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## BLASTOGENESIS AND INTERLEUKIN-2 RECEPTOR EXPRESSION ASSAYS IN THE HARBOR SEAL (*PHOCA VITULINA*)

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**ABSTRACT:** Two in vitro functional assays were developed to evaluate mitogen-induced responses of peripheral blood mononuclear leukocytes (PBML) from free-ranging harbor seals, *Phoca vitulina*. Lymphocyte proliferation was measured by a standard blastogenesis assay following optimization of culture conditions including mitogen concentration, cell density, and incubation time. These optimized parameters, with the exception of incubation time, were subsequently employed to measure lymphocyte activation by analytical flow cytometry using fluorochrome-based identification of cell surface interleukin-2 receptor (IL-2r) expression. Baseline values established for free-ranging harbor seals had extensive animal variability; there was evidence that the samples were derived from a group of animals with a normal distribution. Positive correlations were observed between blastogenesis assays, and between blastogenesis and activation assays, when using pokeweed or concanavalin A as the stimulus. However, no relationship was found in the expression of the IL-2r induced by these mitogens. This result supports the contention that the two mitogens stimulate different lymphocyte subpopulations. This was observed only with the IL-2r expression assay because of its unique ability to measure the number of T lymphocytes initially activated rather than the ultimate number of progeny cells identified by blastogenesis. Both assays, used concurrently, should provide a more comprehensive representation of lymphocyte competence and serve as a measure of animal health.

**Key words:** Harbor seal, *Phoca vitulina*, blastogenesis, interleukin-2 receptor, mitogen.

### INTRODUCTION

Interest in the health of marine mammals has increased in recent years, due in part to the attention given to human influence on the environment and an increased awareness in the number of mass strandings and epizootics of coastal species. For harbor seals (*Phoca vitulina*) in particular, in addition to reports of mass mortalities, the number of wild pups admitted to The Marine Mammal Center (Marin Headlands, Sausalito, California), a pinniped rehabilitation center that responds to 1,600 km of coastline, has increased gradually since 1984 (Gerber et al., 1993).

The immune system represents one indicator of health, and since its analysis is made possible via the accessibility of peripheral blood, measures of immunologic status should be a useful gauge of marine

mammal well-being. Development of leukocyte functional assays, monoclonal antibodies specific for leukocyte differentiation and activation antigens, and molecular complementary DNA probes for cytokine analysis will enable a thorough phenotypic and functional examination of the harbor seal immune system. Past studies involving harbor seals often have consisted of either clinical evaluations of pups in areas where strandings and mortalities are frequently observed (Steiger et al., 1989) or investigations of infectious agents to which seals are susceptible. In particular, viral infections of seals have received in-depth attention; characterization of various phocid viruses (Barrett et al., 1993), their role in mass mortalities (Osterhaus et al., 1989), and clinical or histopathologic manifestations resulting from viral infection (Hofmeister et al., 1988) have been published. In addition, some toxicological

work specifically related to exposure of harbor seals to various environmental contaminants has been reported (Himeno et al., 1989).

Few immunologic studies have been reported for harbor seals. Isotype analysis and quantification of immunoglobulins in seal peripheral blood have been investigated (Carter et al., 1990) as well as the histology of seal lymphoid tissue (Schumacher and Welsch, 1990). Cellular immune studies are less common (Ross et al., 1993), although recently, de Swart et al. (1993) evaluated antigen-specific and mitogen-induced T and B cell responses using peripheral blood mononuclear cells from harbor seals.

Proliferation, as measured by classical blastogenesis assays, is only one of many responses that lymphocytes can make to stimuli and provides a limited perspective on overall immune status. We describe an additional means of measuring immune competence by identifying expression of a lymphocyte activation antigen following mitogen stimulation. This assay employs flow cytometric evaluation of interleukin-2 receptor (IL-2r) expression on individual T lymphocytes and is not dependent upon cellular proliferation. Both the lymphocyte activation and proliferation assays were standardized and used to begin establishing baseline immunologic values for apparently healthy free-ranging harbor seals.

