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The development of methods for immunophenotypic and lymphocyte function analyzes for assessment of Southern sea otter (*Enhydra lutris nereis*) health

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Abstract

The Southern sea otter (*Enhydra lutris nereis*) is listed as threatened under the Endangered Species Act. The population began a pattern of slow decline in 1995. The decline was attributed to high adult mortality rates with infectious disease being the major cause of death. Multiple pathogens were implicated in these deaths including opportunistic pathogens such as *Coccidiodes immitis* and *Toxoplasma* sp. These findings suggested that the immunological health of mature animals in this population might be compromised. The primary goal of this study was to establish techniques for assessing phenotypic and functional baseline data for peripheral blood mononuclear cells (PBMC) in free-ranging sea otters. Standard total and differential white blood cell counts were augmented by emumeration of T and B lymphocyte subsets. Lymphocyte function was determined by both mitogen-induced proliferation and expression of IL-2 receptors. In addition to establishing normal ranges for adult animals, age-related changes were identified in B lymphocyte numbers and cell-surface density of major histocompatability complex class II (MHC II) proteins. The predominant lymphocyte subpopulation in Southern sea otters is the T lymphocyte. Substantial variation among individual animals was observed within the B lymphocyte population both in cell number and density of MHC II expression. Pups had greater numbers of T and B lymphocyte, as well as, greater MHC II expression on B lymphocytes than adults. Mitogen-induced proliferation of peripheral blood mononuclear cells (PBMC) was variable among individual animals with no significant difference in cell response between age class and gender. Concanavalin (ConA) was a more effective mitogen in stimulating proliferation and interleukin (IL)-2 receptor expression than pokeweed. This data can be used to augment routine hematology profiles and aid in the identification of animals with immunologic perturbations.

Keywords: Southern sea otter; Immunological assays; Lymphocyte function; Immunophenotype

1. Introduction

The Southern sea otter (*Enhydra lutris nereis*) population inhabits the Monterey Bay National Marine Sanctuary, a federally protected marine reserve off California's central coast. Once hunted

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to near extinction, the population has slowly recovered; yet, the numbers still remain well below the estimated carrying capacity for the sanctuary (Laidre et al., 2001). Therefore, the Southern sea otter remains listed as threatened under the Endangered Species Act.

In 1995, after a decade of growth, the Southern sea otter population began a pattern of slow decline associated with high adult mortality rates. This decline continues to date despite birth rates comparable to the stable Alaskan northern sea otter (Riedman et al., 1994). As a result of these findings, research efforts were initiated to identify causes of mortality. Infectious diseases were identified as a major cause of sea otter death during the period from 1992 to 1996 (Thomas and Cole, 1996). Multiple pathogens were implicated in these deaths. Occasionally, unusual pathogens such as *Coccidiodes immitis* and *Toxoplasma* sp. were identified in sea otter carcasses (Cole et al., 2000). These finding were surprising, since these agents often cause opportunistic infections in immunosuppressed humans and domestic animals and were thought to be uncommon marine pathogens. A recent epidemiological study conducted by University of California, Davis and California Department of Fish and Game researchers examined the necropsy data collected between 1998 and 2001 and found that this unusual mortality pattern has persisted (C. Kreuder, pers. commun.).

The decline in the Southern sea otter population is just one example of an increasing trend worldwide in reports of disease and mass mortality events affecting marine mammals (Harvell et al., 1999). The problems facing marine mammal populations, including the Southern sea otter, are likely multi-factorial. Several factors have been implicated as likely contributors such as changing environmental conditions, either those induced naturally or through human activities, as well as, altered host factors. In an attempt to better understand this problem in the sea otter population, many investigators are measuring chemical contaminant levels in tissues, markers of exposure to infectious agents, hematology and serum chemistry parameters, pup survival, and population demographics (Ballachey et al., 1996a,b; Kannan et al., 1998). Specific toxic chemicals (organochlorine pesticides, polychlorinated biphenyls, and butyltins) have been documented in Southern sea otter carcasses (Nakata et al., 1998; Kannan et al., 1998); however, there is insufficient evidence to linking their presence to mortality. In addition, immunosuppressive viruses have been isolated from several marine mammal species (Kennedy, 1998; King et al., 1998, 2001; Lipscomb et al., 2000); although, none to date have been isolated from a Southern sea otter. Despite recent research efforts, epidemiological studies in sea otters as well as other marine mammals still lag behind investigations in terrestrial mammals due in part to a lack of essential tools needed to investigate and understand the mechanisms of host disease resistance (Harvell et al., 1999).

Concern regarding the immunological health of the Southern sea otter population and the need for more sensitive diagnostic tests and research tools stimulated the present studies to develop immune function assays for use in this species. The primary goal was to establish baseline values for leukocyte subpopulations and immune function, neither of which is currently available for the sea otter. A second goal was to assess whether immunological tests could be used to identify otters in this population with immunological perturbations that could potentially increase their susceptibility to infectious disease.

2. Materials and methods

2.1. Animals

Opportunistic blood samples were obtained from two groups of free-ranging Southern sea otters (*Enhydra lutris nereis*) by personnel of the California Department of Fish and Game (CDFG). The first group consisted of samples from 21 animals (8 adult females, 5 sub-adult females, 11 adult males) collected as part of a study examining the health of free-ranging Southern sea otters within the Monterey Bay National Marine Sanctuary. These samples were collected from March to October 1998, and since no sea otter-specific leukocyte markers were available at this time, the blood samples were used in lymphocyte function assays only. After identifying cross-reactive antibodies suitable for sea otter leukocyte cell surface antigens, samples from a second group of animals were collected between January 1999 and October 2000 (seven mother-pup pairs and seven additional adult females) and used in immunophenotypic

analyses. The CDFG age classification system is based on dentition, body length, weight and pelage (unpublished data). In this system pups range from 0 to 20 weeks of age, sub-adults from 1 to 3 years, and adults >3 years.

2.2. Blood sample collection

Adult and sub-adult otters were captured using Wilson traps or dipnet capture methods (Bayha and Hill, 1990), anaesthetized using a standard fentanyl and diazepam drug combination (Williams et al., 1981), and recovered by naltrexone reversal. The pups were manually restrained and received no sedative agents. Blood was collected from either the jugular vein or femoral vein into blood collection tubes for either immunophenotypic analysis by flow cytometry (acid citrate dextrose solution B collection tubes, Becton Dickinson, Trenton, NJ) or for PBMC isolation and lymphocyte function assays (CPT tubes containing layered sodium citrate, polyester gel, and ficoll hypaque solution, Becton Dickinson, Trenton, NJ). Additional blood samples were collected into EDTA tubes (Becton Dickinson, Trenton, NJ) for differential counts of white blood cells (performed by IDEXX Veterinary Services Inc., West Sacramento, CA) and into serum clot tubes (Vacutainer, Becton Dickinson, Trenton, NJ) for serum clinical chemistry, serology, toxicology, and to measure interleukin 6 (IL-6) activity by bioassay. The serum samples for IL-6 assay were collected sterilely and handled in sterile fashion and immediately stored at −80 °C.

