen in harbor seals (de Swart *et al.*, 1993). Cultures were incubated at 37°C in 5% CO₂ for 57 h before the addition of ³H-thymidine (spec. act. 6.7 Ci/mmol; PerkinElmer Life Sciences, Boston, MA), which was added at 1 μ Ci/well in 0.02 ml RPMI 1640. Following 15 additional hours of incubation, for a total incubation time of 72 h, cells were harvested onto filter paper using a Tomtech cell harvester (PerkinElmer, Boston, MA, USA). Filters were oven-dried and placed into bags with 11 ml of scintillation cocktail. Counts per minute (CPM) were determined by a Betaplate scintillation counter (PerkinElmer, Boston, MA, USA). Incorporation of radio-labeled thymidine represented DNA synthesis and cellular proliferation. All treatments and controls were performed in duplicate for mitogen-stimulated and unstimulated cultures for each individual. Background blanks were subtracted from each sample CPM and measurements from duplicate wells were averaged before analysis. A stimulation index (SI) was calculated as the ratio of CPM in mitogen-stimulated cultures to that in unstimulated cultures. The reduction (%) in lymphoproliferation for B[a]P-treated cultures

relative to media controls was calculated as: $(1 - (B[a]P\ SI / \text{media SI})) \times 100\%$.

Cytotoxicity

Because the objective was to identify impacts of the model PAH and PCB toxic agents on cellular functioning, it was important to rule out the possibility of direct cytotoxicity. If B[a]P or PCBs had cytotoxic effects on T lymphocytes, we would expect to see reduced cell viability. To test for this effect, we examined viability of cells exposed to model agents vs. vehicle after 48-h incubation using duplicate lymphocyte cultures from 8 seals. Cell viability was assessed as described above.

Data Analysis

Stimulation indices were log-transformed before all statistical tests, to meet assumptions of normality. To compare proliferative responses between cells treated with vehicle versus B[a]P/PCBs among seals, it was necessary to control for individual differences in baseline proliferation (indicated by media SI), which varied among seals and affected the extent of proliferative response when treated (and therefore detection of a suppressive effect of the model compound). Moreover, if B[a]P/PCBs but not the vehicle suppressed proliferation, we would expect to see an interaction between treatment effects and baseline proliferation due to systematically larger differences between the two treatments associated with higher baseline proliferation. We used a General Linear Model with “treatment” (i.e. 0.1, 1.0, and 10 uM B[a]P, 10 uM CB-156, 10 uM CB-80, and vehicle) SI as the factor and media control SI as a covariate. The treatment \times media interaction term was used to indicate differences between the two treatments

(i.e. differences between treatment vs. baseline slopes; test of homogeneity of regression (Tabachnick and Fidell, 1989)). The value of the slopes indicated the degree of suppression, with slope = 1 indicating no difference in proliferation between baseline and treated, and slope < 1 indicating a suppressive effect. Two-tailed *t*-tests were used to compare SI of males vs. females for agent-treated and vehicle-treated cultures. A two-tailed, paired samples *t*-test was used to compare the number of viable cells in agent-treated vs. vehicle-treated cultures. All statistical analyses were performed using SYSTAT (version 9, SPSS, Inc.) with an alpha of 0.05 for statistical significance.

RESULTS

Blood samples were collected from 19 seals. Sample cultures not showing stimulation in response to mitogen (*n* = 2 seals) were excluded. The remaining 17 samples included 7 from San Francisco Bay, CA, collected 7/15/01–7/16/01, 5 from Elkhorn Slough (Monterey Bay, CA) collected on 5/18/01, and 5 from Pebble Beach (Monterey Bay, CA) collected on 5/16/01 and 2/7/02 (Table I). All animals appeared healthy at time of capture. Cells taken from all 17 individuals were used in the 10 uM B[a]P and CB-156 experiments. Due to limited sample volumes, however, only 9 seals were included in the experiments using CB-80 and lower concentrations (1.0 and 0.1 uM) of B[a]P.

A general trend of depression of lymphoproliferation was evident in all treatments relative to the vehicle control (Fig. 1), however the General Linear Model procedure failed to reveal significant effects of the two PCBs and lower concentrations of B[a]P (all *p* > 0.7). Therefore, the remainder of this discussion will focus on the experiment using 10 uM B[a]P.