#### MATERIALS AND METHODS

Blood samples for assay optimization were obtained on 27 January 1993 from six apparently healthy captive harbor seals. Animals included three pups (two males and one female) and three adults (two males and one female). Wild animals were captured in Mowry Slough in San Francisco Bay, California (USA) (37°28'N to 37°29'N, 122°0'W to 122°2'W) on 9 September 1992; Seal Rock near Monterey, California (36°35'N, 121°57'W) on 27 September 1992; Gertrude Island in Puget Sound, Washington (USA) (47°13'N, 122°40'W) on 15 November 1992 and 21 June 1993; Whitcomb Flats, Washington (46°55'N, 124°4'W) on 15 June 1993; and the Umpqua River, Oregon (USA) (43°41'N, 124°5'W) on 17 June 1993. Seventeen adults (14

males and three females) and 23 subadults (16 males and seven females) were sampled. Length, girth, and weight of adults averaged 149 cm, 108 cm, and 78 kg, respectively, with ranges of 124 to 172 cm, 101 to 112 cm, and 52 to 111 kg. Length, girth, and weight of subadults averaged 124 cm, 88 cm, and 44.5 kg, respectively, with ranges of 95 to 140 cm, 70 to 100 cm, and 30 to 60 kg. In some instances, animals were heavy but short or unusually long but light. Therefore, although length, girth, and weight were measured, age classification was subjectively based on overall size and appearance of the animal. None of the females were pregnant or lactating.

Blood was drawn from the extradural vein and collected into sterile tubes containing acid citrate dextrose (Becton Dickinson Vacutainer Systems, Rutherford, New Jersey, USA). Samples were shipped overnight on wet ice to the laboratory and were evaluated within 24 hr of blood collection. All samples collected from a given location on a given date were evaluated simultaneously, and all samples, regardless of collection date, were evaluated in an identical fashion. Mononuclear cells were isolated by centrifugation of blood for 20 min at  $700 \times G$ . Buffy coats were diluted 1:2 in phosphate-buffered saline (PBS), pH 7.4, and mononuclear cells were further purified by density gradient centrifugation using 5 ml Histopaque-1077 (Sigma Diagnostics, St. Louis, Missouri, USA) per 10 ml of whole blood. Gradients were centrifuged for 30 min at  $700 \times G$ . The resulting interface was removed and washed twice in PBS with 5 min centrifugations at  $250 \times G$ . Cell pellets were resuspended to  $2 \times 10^6$ /ml in Dulbecco's Modified Eagle Media containing 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM minimal nonessential amino acids, 10  $\mu$ g/ml gentamicin, 5 unit/ml penicillin, 50  $\mu$ g/ml streptomycin (media and supplements were from Gibco Laboratories Life Technologies, Inc., Grand Island, New York, USA) and 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah, USA).

Six captive animals were used to determine optimal conditions for the immunologic assays; however, all titrations (cell number, mitogen concentration, and incubation time) could not be conducted on all six animals due to limited blood volume. Thus, cells were analyzed in the following priority: blastogenesis to determine optimal mitogen concentration, blastogenesis to titrate incubation time, IL-2r expression assay kinetics, and blastogenesis to determine optimal cell concentration.

Blastogenesis assays were performed as follows. Peripheral blood mononuclear leukocytes (PBML) were cultured in 96-well microtiter