2.3. Analytical antibodies

Because there are no available sea otter-specific antibodies that can differentiate leukocyte subsets, antibodies developed for other species were used in this study (Table 1). The cross-reactivity of these antibodies with sea otter leukocyte antigens was validated in preliminary studies by immunohistochemical staining of sea otter lymphoid tissues including spleen, lymph node and thymus (data not shown).

2.4. Immunofluorescence staining and flow cytometric analysis

Cell preparations were stained and analyzed by flow cytometry using a modified protocol to that previously described (Blanchard-Channell et al., 1994). Briefly, for single labeling, 1×10^6 leukocytes were washed in cytoflow buffer composed of phosphate-buffered saline (PBS) pH 7.4, 0.01% (v/v) 0.5 M EDTA (pH 8.0), 0.001% (w/v) Na Azide, 0.5% (w/v) BSA and then incubated with hybridoma supernants of anti-MHC II mAb. After washing, $F(ab')_2$ goat anti-mouse IgG (H + L) allophycocyanin (APC)-conjugated antibody (Caltag, South San Francisco, CA) was added to the tubes and incubated. Following incubation the cells were washed, fixed and permeabilized for intracellular labeling (DAKO IntraStain kit, Carpenteria, CA), and incubated according to the kit instructions with 5 μl of R-phycoerythrin (PE)-conjugated anti-CD79α and 1 μl fluorescein isothiocyanate (FITC)-conjugated anti-CD3ϵ. Incubations were performed at 4 °C in the dark for 30 min. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Antibody lots used were kept constant throughout the experiment. Non-specific binding of mouse Ig cells was determined by using an isotype-matched irrelevant antibody (*Borrelia coriaceae*). Appropriate irrelevant antibodies were used for internal staining controls. Events were collected using a

Table 1
Specifications of monoclonal and control antibodies

Name	Specificity	Publication
Rat anti-mouse CD3 epsilon antibody[a]	Mature T cells	Tomonari (1988)
Mouse anti-human CD79α (clone HM57)[b]	B cells (except plasma cells)	Mason et al. (1991)
Mouse anti-bovine MHC II (UCDBov171D3)[c]	B cells, monocytes, variable expression on activated T cells	Taylor et al. (1993)
Mouse anti-*B. coriaceae*[c]	*B. coriaceae* antigen	Blanchard-Channell et al. (1994)

[a] Serotec Inc., Washington, DC.
[b] Dako, Carpenteria, CA.
[c] Laboratory for Marine Mammal Immunology, School of Veterinary Medicine, University of California, Davis, CA.

live gate set to collect all cells. When 10,000 events were collected in the lymphocyte gate, determined by relative size (forward scatter) and complexity (side scatter) characteristics, collection was stopped. Markers were adjusted according to proper controls. The percentage of B cells expressing the antigen presentation molecule, MHC II, was based on the staining pattern of an irrelevant control antibody. Density of surface protein expression was measured as the geometric mean of the *x*-axis. The data was processed using relative fluorescent intensities against Ig class-matched controls. The flow cytometer was calibrated for four color analysis with the same lot of fluorochrome labeled beads prior to the analysis of each sample set for consistency. Analysis was performed using Cell Quest data analysis software (Becton Dickinson) and Flowjo (Treestar, San Carlos, CA). The results are the means ± S.D. of adults versus pups.

2.5. Isolation and cryopreservation of PBMC

Blood collected in CPT vacutainer tubes were centrifuged (1800 × *g* for 20 min). The supernatant/buffer was discarded and the isolated mononuclear cells were resuspended in sterile PBS with 0.5 M EDTA, pH 7.4. The cells were then centrifuged (250 × *g* for 8 min). Supernatant was discarded and the cells were resuspended in freezing media (10% Dulbecco's Modified Eagles Media [DMEM], 10% DMSO, 80% fetal calf serum [FCS]) and rate frozen in Nalgene cryojars (Nalgene, Rochester, NY) to −80 °C. Within 2–7 days cells were transferred to liquid nitrogen. Cryopreservation of the PBMCs enabled cells collected from animals on different dates to be preserved, and assayed under standardized conditions that minimized variability in experimental conditions at a later date.

2.6. Mitogen-induced proliferation

PBMCs were cultured in Dulbecco's Modified Eagles Media (DMEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml gentomycin and 10% FCS (Atlanta Biologic, Norcross, GA). All cell culture media and supplements, except for FCS, were purchased from Gibco, Invitrogen Corp., Carlsbad, CA. Mitogen stocks of concanavalin A (ConA) and pokeweed mitogen (PWM) (Sigma Chemical Company, St. Louis, MO) were reconstituted in non-supplemented DMEM to 1 mg/ml stock solutions and used to make dilutions. Cryopreserved cells were rapidly thawed in a 37 °C water bath. The PBMCs were resuspended in sterile PBS with 0.5 M EDTA, pH 7.4 and centrifuged at 250 × *g* for 8 min. The supernatant was aspirated and the PBMCs were resuspended in supplemented DMEM. An aliquot of cells was stained with ethidium bromide/acridine orange and counted with a fluorescent microscope to confirm cellular viability. PBMCs were resuspended to a final cell suspension of 2×10^6 live PBMC/ml in supplemented DMEM.

Proliferation assays were performed in 96-well tissue culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). PBMCs were added to each well at a volume of 100 μl/well (2×10^5 cells). Cells from each animal sample were evaluated in triplicate for each mitogen concentration using both ConA and PWM. Mitogens were serially diluted (starting from a 1 mg/ml stock solution) and added to the appropriate wells at a volume of 100 μl/well. "High" and "low" concentrations of each mitogen were determined using a standard dose response curve. The high concentration represented the dilution that gave maximal stimulation while the low concentration was the lowest dilution that approached a maximal response; lymphocyte responses declined dramatically to dilutions of mitogen below the "low" concentration. The high and low final concentrations for ConA and PWM mitogen were found to be as follows: ConA 1.0, 0.1 μg/ml and PWM 0.1, 0.01 μg/ml. Negative control wells contained 100 μl of media only. Pre-tested cryopreserved bovine PBMCs were used as an internal standard. They were cultured and stimulated under the same conditions. Tissue culture plates were incubated at 37 °C in 5% CO_2. After incubating for 96 h, 20 μl of bromodioxyuridine (BrdU) was added to each well for a final concentration of 10 μM. Colorimetric immunoassay was used for the quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis (Roche, Boerhringer Mannheim, cell proliferation ELISA, BrdU [colorimetric]). Tissue culture plates were incubated at 37 °C in 5% CO_2 for an additional 24 h (a predetermined length of time for optimal BrdU

incorporation into proliferating sea otter PBMCs, data not presented). After 24 h, the tissue culture plates were centrifuged at 300 × *g* for 10 min and labeled as per manufacturer's instructions. Absorbance of the samples was immediately measured in an UVmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm (reference wavelength: 650 nm). Data is summarized as the mean of three replicates and standard deviation.