TABLE I Sample characteristics for 17 seals, baseline (media controls) stimulation indices (SI), and reduction in proliferation (relative to baseline) due to exposure to the model PAH (10 uM B[a]P). Samples arranged in order of increasing baseline SI

Capture site*	Sex	Age [†]	Mass (kg)	SL [‡] (cm)	Media SI	Reduction (%)
ES	F	P	25	87	1.1	No reduction
ES	M	P	23	94	2.5	No reduction
ES	M	P	29	94	2.6	No reduction
Bay	M	S	37	111	3.2	No reduction
PB	M	P	27	86	3.3	56.0
Bay	F	S	37	123	6.0	43.5
Bay	F	P	20	89	6.4	34.1
Bay	F	A	50	122	6.5	56.3
Bay	F	S	44	117	8.3	37.8
Bay	M	P	26	96	9.3	55.7
ES	M	S	63	133	15.0	54.0
Bay	F	S	42	117	16.5	44.2
ES	M	S	58	131	22.1	60.5
PB	F	P	21	89	26.3	89.0
PB	M	P	27	90	29.8	73.7
PB	F	P	21	80	30.2	92.0
PB	F	Y	30	102	32.2	96.9

* Capture site: SFB, San Francisco Bay; ES, Elkhorn Slough; PB, Pebble Beach.

[†] Estimated age: P, pup; Y, yearling (1–2 years); S, subadult; A, adult.

[‡] Standard length (straight-line length measurement from tip of nose to tip of tail).

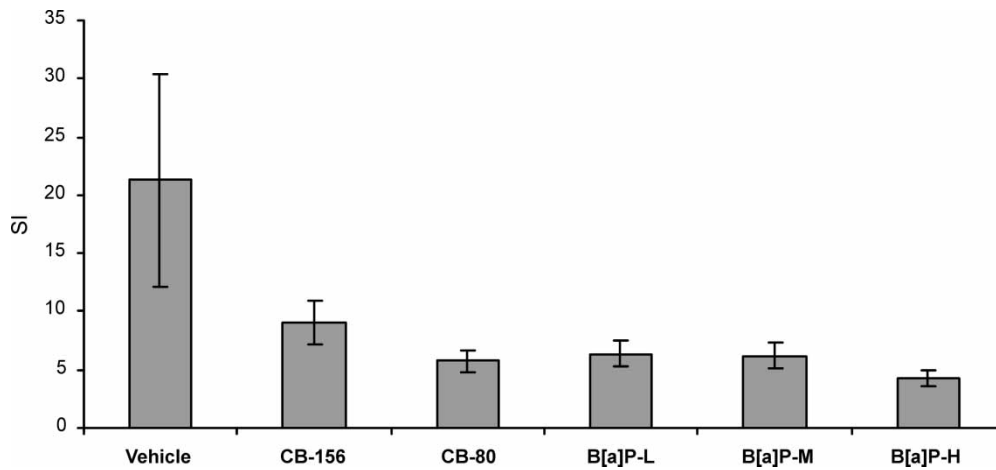


FIGURE 1 Lymphoproliferation of harbor seal T cells following *in vitro* exposure to vehicle (10 μ M DMSO), CB-156 (10 μ M in DMSO), CB-80 (10 μ M in DMSO), and low (L; 0.1 μ M), medium (M; 1.0 μ M) and high (H; 10 μ M) B[a]P. Data shown are means (\pm SE) of stimulation indices (SI; ratio of mitogen-stimulated CPM to unstimulated CPM) based on 17 harbor seals for vehicle, CB-156, and B[a]P-H, and 9 seals for CB-80, B[a]P-M, and B[a]P-L.

The General Linear Model procedure revealed a significant interaction between treatment (B[a]P or vehicle) SI and baseline (media) SI (Fig. 2; $F_{1,30} = 6.50$, $p = 0.02$), indicating significantly different relationships between the two treatments relative to media controls. The regression of SI in B[a]P-treated cultures relative to media controls produced a slope significantly and substantially less than 1 (slope estimate = 0.14, 95% CI: -0.15 – 0.43), indicating a clear suppressive effect of the model PAH. In contrast, the vehicle-media SI slope did not differ significantly from 1 (slope estimate = 0.85, 95% CI: 0.38 – 1.32), indicating no suppressive effect of the vehicle.

After stimulation by the mitogen, exposure to 10 μ M B[a]P generally depressed the proliferative response, although large variation among individuals was apparent (Fig. 3). Because vehicle SI did not differ significantly from media SI and because there was less variance among media SI values, we calculated reduction in proliferation

in B[a]P-treated cultures relative to media SI (baseline) for each individual. B[a]P-depressed proliferation of T lymphocytes in response to mitogen was evident for all individuals except those with the lowest baseline stimulation (Table I). The average reduction in lymphoproliferation due to exposure to the model PAH was 61% (range 34–97%). Because age was a potentially confounding factor, we also examined pups only (the only age class present at all 3 locations). When only pups were considered ($n = 6$), the depression of proliferation in B[a]P-treated cultures was even greater (67% reduction). Stimulation indices (log-transformed) did not differ significantly between sexes for B[a]P-treated ($p = 0.31$) or vehicle-treated ($p = 0.89$) cultures.