plates (Costar Corporation, Cambridge, Massachusetts, USA) containing 0.2 ml total volume with either concanavalin A (Con A), phytohemagglutinin-L (PHA), pokeweed mitogen (PWM), or no mitogen (all mitogens from Sigma Chemical Company, St. Louis, Missouri). All variable conditions were evaluated in triplicate. The various mitogen concentrations tested were 20, 10, 2.5, 0.5, and 0.1  $\mu\text{g}/\text{ml}$  for Con A; 25, 12.5, 5, 2.5, and 1  $\mu\text{g}/\text{ml}$  for PHA; and 12.5, 2.5, 0.5, 0.1, and 0.02  $\mu\text{g}/\text{ml}$  for PWM. Five different cell concentrations were tested ranging from  $8 \times 10^5$  to  $5 \times 10^4$  cells per well, with the concentrations decreasing two-fold within that range. Cultures were incubated at 37 C in 5%  $\text{CO}_2$  for 12, 24, 48, 72, 96, or 120 hr prior to the addition of  $^3\text{H}$ -thymidine (specific activity 2 curies/mmol, Amersham Life Science, Arlington Heights, Illinois, USA). Invariant conditions employed for assay optimization included a cell concentration of  $2 \times 10^5$ /well, incubation time of 72 hr, and mitogen concentrations for Con A at 2.5  $\mu\text{g}/\text{ml}$ , PHA at 12.5  $\mu\text{g}/\text{ml}$ , and PWM at 0.5  $\mu\text{g}/\text{ml}$ ; these invariant conditions were also used to establish baseline values for free-ranging animals.

Tritiated thymidine was added at 1  $\mu\text{Ci}$  per culture well in 0.02 ml media. Following 15 hr of incubation, cells were harvested onto glass fiber filters using a PHD $\text{\textcircled{R}}$  harvester (both from Cambridge Technology, Inc., Watertown, Massachusetts). Individual filters for each well were placed into polyethylene scintillation vials (Kimble Glass through Fisher Scientific, Pittsburgh, Pennsylvania, USA) with three ml liquid scintillation cocktail (Ready Safe, Beckman Instruments, Inc., Fullerton, California). Counts per minute (CPM) were determined by a model LS-3801 Beckman scintillation counter (Beckman Instruments, Inc., Palo Alto, California).

The IL-2r expression assays were conducted as follows. One million PBML were dispensed into 24-well tissue culture plates (Costar Corporation) and incubated at 37 C with 5%  $\text{CO}_2$  in a total volume of 1 ml with either 2.5  $\mu\text{g}/\text{ml}$  Con A or 0.5  $\mu\text{g}/\text{ml}$  PWM. After 0, 6, 12, 24, 36, and 48 hr, cells were harvested, and  $2 \times 10^5$  of these were incubated on ice with either phycoerythrin (PE)-conjugated human recombinant IL-2 or PE-conjugated streptavidin (both from Fluorokine Kit, R&D Systems, Minneapolis, Minnesota, USA) for 60 min (Taylor et al., 1992) as per the manufacturer's instructions. The streptavidin-PE control was used to verify the absence of nonspecific binding. The PBML obtained from free-ranging animals were evaluated in an identical manner and with the same conditions but always were harvested at 36 hr. Stained cells were analyzed at 488 nm on a FACStar-Plus flow cytometer (Becton Dickinson

Immunocytometry Systems, Mountain View, California) with an Innova 90 5-W argon ion laser at 200 mW (Coherent, Palo Alto, California). Ten thousand events were collected in list mode and a live gate was set to include only lymphocytes and monocytes as identified by their forward and side light scatter characteristics. Cells lacking the IL-2r were set below the first log of fluorescence using the negative control. Cells identified as expressing the IL-2r at a moderate density were those falling between the first and second log of fluorescence on a dot plot and represented by the first positive peak on a histogram. Cells densely expressing the IL-2r were identified as those above the second log of fluorescence on a dot plot and observed on a histogram as the second positive peak. Cell size was compared to the density of IL-2r expression by analysis of dot plots consisting of forward scatter versus fluorescence.

All statistical tests were based on Kuo et al. (1992). Student's *t*-tests were performed to identify potential differences between proliferative responses and IL-2r expression of free-ranging subadults versus adults and males versus females. A *P* value of less than 0.05 was considered to have statistical significance. Pearson product moment correlation was used to identify a possible correlation between responses induced by the various mitogens. In addition, this test was used to determine the relationship between IL-2r expression and blastogenesis when either Con A or PWM was used as the mitogen. The Kolmogorov-Smirnov normality test was used to determine if the data were drawn from a population with a normal distribution.