2.7. Interleukin-2 receptor (IL-2R) expression

Isolated PBMCs were analyzed for IL-2R expression after incubation with ConA and PWM. Cells were added to a 24-well tissue culture plate (Falcon, Becton Dickinson) at 250 μl/well (5×10^5 cells/well). ConA was added to a final concentration of 5.0 μg/ml and PWM to 5.0 μg/ml. Cells were stained as per manufacturers instructions with the Fluorokine kit (R&D Systems, Minneapolis, MN) and analyzed for receptor expression at 20 h using flow cytometry. Briefly, cells were removed from the wells, washed in PBS, centrifuged, and the supernatant discarded. Biotinylated *r*hIL-2 was added to the leukocyte samples and incubated on ice for 1 h. An equivalent volume of streptavidin conjugated FITC was added to each sample and incubated for 30 min as per manufacturer's instructions. Biotinylated trypsin was used as a negative control reagent. Leukocytes were washed in RDF1 buffer (R&D Systems) and resuspended in PBS for FACS analysis (Becton Dickenson Immunocytometry Systems, Mountain View, CA). Forward and side scatter features were used to set a live gate for collection of 10,000 events, representing the lymphocyte/monocyte population. Data was analyzed using Winlist software (Verity Software House, Topsham, ME).

2.8. Interleukin-6 (IL-6) bioassay

The detection of sea otter IL-6 was determined using the IL-6 dependant B9 mouse hybridoma bioassay using a protocol previously described (King et al., 1996; Mosmann, 1983). Only samples collected from the 21 free-ranging adult and sub-adult animals captured in 1998 and one sample (30 November 2000) from the Monterey Bay Aquarium animal were available for evaluation. This assay is based on measuring the survival and stimulated growth of IL-6 dependent B9 hybridoma cells following exposure to the sample of interest. In brief, 2×10^4 hybridoma cells/ml were cultured in RPMI media with 5% FCS in 96-well plates. Sea otter serum samples were prepared in a dilution series and added in duplicate to the cells. A dilution series of *r*hIL-6 was used as a standard for comparison. After 72 h of incubation at 37 °C, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO) was added to the wells. MTT is cleaved by active mitochondria and forms a dark blue product. The reaction was stopped after 4 h by adding lysis solution (20% SDS, 0.02N HCl). The stop reaction was allowed to proceed overnight before the absorbance at 590 nm was measured in an UVmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA). Dose response curves were developed for all positive serum in concert with the IL-6 standard. Parallel line analysis was used to quantify biological activity for the positive sea otter samples (Mire-Sluis and Thorpe, 1998).

2.9. Statistical analysis

Differences between relevant groups were calculated for either the phenotypic and functional parameters when samples permitted, by one-way analysis of variance (ANOVA) using the PROC GLM command of SAS for unbalanced data (SAS 8.0). Statistically significant differences between the free-ranging sea otter groups were calculated for normally distributed parameters as determined by the Kolmogorov–Smirnov normality test. Statistical comparisons utilized a minimum significance level of $P < 0.05$. Descriptive statistics for the IL-2 receptor expression data was examined using box plots of the median, interquartile range, and outliers (NCSS Statistical Software, Kaysville, UT). A two sample *t*-test was also used to compare response to mitogens using a minimum significance level of $P < 0.05$.

3. Results

Health status was determined by physical examination, using routine hematological analysis, serum chemistry; and, in several animals, serum levels of

IL-6 (Funke et al., 2003). Serum clinical chemistry values were all within normal age class limits for free-ranging Southern sea otters (Williams et al., 1992). Using all of these measures of health, there was no evidence of disease in any of the free-ranging animals examined, suggesting that this was a healthy population, and therefore acceptable for beginning to define immunological health parameters in this species.

3.1. Complete blood counts and serum chemistry

A complete blood count, using standard methodology, was used to determine and compare leukocyte and lymphocyte numbers between different age groups. The overall mean leukocyte ($7.140 \pm 2.065 \times 10^3$/μl, mean ± S.D.) and lymphocyte counts ($2.526 \pm 1.343 \times 10^3$/μl, mean ± S.D.) were consistent with those reported for other species. In addition, there were no differences in either total leukocyte or lymphocyte numbers between adults and pups (Table 2).

3.2. Immunophenotypic analysis of circulating blood leukocytes

The peripheral blood leukocytes were labeled with cross-reactive antibodies recognizing cell surface markers that distinguish the major lymphocyte subsets and with an antibody that identifies antigen presenting cells (MHC class II). These antibodies were useful in differentiating distinct populations of T and B lymphocytes (Fig. 1). The predominant lymphocyte subpopulation was T cells ($1.808 \pm 922 \times 10^3$/μl, mean ± S.D.), with a lower number of B cells ($168 \pm 166 \times 10^3$/μl, mean ± S.D.) (Table 2, Fig. 1A). While a

Table 2
Number and phenotypic characterization of peripheral blood lymphocytes in Southern sea otters, adults and pups

Lymphocyte subset	Age group	*n*	Mean	S.D.[a]	Min[b]	Median	Max[c]	95% CI[d]
Leukocytes (cells/μl)[e]	Adults	19	6928	1960	4200	6400	11300	5953–7902
	Pups	7	7686	2385	4900	6800	10600	5479–9891
Lymphocytes (cells/μl)[e]	Adults	19	2272	1098	924	1818	4440	1726–2818
	Pups	7	3180	1763	1320	2516	6254	1550–4810
B cells (cells/μl)	Adults	19	168	166	70	109	800	85–251
	Pups	7	525*	241	ND[f]	ND	ND	ND
T cells (cells/μl)	Adults	12	1808	922	797	1591	3501	1188–2427
	Pups	3	3437*	2009	ND	ND	ND	ND
B/T cell ratio	Adults	12	0.10	0.06	0.05	0.08	0.28	0.05–0.14
	Pups	3	0.19	0.06	ND	ND	ND	ND
MHC II+ B cells[g]	Adults	10	73	11	55	70	92	65–81
	Pups	3	90*	9	ND	ND	ND	ND
B cell MHCII expression[h]	Adults	10	43	19	24	34	84	29–56
	Pups	3	76*	11	ND	ND	ND	ND
MHC II+ T cells[g]	Adults	10	54	13	35	52	72	45–63
	Pups	3	43	11	ND	ND	ND	ND
T cell MHCII expression[h]	Adults	10	27	10	13	29	43	20–33
	Pups	3	21	9	ND	ND	ND	ND

[a] S.D. = standard deviation.
[b] Min = lowest value.
[c] Max = highest value.
[d] CI = confidence interval.
[e] CBC derived value.
[f] ND = not determined.
[g] Percentage of cells expressing MHCII on the cell surface.
[h] *x* geometric mean fluorescense represents level of MHCII molecule density on the surface of lymphocytes subset.
* Statistically significant differences between adults and pups ($P < 0.05$ by ANOVA).