Small sample sizes and unequal representation of age classes across locations prevented the determination of independent effects of capture site and age. Because mass and standard length were necessarily highly correlated with age class, these also were not assessed as independent

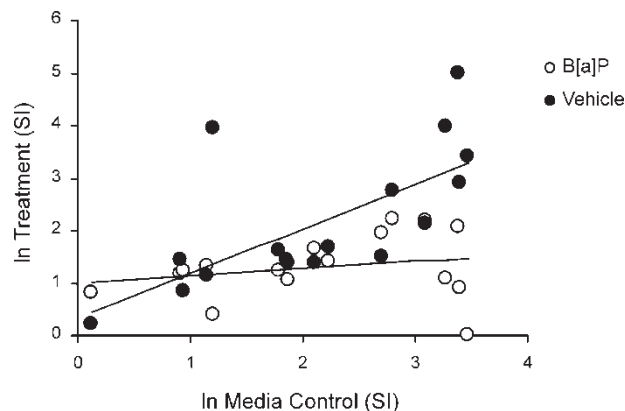


FIGURE 2 Stimulation indices (SI; ln-transformed) from 17 individual harbor seals for 10 μ M vehicle- and B[a]P-treated cultures with linear regressions relative to baseline (media control) proliferation.

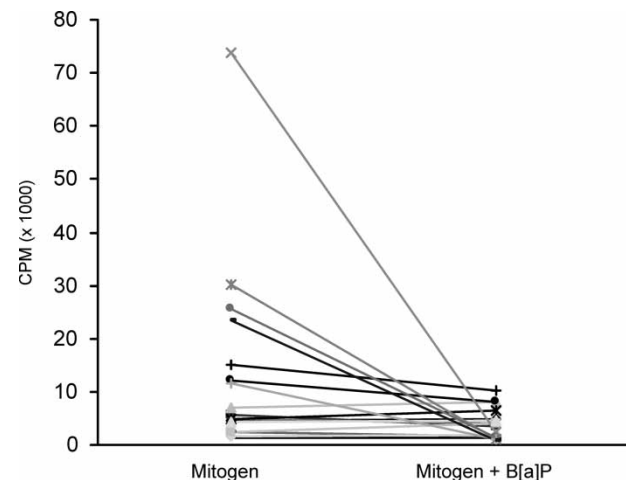


FIGURE 3 T cell proliferation in lymphocytes from 17 harbor seals. Cells were exposed *in vitro* to mitogen and vehicle only versus mitogen and 10 μ M B[a]P.

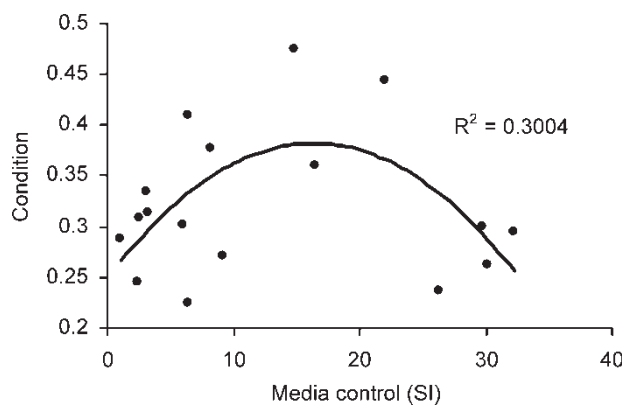


FIGURE 4 Relationship between physiological condition (indexed by the ratio of mass to standard length) and baseline proliferation (stimulation index for media control cultures) of T cells from 17 individual harbor seals. Data are fitted to a polynomial function.

factors. The relationship between physiological condition (based on our index) and baseline stimulation (media SI) was investigated and revealed a polynomial function (Fig. 4), indicating that animals in the greatest physiological condition showed moderate baseline stimulation. These same individuals also tended to be older and least affected by B[a]P treatment (i.e. showed highest SI for 10 μ M B[a]P). Finally, viability in B[a]P-treated lymphocyte cultures did not differ from vehicle controls ($p = 0.39$), indicating that the observed effects of *in vitro* exposure to B[a]P were not caused by direct cytotoxicity.

DISCUSSION

Increasing awareness of the widespread environmental occurrence and persistence of certain organic pollutants has generated great interest, among scientists as well as the general public, in their potential health effects on human and wildlife populations. Previous toxicity studies using laboratory animals indicate that the mammalian immune system is a sensitive target of persistent organic pollutants such as tetrachlorodibenzo-*p*-dioxin, PCBs, and PAHs (Tucker *et al.*, 1986; Ladics *et al.*, 1991; Stack *et al.*, 1999). Many reports have documented the specific suppressive effects of the carcinogenic PAHs on both humoral and cell-mediated immunity in rodents and humans (Ladics *et al.*, 1991; 1992; reviewed in White *et al.*, 1994; Davila *et al.*, 1996; Salas and Burchiel, 1998). Effects included decreased proliferation responses to T-cell mitogens as well as lymphoid cell death, lymphoid organ atrophy, altered lymphocyte subsets, apoptosis, decreased B-cell responses to mitogens and antigens, altered cytokine regulation, and changes in innate immune function. Despite evidence of widespread exposure of marine mammals to PAHs and their potential influence on health, and their recent identification as a research priority by the Marine Mammal Commission (1999), PAHs have received little attention in marine mammal research (Martineau *et al.*, 1994; Fossi *et al.*, 1997).