## RESULTS

Optimal mitogen concentrations for Con A, PHA, and PWM were 2.5  $\mu\text{g}/\text{ml}$  for five of six animals, 12.5  $\mu\text{g}/\text{ml}$  for four of six animals, and 0.5  $\mu\text{g}/\text{ml}$  for five of six animals, respectively (Fig. 1). Five animals were used in the assays measuring CPM over time, although due to a lack of a sufficient number of cells, the response of one animal was only measured to 96 hr. Optimal incubation times for Con A and PWM tended to be 72 hr and  $\geq 120$  hr, respectively (Fig. 2). Optimal incubation time for PHA was highly variable and ranged from 48 hr to  $\geq 120$ . The optimum effect of cell number on CPM ranged between  $2$  and  $4 \times 10^5$  cells per well (Fig. 3).

There was an increase in IL-2r expression from the initial measurement before

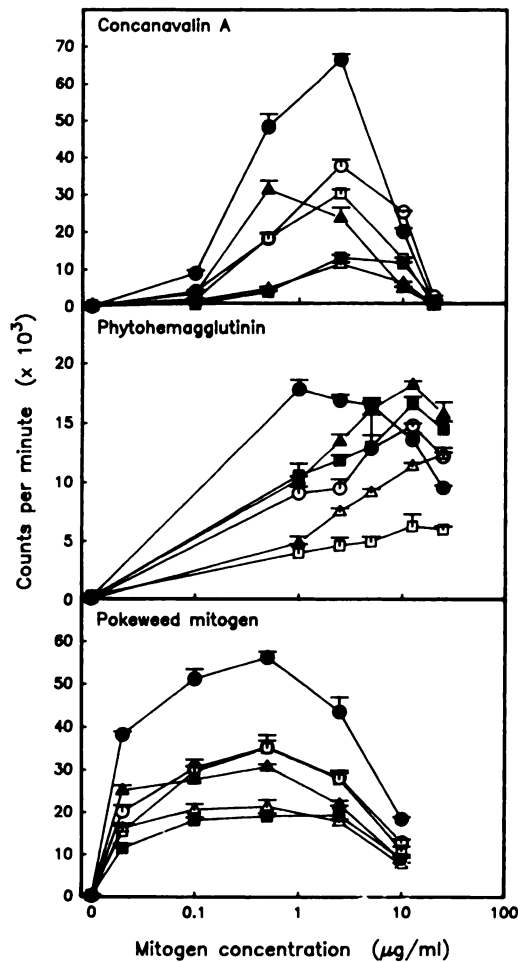


FIGURE 1. Proliferative responses of harbor seal peripheral blood mononuclear leukocytes to increasing mitogen concentrations as determined by  $^3\text{H}$ -thymidine incorporation. Individual animals are represented by the same symbol for each mitogen. Error bars represent standard deviation based on counts per minute from triplicate wells.