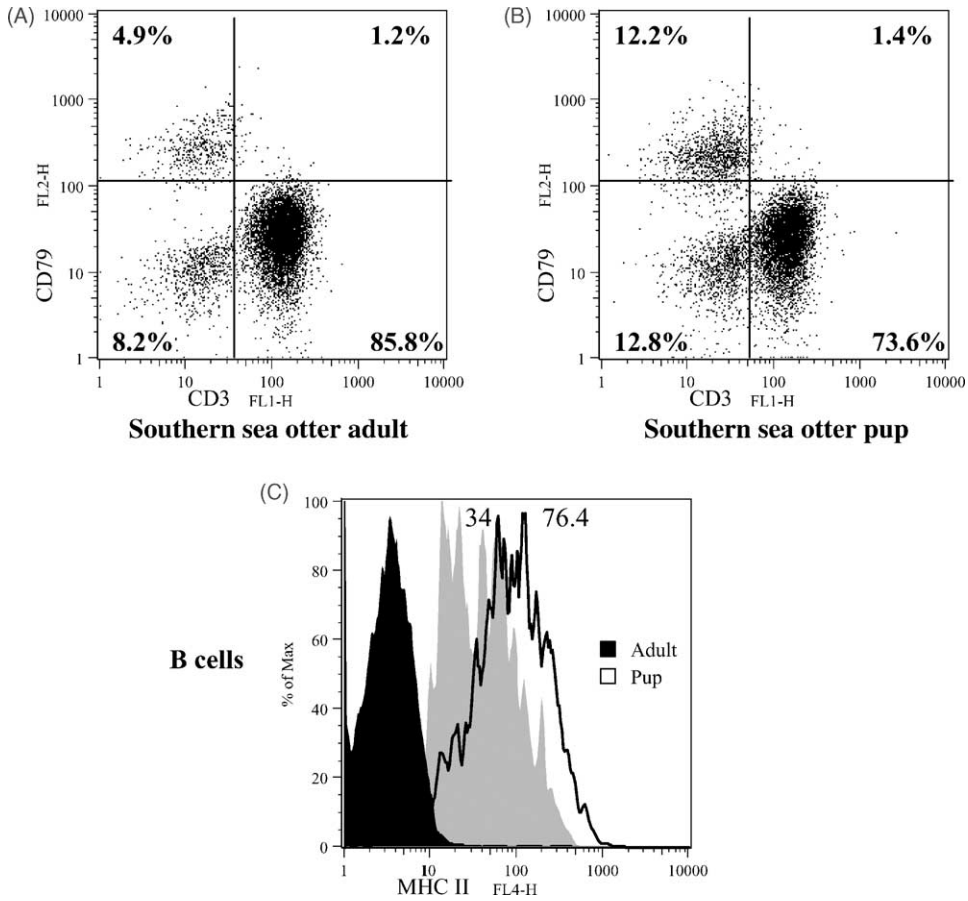

Fig. 1. Immunophenotypic profiles of peripheral blood lymphocytes from representative adult and pup Southern sea otters. Cells were triple-stained with anti-CD79α-PE, anti-CD3-FITC, and anti-MHCII labeled with APC (A–C). In representative dot plots, the upper left quadrant represents the percentage of CD79α+ B cells and lower right quadrant the percentage CD3+ T cells (A and B). Quadrants were adjusted according to standard controls. The overlay histogram (C) shows MHC class II expression density of the B lymphocyte gated cells of a pup (open histogram) versus an adult (gray histogram). The black histogram corresponds to background staining with fluorescent conjugate alone. The numeric values represent the *x*-axis geometric mean of fluorescence of the cells labeled by anti-MHC II mAb for each animal.

significant population of non-B/non-T cells was identified (Fig. 1A and B), the phenotype of these cells could not be determined with the available antibody panel. Table 2 represents the general reference range for absolute leukocyte and lymphocyte numbers, lymphocyte subset numbers and MHC class II expression in this population of adult and pup Southern sea otters. The proportion of cells expressing MHC class II molecules, and the density of this expression was significantly higher for B cells than T cells (Table 2). While the range of T cell numbers, the proportion of MHC II positive B and T cells, and the density of T cell MHC II expression were relatively narrow, the broad ranges for B cell numbers and density of B cell MHC II expression indicated substantial individual variation for the latter values (Table 2). Reference ranges for lymphocyte subset numbers and MHC class II expression could not be established for pups ($n = 3$) or sub-adults ($n = 0$) because of the limited sample size. However, statistically significant age-associated differences in lymphocyte subset numbers and MHC class II expression were detected (Table 2). The mean B and T lymphocyte numbers, mean percentage of B cell expressing MHC class II molecules, and the mean density of MHC class II expression on B cells, were significantly

increased in the pups over adults (Table 2, Fig. 1C). The disproportionate increase in B over T cells in the pups (Table 2) was reflected in an increased B/T ratio.

3.3. Mitogen-induced PBMC proliferation

The average response of PBMC cell cultures to stimulation with high and low concentrations of ConA were 0.353 ± 0.257 (S.D.) OD (response range 0.139–1.017 OD) and 0.206 ± 0.151 (S.D.) OD (range 0.064–0.589 OD), respectively (Fig. 2A). The responses to high and low concentrations of PWM were 0.204 ± 0.144 OD (range 0.044–0.563 OD) and 0.122 ± 0.085 OD (range 0.033–0.432 OD), respectively (Fig. 2B). Wide individual animal variation in response to mitogen stimulation occurred with both mitogens at both concentrations (Fig. 2A and B). In general, within individual animals, ConA induced a greater proliferation response as compared to PWM, and a broader range of responses between individual animals (Fig. 2). The average response ratio (response to a high concentration of mitogen/response to low concentration of mitogen) and standard deviation was measured for both ConA (2.2 ± 2.3 S.D., range 0.8–12) and PWM (1.8 ± 0.83 S.D., range 0.8–3.4), respectively (Fig. 2C). No significant difference in