Here, we exposed harbor seal lymphocytes *in vitro* to model pollutants and examined the effects on one measure of cell-mediated immunity—the proliferation of T cells in response to mitogen stimulation. Although the *in vitro* approach utilized in this investigation represents an extreme reductionism relative to the very complex situation in the intact organism, it can provide insight into specific effects of model agents. Despite the variability associated with our samples from free-ranging harbor seals, we observed a clear suppressive effect of B[a]P exposure on T cell mitogenesis. The greatest effect on proliferation due to treatment with B[a]P was seen in cultures with relatively high stimulation responses; such samples may represent individuals whose peripheral lymphocytes were not already activated by immunotoxic or infectious agents. The lack of evidence of cytotoxicity indicates a direct effect of the model PAH on T cell function. Low statistical power may have decreased our ability to confirm depressive effects of the PCBs and lower concentrations of B[a]P.

Although studies such as the present one advance our understanding of possible immunotoxic effects of PAH at the cellular level, the mechanisms of PAH immunotoxicity are not yet fully understood (White *et al.*, 1994). In this study, only the parent compound was used and no attempt was made to determine if the immunosuppressive moiety was B[a]P or a metabolite(s). Various mechanisms of action of PAH immunotoxicity have been proposed, many of which involve formation of reactive metabolites via cytochrome P450 metabolism. Activation of B[a]P by P450-mediated metabolism to a dihydrodiol epoxide or radical cation metabolite occurs in splenic macrophages in the mouse (Ladics *et al.*, 1992). Human peripheral lymphocytes also possess cytochrome P450s, which metabolize PAHs (Davila *et al.*, 1995). Recent work indicates that several marine mammal species possess hepatic P450 enzymes with broadly similar activity to that of laboratory species (Hart *et al.*, 1996; Addison *et al.*, 1998; Stegeman *et al.*, 1998). It is reasonable to postulate that in the harbor seal, extrahepatic tissues such as peripheral blood leukocytes also may have this oxidative capacity. In this study, cell preparations contained macrophages in addition to B and T lymphocytes; these cell types were likely capable of biotransformation of B[a]P to metabolites which may have served as the proximate toxicant(s).

Previous investigations of contaminant-induced immune suppression in marine mammals have typically correlated impaired immune responses or less specific biomarkers with tissue levels of polyhalogenated aromatic hydrocarbons (PHAHs) such as the organochlorine pesticides and PCBs (Reijnders, 1994; Lahvis *et al.*, 1995; Simms *et al.*, 2000). While providing circumstantial evidence for immune suppression, causal relationships and mechanisms of action could not be established. Experimental research has been limited to studies of captive harbor seals fed environmentally contaminated fish, which indicated immune suppression related to PCBs

(Brouwer *et al.*, 1989; de Swart *et al.*, 1995; Ross *et al.*, 1996). However, presence and levels in fish of other potentially immunotoxic compounds such as metals and PAHs were not assessed.

Our work complements previous *in vivo* research by experimentally demonstrating a depressive effect of exposure to a model marine pollutant on an important lymphocyte function in cell-mediated immunity. The B[a]P exposure showing depressive effects *in vitro* in the present study (10 μ M) is equivalent to 2.5 ppm, a concentration similar to that found in brain and liver (DNA adducts of B[a]P) of beluga whales of the St Lawrence Estuary (Quebec, Canada) and in liver (sum of 8 PAH) of South American fur seals of the Rio Grande, Brazil (Martineau *et al.*, 1994; Fossi *et al.*, 1997). Taken together, the data indicate that PAHs as well as PHAHs are immunotoxic marine contaminants with the potential to affect host resistance to disease in free-ranging pinnipeds and probably other marine mammals (and possibly human populations).

In conclusion, our findings indicate an immunosuppressive effect of the prototypic PAH on T-cell function and hence imply impairment of host resistance against viral pathogens *in vivo*. To our knowledge, this is the first study to investigate PAH exposure in harbor seals and directly demonstrate immunotoxic effects of a model marine pollutant on functional responses of lymphocytes in a marine mammal species. Future work in this area should include investigation of the possible effects of PAHs on other T-cell-mediated responses and attempt to illuminate the interactive effects of age, physiological condition, capture site (environment), and other potential factors at play in a free-ranging, outbred population.

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