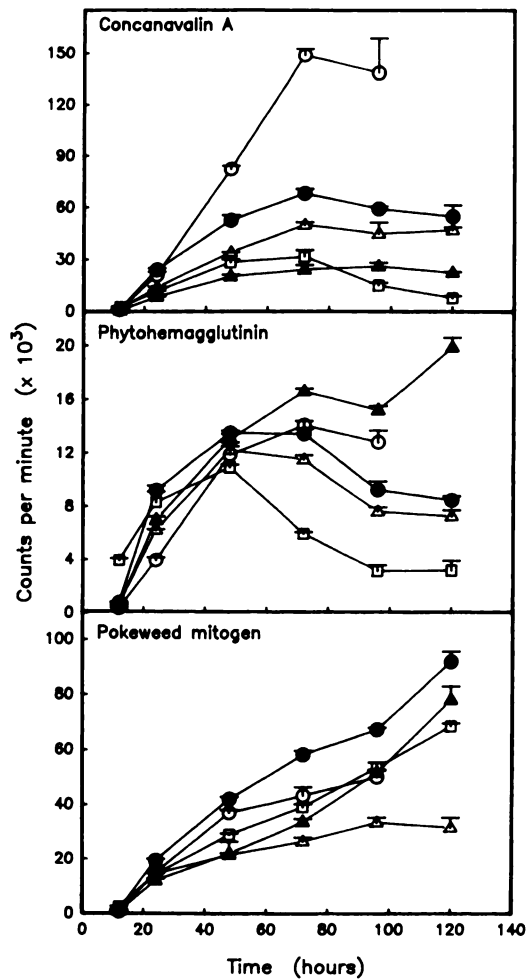


FIGURE 2. Proliferative responses of harbor seal peripheral blood mononuclear leukocytes over time as measured by  $^3\text{H}$ -thymidine incorporation. Individual animals are represented by the same symbol for each mitogen. Error bars represent standard deviation based on counts per minute from triplicate wells.

cells had been cultured with mitogen until it reached a peak for most animals following 48 hr in culture (Fig. 4A). The temporal increase in IL-2r expression was observed for cells cultured in either Con A or PWM, although the kinetics of expression differed; while the percentage of cells induced to express the receptor by PWM was initially more rapid (12 hr time-point), the percentage of cells ultimately activated by Con A always exceeded that of PWM. Typically, cells within the cultures could

be categorized into three distinct populations based upon the amount of fluorescence as measured by flow cytometry: cells lacking the IL-2r, cells expressing the IL-2r at a low to moderate density, and cells expressing high density IL-2r. These populations are represented by the peaks on a histogram at 36 hr of culture with either Con A (Fig. 4B) or PWM (Fig. 4C) respectively. Although the kinetics of expression differed, cell surface density profiles at 36 hr were similar between mito-

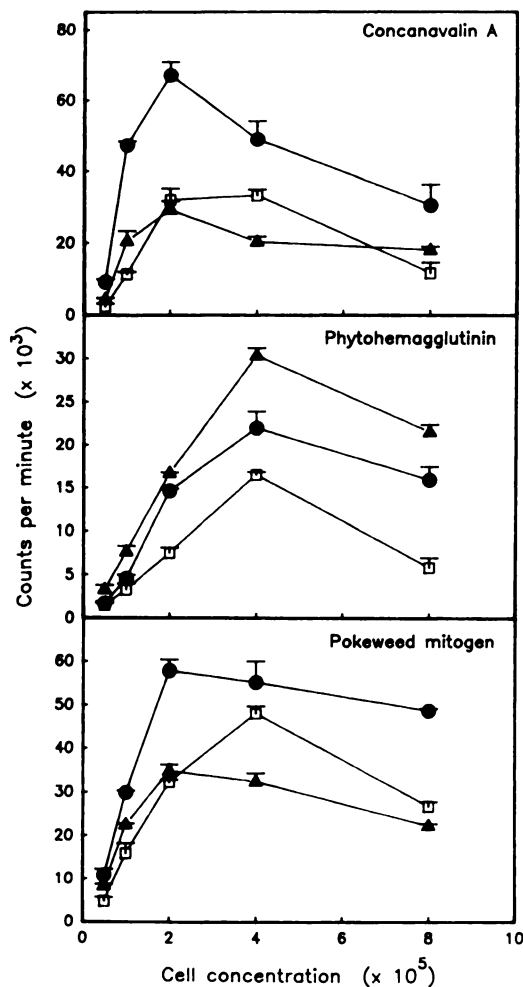


FIGURE 3. Proliferative responses of harbor seal peripheral blood mononuclear leukocytes as a function of cell concentration measured by  $^3\text{H}$ -thymidine incorporation. Individual animals are represented by the same symbol for each mitogen. Error bars represent standard deviation based on counts per minute from triplicate wells.

gens (Fig. 4). A correlation appeared to exist between cell size and IL-2r density, with the largest blasts having the most dense receptor expression and the smallest having few to no receptors (data not illustrated).