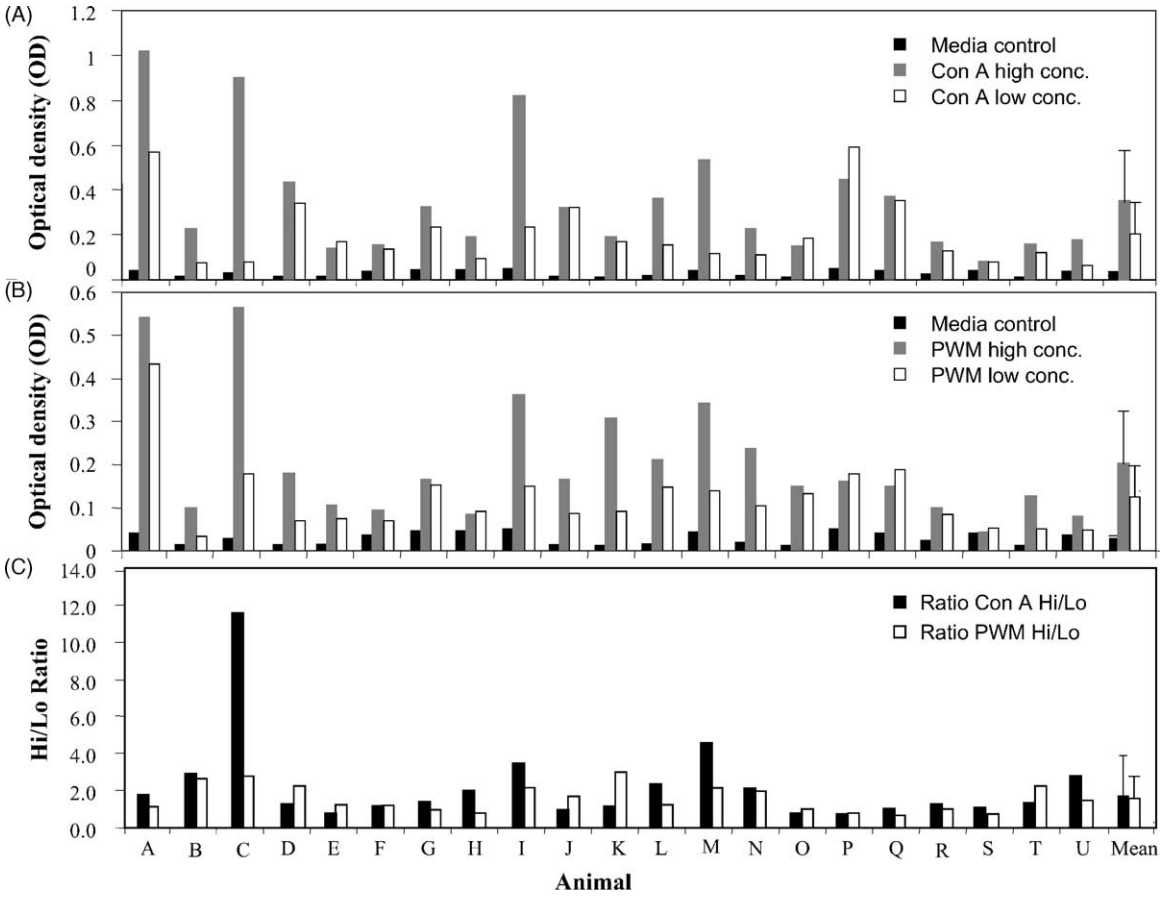

Fig. 2. Mitogen-induced PBMC proliferation responses for individual free-ranging Southern sea otters. The mean response and standard deviation are represented by the bar graph and individual animals are represented by letters (*x*-axis). The response to the media control (black bar), high concentration of mitogen (gray bar) and low concentration of mitogen (white bar) were measured in optical density (A and B). The ratio of the response to the high concentration of ConA/low concentration of ConA (black bar) and the response to the high concentration of PWM/low concentration of PWM (white bar) are represented (C). Error bars represent S.D.

PBMC cell response to mitogen was identified between age classes (sub-adult versus adult), gender, or capture location sites.

3.4. Interleukin-2 receptor (IL-2R) expression

Mitogen-induced IL-2 receptor expression was measured in PBMC cultures from adult and sub-adult sea otters. The ability to upregulate IL-2R expression is an early indicator of cellular activation. ConA stimulation produced a greater percentage of IL-2 receptor positive cells with a narrower spread of values (median 50.8%, interquartile range 61.2–53.1%) as compared to that induced by stimulation with PWM (median 31.7%, interquartile range 46.4–29.3% (Fig. 3). Three individuals were identified as ''mild outliers'' with respect to their response to stimulation with ConA (Fig. 3). This categorization was based on the fact that their IL-2 receptor expression fell outside the 25–75th interquartile range (IQR), but within three IQRs (NCSS, Kaysville, Utah). Two outliers were 37 and 36% IL-2R positive cells [animals Q (98019) and C (98004), respectively]. An additional outlier [animal G (98030)] with a high response (68%) was also detected (Fig. 3).

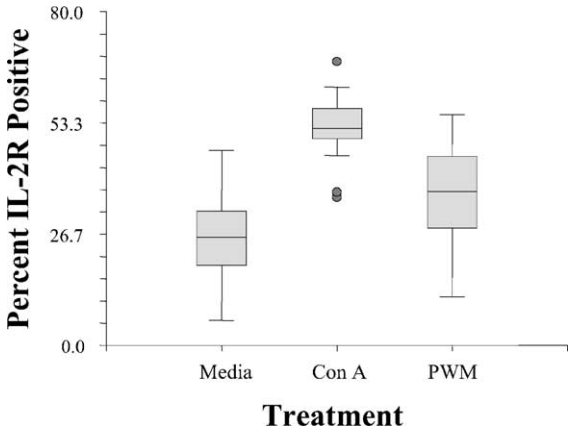

Fig. 3. The percentage of interleukin 2 receptor (IL-2R) positive PBMCs from adult and sub-adult Southern sea otters. The box plot graph shows the median percent positive cells, interquartile range (IQR) (rectangle), upper adjacent values (less than or equal to the 75th percentile plus 1.5× IQR), lower adjacents (greater than or equal to the 25th percentile minus 1.5× IQR), and mild outliers for responses to media alone, ConA (5.0 μg/ml), and PWM (5.0 μg/ml) stimulation (*x*-axis). Upper and lower mild outlier values were only detected by ConA stimulation. Response to both mitogens, as compared to media controls, were significantly increased, $P < 0.05$.

3.5. Interleukin-6 (IL-6) quantification

Serum IL-6 values for all free-ranging animals assayed were less than the detectable limits of the B-9 bioassay (<1.5 U) (data not shown). These results suggest that the free-ranging animals examined did not have an active inflammatory process present.

3.6. Statistical analysis

Except for mitogen-induced proliferation data, all other results passed the Kolmogorov–Smirnov normality test; thus, the free-ranging Southern sea otter samples were representative of a normally-distributed group.

4. Discussion

The recent decline in the Southern sea otter (*Enhydra lutris nereis*) population (Riedman et al., 1994) combined with the concurrent high incidence of mortality associated with infectious disease in adult animals (Thomas and Cole, 1996), has stimulated concern regarding the immunological health of the Southern sea otter population. Very limited information with regard to normal immune system parameters in the Southern sea otters exists. Therefore, a panel of immunlogical tests that are commonly used to detect immunological dysfunction in other species was adapted for use in studying the Southern sea otter. Previous studies in seals, beluga whales and bottlenose dolphins have demonstrated that immune function assays could be successfully adapted for application in marine mammals (Bernier et al., 2000; De Guise et al., 1995; De Swart et al., 1995; DiMolfetto-Landon et al., 1995; Erickson et al., 1995; Romano et al., 1992; Ross et al., 1996). These studies were extended to include development and characterization of monoclonal antibodies specific for leukocyte surface antigens in cetaceans (Beineke et al., 2001; De Guise et al., 1998, 2002, 2003); however, application on free-ranging populations is as yet quite limited. The current study identified monoclonal antibodies developed for other species that were cross-reactive with sea otter

leukocytes antigens. These were successfully used to define baseline values for circulating numbers of T and B lymphocytes, and cell-surface expression of MHC class II proteins, in free-ranging animals.

The absolute leukocyte and lymphocyte numbers for the adults in our study are comparable to those previously reported (Williams et al., 1992). The mean leukocyte and lymphocyte numbers in pups were equal, or slightly elevated, as compared to the adults in the present study. This is in contrast to that previously reported by Williams et al. (1992) in which leukocyte and lymphocyte numbers were found to be slightly lower in pups as compared to juveniles and adults. We would hypothesize that the pups examined in the prior study were unhealthy and/or premature, since they were found beached and sampled upon arrival at the rehabilitation center. A characteristic process of neonatal lymphocytosis, by which low lymphocyte numbers in the newborn increase significantly to reach levels equal or above adult values during the immediate post-natal period, has been well documented in both veterinary species and humans (Bortnick et al., 1999; Faldyna et al., 2001; Gajl-Peczalska et al., 1974; Smith et al., 2002). Since the pups in the present study were free-ranging, and with their mothers at the time of sampling, it is highly probable that the leukocyte values reported here more accurately represent normal ranges for a healthy population.

Analytical flow cytometry and cross-reactive monoclonal antibodies were used to deliniate lymphocyte subsets and expression of MHC class II proteins. Alterations in immunophenotype can be useful indicator(s) of a variety of systemic insults to the host (Aldridge et al., 2001; Folds and Normansell, 1999; Vos and Van Loveren, 1998). The primary aim of our study was to examine the composition and function of circulating immune cells of the Southern sea otter in greater detail and to establish baseline immunological parameters for this species (Table 2). This is prerequisite to the application of advanced immunologic techniques for identifying the influence of environmental stressors on sea otter health. The immunological analyses performed in this study revealed a similar overall pattern of lymphoid subpopulations as that reported in other species, with only relatively minor species variation. The absolute numbers of T and B cells for the adult sea otter population closely resembled reference ranges established for adult humans (Folds and Normansell, 1999). The relatively high proportion of T to B lymphocytes identified in the otters was similar to that previously described in domestic veterinary species (Bortnick et al., 1999; Faldyna et al., 2001; Greeley et al., 2001; Smith et al., 2002). Unlike human and murine species, a large percentage of sea otter peripheral blood T cells expressed MHC class II proteins (Table 2). Such expression has similarly been reported in other marine mammal species (beluga whales, bottlenose dolphins, and harbor porpoises) as well as in domestic species such as swine, cats, dogs, and horses (Beineke et al., 2001; Bernier et al., 2000; Lunn et al., 1993; Rideout et al., 1992; Romano et al., 1992).

The age-associated increase in the number of circulating T- and B-lymphocytes in the pups as compared to the adults is similar to that reported in other species including humans, dogs, cats and horses (Bortnick et al., 1999; Faldyna et al., 2001; Greeley et al., 2001; Hicks et al., 1983; Smith et al., 2002). Interestingly, not only were the B cells increased in number in the pup, but there was also a higher density of MHC II expression on their surface compared to adults (Table 2). An age-associated change in MHC II on B cells has been described in studies in mice and humans, in which down-regulation of this molecule occurred in the elderly (Janick-Buckner et al., 1991). There is however, limited information on the changes in MHC II expression levels in the post-natal period. The higher level of expression in the pups compared to adults may be either developmental or related to environmental factors such as pathogen exposure. The increased expression of MHC II on B cells in the absence of an increase on T cells in the sea otter pups further suggests that this change was less likely to be related to pathogen exposure and more likely representative of a normal developmental adaptation of the immune system. These observations have important health implications since it would appear that, similar to human infants and the young of other domestic species, sea otter pups are at a vulnerable stage of immunological development and may be particularly sensitive to chemical and microbial changes in their environment. This information could be important in making decisions directed at restricting human usage or effluent input in areas where young animals may be present.

The number of B cells and level of cell-surface MHC II expression was also variable between individuals within the adult group. Since this study was performed on a population of free-ranging adults, high variability between individuals may be expected. However, the variability for B cell number and B cell MHC II expression was significantly greater than for other immunological parameters, suggesting that these parameters may be inherently more sensitive to external or internal stressors specific to each individual sea otter. These findings highlight the potential benefits of performing detailed immunophenotypic analyses in wildlife, in that they can be used to identify animals with subtle immune alterations that might indicate greater risk of morbidity or mortality.

Immunophenotypic analysis, although beneficial, is limited in that it is not direct measure of immune function, and therefore, should be used in conjunction with more specific tests of mononuclear cell function. Mitogen-induced cellular proliferation and interleukin-2 receptor (IL-2R) expression was used in this study for assessing mononuclear cell function; IL-2R expression measures the ability to mobilize membrane proteins involved in lymphocyte activation, while mitogen-induced proliferation measures the ability of lymphocytes to divide (Erickson et al., 1995). A wide range in the proliferative response of different individuals was identified. In general, this variation was greater with ConA stimulation, probably due to the greater stimulation values for this mitogen as compared to the PWM-induced response. This wide variation was actually advantageous, in that animals with levels of proliferation further from the mean of the population could be identified more readily than with PWM stimulation. Since no real additional information was gained from using two mitogens, we would recommend using only ConA if limited sample is available.

Two concentrations of each mitogen were employed in the present study and referred to as high or low concentrations. These concentrations were arrived at by serial titration of each mitogen. The ''high'' concentration was that which routinely gave the most optimal response. The ''low'' concentration represented that which still induced a substantial response but any further reduction in concentration resulted in a dramatic decrease in stimulation. This ''low'' concentration was employed in an attempt to identify subtle decreases in lymphocyte function as apposed to outright lymphocyte suppression. Previous unpublished studies in horses, dolphins, and killer whales (Horohov, D., Stott, J.L., unpublished data) suggest that physiological stress can induce a response pattern characterized by good response to a high concentration of mitogen, yet failure to respond to a low concentration of the same mitogen. This aberration manifests as an elevated response ratio of high to low. Failure to respond to both high and low concentrations of mitogens was interpreted as an indication of substantial immunosuppression (Folds and Normansell, 1999). Harbor seals fed contaminated herring from the Baltic Sea had significantly decreased T lymphocyte responses to high doses of mitogen (De Swart et al., 1995). In contrast, recent studies reported an elevated response to both high and low concentrations of mitogen associated with a heightened state of lymphocyte activation induced by immunotoxic chemicals (McMurry et al., 1999; Schwartz et al., 2004). Three animals were identified in the present study with abnormal lymphocyte proliferation response patterns; animal A responded with a heightened response to both concentrations of mitogen, animal C responded well to a high concentration of mitogen but failed to respond to the low concentration, while animal S failed to respond to either mitogen concentration (Fig. 2). Interpretation of these results is limited as all otters in this study appeared healthy and follow up studies were not possible. A hypothesis that these three animals were immunologically perturbed could have been strengthened by additional analyses. One of these animals (animal C, 98004) did, however, demonstrate a low IL-2R expression on peripheral blood lymphocytes, providing additional evidence of a subtle immunologic perturbation. Unfortunately, immunophenotypic analyses were not performed on these three animals because of the opportunistic nature of sample availability at the time the study was conducted. For consistency, and in the absence of information regarding the significance of such proliferation patterns in the sea otter, we included these animals as the outer limits of our normal reference range.