Several considerations were made in selecting blastogenesis conditions for assessing free-ranging harbor seals, including the average optimal value, the limited volume of blood generally available for analysis,

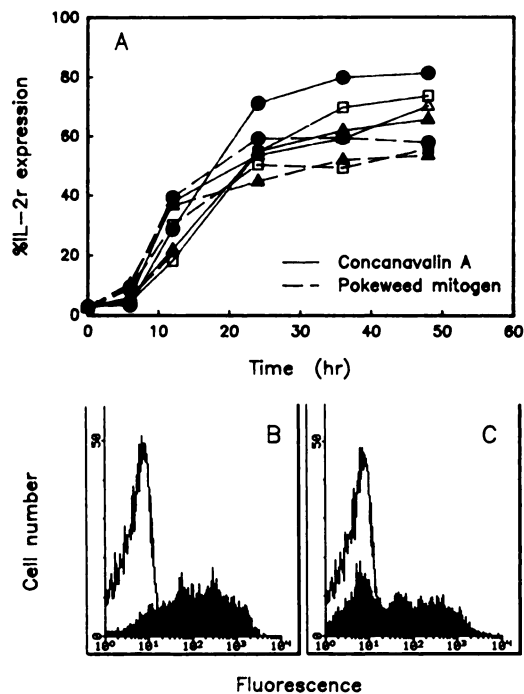


FIGURE 4. A) Kinetics of interleukin-2 receptor (IL-2r) expression by harbor seal peripheral blood lymphocytes. Cells were cultured for the indicated number of hours with either  $2.5 \mu\text{g}/\text{ml}$  concanavalin A (solid line) or  $0.5 \mu\text{g}/\text{ml}$  pokeweed mitogen (broken line). Individual animals are represented by the same symbol. B) Interleukin-2 receptor (IL-2r) density after 36 hr incubation with  $2.5 \mu\text{g}/\text{ml}$  concanavalin A as determined by flow cytometry. The histogram representing the negative control stained with streptavidin-phycoerythrin (SA-PE) (open) is overlaid with the histogram representing IL-2-PE-stained cells (closed). C) Interleukin-2 receptor (IL-2r) density after 36 hr incubation with  $0.5 \mu\text{g}/\text{ml}$  pokeweed mitogen as determined by flow cytometry. The histogram representing the negative control stained with streptavidin-phycoerythrin (SA-PE) (open) is overlaid with the histogram representing IL-2-PE-stained cells (closed).

and the ease of routine laboratory analysis. The conditions used ( $2 \times 10^5$  cells incubated for 72 hr in either  $2.5 \mu\text{g}/\text{ml}$  Con A,  $12.5 \mu\text{g}/\text{ml}$  PHA, or  $0.5 \mu\text{g}/\text{ml}$  PWM) represented a compromise based upon these considerations. Similarly, although the percentage of cells expressing the IL-2r was slightly greater at 48 hr, a 36 hr timepoint was selected for assessment of free-ranging harbor seal PBML.

Proliferative (blastogenesis) and activation (IL-2r expression) responses for wild harbor seals manifesting no signs of clinical illness were variable (Table 1). In general, Con A elicited a greater PBML response than either PHA (blastogenesis assay only) or PWM (both assays). Proliferative responses of individual animals were consistent; they were either relatively high, intermediate, or low regardless of the mitogen used as indicated by positive correlations (Con A and PHA, correlation coefficient = 0.553,  $P < 0.001$ ; Con A and PWM, correlation coefficient = 0.556,  $P < 0.001$ ; PHA and PWM, correlation coefficient = 0.694,  $P < 0.001$ ). However, no significant relationship existed between Con A- and PWM-induced activation responses of individual animals as measured by IL-2r expression (correlation coefficient = -0.051,  $P > 0.05$ ).