Immunity to any given pathogen is dependent on the host's ability to organize and focus a diverse array of immune cells and soluble proteins against a pathogen. This study describes a panel of immunologic tests that can be used to assess host defense(s) in the sea otter.

Animals that were not otherwise detectable as outliers by routine hematology and serum chemistry analysis could be identified using these more advanced immunological techniques. Similar analyses have proven invaluable when used in controlled studies performed in other species (De Swart et al., 1995; Greeley et al., 2001; Ross et al., 1996; Vos and Van Loveren, 1998; Schwartz et al., 2004). While the validation of these tests in controlled longitudinal studies in captive and free-ranging sea otters is still necessary, they show promise in identifying individuals with immunologic perturbations. The ability to study such immunological parameters, in conjunction with markers of chemical and pathogen exposure, should allow the performance of epidemiological studies examining the influences of environmental and anthropogenic factors on the health of the Southern sea otter population.

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References

Aldridge, B.M., King, D.P., Kennedy-Stoskopf, S., Stott, J.L., 2001. Immunology. In: Dierauf, C.A., Gulland, F.M.D. (Eds.), CRC Handbook of Marine Mammal Medicine, 2nd ed. CRC Press, Ohio, pp. 237–248.

Ballachey, B.E., Bodkin, J.L., Howlin, S., Kloecker, K.A., Monson, D.H., Rebar, A.H., Snyder, P.W., 1996a. Hematology and Serum Chemistry of Sea Otters in Oiled and Unoiled Areas of Prince William Sound, Alaska, 1996–1998. *Exxon Valdez* Oil Spill State/Federal Natural Resource Damage Assessment Report.

Ballachey, B.E., Stegeman, J.J., Snyder, P.W., Blundell, G.M., Bodkin, J.L., Dean, T.A., Duffy, L.K., Esler, D., Golet, G., Jewett, S., et al., 1996b. Oil Exposure and Health of Nearshore Vertebrate Predators in Prince William Sounds Following the 1989 *Exxon Valdez* Oil Spill. *Exxon Valdez* Oil Spill State/Federal Natural Resource Damage Assessment Report.

Bayha, K., Hill, K., 1990. Overall capture strategy. US Fish and Wildlife Service Biological Report, pp. 41–60.

Beineke, A., Siebert, U., Wunschmann, A., Stott, J.L., Prengel, I., Kremmer, E., Baumgartner, W., 2001. Immunohistochemical investigation of the cross-reactivity of selected cell markers from various species for characterization of lymphatic tissues in the harbour porpoise (*Phocoena phocoena*). J. Comp. Pathol. 125, 311–317.

Bernier, J., De Guise, S., Martineau, D., Beland, P., Beaudet, M., Fournier, M., 2000. Purification of functional T lymphocytes from splenocytes of the beluga whales (*Delphinapterus leucas*). Dev. Comp. Immunol. 24, 653–662.

Blanchard-Channell, M., Moore, P.F., Stott, J.L., 1994. Characterization of monoclonal antibodies specific for equine homologues of CD3 and CD5. Immunology 82, 548–554.

Bortnick, S.J., Orandle, M.S., Papadi, G.P., Johnson, C.M., 1999. Lymphocyte subsets in neonatal and juvenile cats: comparison of blood and lymphoid tissues. Lab. Anim. Sci. 49, 395–400.

Cole, R.A., Lindsay, D.S., Howe, D.K., Roderick, C.L., Dubey, J.P., Thomas, N.J., Baeten, L.A., 2000. Biological and molecular characterizations of *Toxoplasma gondii* strains obtained from Southern sea otters (*Enhydra lutris nereis*). J. Parasitol. 86, 526–530.

De Guise, S., Martineau, D., Beland, P., Fournier, M., 1995. Possible mechanisms of action of environmental contaminants on St. Lawrence beluga whales (*Delphinapterus leucas*). Environ. Health Perspect. 103 (Suppl. 4), 73–77.

De Guise, S., Erickson, K., Blanchard, M., Dimolfetto, L., Lepper, H., Wang, J., Stott, J.L., Ferrick, D.A., 1998. Characterization of a monoclonal antibody that recognizes a lymphocyte surface antigen for the cetacean homologue to CD45R. Immunology 94, 207–212.

De Guise, S., Erickson, K., Blanchard, M., DiMolfetto, L., Lepper, H.D., Wang, J., Stott, J.L., Ferrick, D.A., 2002. Monoclonal antibodies to lymphocyte surface antigens for cetacean homologues to CD2, CD19 and CD21. Vet. Immunol. Immunopathol. 84, 209–221.

De Swart, R.L., Ross, P.S., Timmerman, H.H., Vos, J.G., Osterhaus, A.D.M.E., 1995. Impaired cellular immune response in harbour seals (*Phoca vitulina*) feeding on environmentally contaminated herring. Clin. Exp. Immunol. 101, 480–486.

DiMolfetto-Landon, L., Erickson, K.L., Blanchard-Channell, M., Jeffries, S.J., Harvey, J.T., Jessup, D.A., Ferrick, D.A., Stott, J.L., 1995. Blastogenesis and interleukin-2 receptor expression assays in the harbor seal (*Phoca vitulina*). J. Wildl. Dis. 31, 150–158.

Erickson, K.L., DiMolfetto-Landon, L., Wells, R.S., Reidarson, T., Stott, J.L., Ferrick, D.A., 1995. Development of an interleukin-2 receptor expression assay and its use in evaluation of cellular immune responses in bottlenose dolphin (*Tursiops truncatus*). J. Wildl. Dis. 31, 142–149.

Faldyna, M., Leva, L., Knotigova, P., Toman, M., 2001. Lymphocyte subsets in peripheral blood of dogs—a flow cytometric study. Vet. Immunol. Immunopathol. 82, 23–37.

Folds, J.D., Normansell, D.E., 1999. Pocket Guide to Clinical Immunology. American Society for Microbiology Press, Washington, DC.

Funke, C., King, D., McBain, J., Adelung, D., Stott, J., 2003. Expression and functional characterization of killer whale (*Orcinus orca*) interleukin 6 (IL-6) and development of a competitive immuno-assay. Vet. Immunol. Immunopathol. 93, 69–79.

Gajl-Peczalska, K.J., Hallgren, H., Kersey, J.H., Zusman, J., Yunis, E.J., 1974. Letter: B lymphocytes during ageing. Lancet 2, 163.

Greeley, E.H., Ballam, J.M., Harrison, J.M., Kealy, R.D., Lawler, D.F., Segre, M., 2001. The influence of age and gender on the immune system: a longitudinal study in Labrador Retriever dogs. Vet. Immunol. Immunopathol. 82, 57–71.

Harvell, C.D., Kim, K., Burkholder, J.M., Colwell, R.R., Epstein, P.R., Grimes, D.J., Hofmann, E.E., Lipp, E.K., Osterhaus, A.D.M.E., Overstreet, R.M. et al., 1999. Emerging marine diseases, climate links and anthropogenic factors. Science 285, 1505–1510.

Hicks, M.J., Jones, J.F., Minnich, L.L., Weigle, K.A., Thies, A.C., Layton, J.M., 1983. Age-related changes in T- and B-lymphocyte subpopulations in the peripheral blood. Arch. Pathol. Lab. Med. 107, 518–523.

Janick-Buckner, D., Briggs, C.J., Meyer, T.E., Harvey, N., Warner, C.M., 1991. Major histocompatibility complex antigen expression on lymphocytes from aging strain A mice. Growth Dev. Aging 55, 53–62.

Kannan, K., Guruge, K.S., Thomas, N.J., Tanabe, S., Giesy, J.P., 1998. Butyltin residues in Southern sea otters (*Enhydra lutris nereis*) found dead along California coastal waters. Environ. Sci. Technol. 32, 1169–1175.

Kennedy, S., 1998. Morbillivirus infections in aquatic mammals. J. Comp. Pathol. 119, 201–225.

King, D.P., Schrenzel, M.D., McKnight, M.L., Reidarson, T.H., Hanni, K.D., Stott, J.L., Ferrick, D.A., 1996. Molecular cloning and sequencing of interleukin 6 cDNA fragments from the harbor seal (*Phoca vitulina*), killer whale (*Orcinus orca*) and Southern sea otter (*Enhydra lutris nereis*). Immunogenetics 43, 190–195.

King, D.P., Parselles, R., Gulland, F.M., Lapointe, J.M., Lowenstine, L.J., Ferrick, D.A., Stott, J.L., 1998. Antigenic and nucleotide characterization of a herpesvirus isolated from Pacific harbor seals (*Phoca vitulina richardsii*). Arch. Virol. 143, 2021–2027.

King, D.P., Lie, A.R., Goldstein, T., Aldridge, B.M., Gulland, F.M., Haulena, M., Adkison, M.A., Lowenstine, L.J., Stott, J.L., 2001. Humoral immune responses to phocine herpesvirus-1 in Pacific harbor seals (*Phoca vitulina richardsii*) during an outbreak of clinical disease. Vet. Microbiol. 80, 1–8.

Laidre, K.L., Jameson, R.J., DeMaster, D.P., 2001. An estimation of carrying capacity for sea otters along the California coast. Mar. Mammal Sci. 17, 294–309.

Lipscomb, T.P., Scott, D.P., Garber, R.L., Krafft, A.E., Tsai, M.M., Lichy, J.H., Taubenberger, J.K., Schulman, F.Y., Gulland, F.M., 2000. Common metastatic carcinoma of California sea lions (*Zalophus californianus*): evidence of genital origin and association with novel gammaherpesvirus. Vet. Pathol. 37, 609–617.

Lunn, D.P., Holmes, M.A., Duffus, W.P., 1993. Equine T-lymphocyte MHC II expression: variation with age and subset. Vet. Immunol. Immunopathol. 35, 225–238.

Mason, D.Y., Cordell, J.L., Tse, A.G., Van Dongen, J.J., Van Noesel, C.J., Micklem, K., Pulford, K.A., Valensi, F., Comans-Bitter, W.M., Borst, J., Gatter, J.C., 1991. The IgM-associated protein mb-1 as a marker of normal and neoplastic B cells. J. Immunol. 147, 2474–2482.

McMurry, S.T., Lochmiller, R.L., McBee, K., Qualls Jr., C.W., 1999. Indicators of immunotoxicity in populations of cotton rats (*Sigmodon hispidus*) inhabiting an abandoned oil refinery. Ecotoxicol. Environ. Safe. 42, 223–235.

Mire-Sluis, A.R., Thorpe, R., 1998. Laboratory protocols for the quantitation of cytokines by bioassay using cytokine responsive cell lines. J. Immunol. Meth. 211, 199–210.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65, 55–63.

Nakata, H., Kannan, K., Jing, L., Thomas, N., Tanabe, S., Giesy, J.P., 1998. Accumulation pattern of organochlorine pesticides and polychlorinated biphenyls in Southern sea otters (*Enhydra lutris nereis*) founded stranded along coastal California, USA. Environ. Pollut. 103, 45–53.

Rideout, B.A., Moore, P.F., Pedersen, N.C., 1992. Persistent upregulation of MHC class II antigen expression on T-lymphocytes from cats experimentally infected with feline immunodeficiency virus. Vet. Immunol. Immunopathol. 35, 71–81.

Riedman, M.L., Estes, J.A., Staedler, M.M., Giles, A.A., Carlson, D.R., 1994. Breeding patterns and reproductive success of California sea otters. J. Wildl. Manage. 58, 391–399.

Romano, T.A., Ridgway, S.H., Quaranta, V., 1992. MHC class II molecules and immunoglobulins on peripheral blood lymphocytes of the bottlenosed dolphin, *Tursiops truncatus*. J. Exp. Zool. 263, 96–104.

Ross, P.S., De Swart, R.L., Timmerman, H.H., Reijnders, P.J.H., Vos, J.G., Van Loveren, H., Osterhaus, A.D.M.E., 1996. Suppression of natural killer cell activity in harbour seals (*Phoca vitulina*) fed Baltic Sea herring. Aquat. Toxicol. 34, 71–84.

Ross, P.S., De Swart, R.L., Timmerman, H.H., Reijnders, P.J.H., Vos, J.G., Van Loveren, H., Osterhaus, A.D.M.E., 1996. Suppression of natural killer cell activity in harbour seals (*Phoca vitulina*) fed Baltic Sea herring. Aquat. Toxicol. 34, 71–84.

Schwartz, J.A., Aldridge, B.M., Mohr, F.C., Stott, J.L., 2004. Immunophenotypic and functional effects of bunker C fuel oil on the immune system of American mink (*Mustela vison*). Vet. Immunol. Immunopathol., in press.

Smith III, R., Chaffin, M.K., Cohen, N.D., Martens, R.J., 2002. Age-related changes in lymphocyte subsets of quarter horse foals. Am. J. Vet. Res. 63, 531–537.

Thomas, N., Cole, R.A., 1996. Endangered Species Update. Paper presented at: Conservation and Management of the

Southern Sea Otter Monterey Bay Aquarium, Monterey, California.

Tomonari, K., 1988. A rat antibody against a structure functionally related to the mouse T-cell receptor/T3 complex. Immunogenetics 28 (6), 455–458.

Vos, J.G., Van Loveren, H., 1998. Experimental studies on immunosuppression: how do they predict for man? Toxicology 129, 13–26.

Williams, T.D., Williams, A.L., Siniff, D.B., 1981. Fentanyl and azaperone produced neuroleptanalgesia in the sea otter (*Enhydra lutris*). J. Wildl. Dis. 17, 337–342.

Williams, T.D., Rebar, A.H., Teclaw, R.F., Yoos, P.E., 1992. Influence of age, sex, capture technique, and restraint on hematologic measurements and serum chemistries of wild California sea otters. Vet. Clin. Pathol. 21, 106–110.