Generally, cultures with the greatest number of high density IL-2r<sup>+</sup> cells were those that had the highest proliferative response as determined by blastogenesis. There was a positive correlation between lymphocyte proliferation and activation assays using both Con A and PWM as stimuli; this correlation was greatest for Con A (correlation coefficient = 0.748,  $P < 0.001$ ) as compared to PWM (correlation coefficient = 0.559,  $P = 0.005$ ). Student's *t*-tests were employed to identify potential differences between lymphocyte responsiveness as functions of age and sex. The only significant differences noted were between subadults and adults when using Con A as the mitogen in a blastogenesis assay ( $P = 0.002$ ). With the exception of PHA-induced blastogenesis, the free-ranging animals were representative of a normal group, based on analysis of all immune function data using the Kolmogorov-Smirnov normality test.

#### DISCUSSION

Blastogenesis assays have been used to assess immunocompetence in a variety of species (Fletcher et al., 1992). Because responses to mitogens vary among species,

TABLE 1. Proliferative (blastogenesis) and activation (interleukin-2 receptor expression) responses of peripheral blood mononuclear leukocytes from free-ranging harbor seals.

Age	Sam- ple size	Mitogen <sup>a</sup>	Mean ( $\pm$ SD) counts per minute	Mean ( $\pm$ SD) %IL-2r expression
Subadults	23	Con A	94,556 $\pm$ 37,392	78 $\pm$ 11
	23	PHA	31,740 $\pm$ 20,682	ND
	23	PWM	41,327 $\pm$ 23,439	46 $\pm$ 17
Adults	17	Con A	56,532 $\pm$ 32,113	71 $\pm$ 10
	17	PHA	27,658 $\pm$ 24,745	ND
	17	PWM	28,477 $\pm$ 21,894	44 $\pm$ 17

<sup>a</sup> Con A, concanavalin A; PHA, phytohemagglutinin-L; PWM, pokeweed mitogen.

<sup>b</sup> ND, not done.

blastogenesis assay conditions must be optimized for the species of interest. However, while optimization increases sensitivity of the assay, and standardization facilitates consistency between experiments and minimizes the volume of blood required, not all animals respond optimally to a restricted set of assay parameters.

Blastogenesis assays are influenced notably by day to day variation (Fletcher et al., 1992); variability in mitogen-induced responses is due partly to the differential expression by lymphocyte subpopulations of the various mitogen-specific surface oligosaccharides. Con A and PHA generally are considered T cell-specific mitogens, while PWM may stimulate both B and T cells (Greaves et al., 1974). A recent report by de Swart et al. (1993) extends this observation to harbor seals. This group identified IL-2-enhanced proliferative (blastogenesis) responses of Con A-, PHA- and PWM-stimulated PBML, providing evidence that all three mitogens stimulate harbor seal T lymphocytes. In addition, they observed antibody production by harbor seal PBML after stimulation with PWM but not with Con A or PHA; thus PWM may activate harbor seal B cells. These studies add support to the notion that variation in the relative numbers of mononuclear leukocyte subpopulations in the

peripheral blood of a given seal can play a major role in the animal variability shown in Table 1. Accordingly, overinterpretation of blastogenesis data generated as the sole measure of immunocompetence should be avoided.

Activation of immune system cells can evoke several responses. Proliferation of stimulated cells, the response measured by blastogenesis, often ensues; but frequently cells respond by mediating effector functions, expressing cytokine receptors, or secreting cytokines or other soluble factors. Such events may not necessarily be followed by cellular proliferation. Assessment of IL-2r expression recently has gained in popularity as a sensitive and meaningful measure of lymphocyte responsiveness to antigen or mitogen exposure that is not dependent upon cell proliferation (Lai et al., 1991; Nash and Mastro, 1993). Availability of phycoerythrin-conjugated human IL-2 and the cross-species reactivity of this lymphokine (Taylor et al., 1992) have permitted its application in measuring IL-2r expression in harbor seals.

We describe an IL-2r expression assay that can be used in conjunction with, or as an alternative to, blastogenesis to assess cellular immunocompetence in harbor seals. There is a rapid acquisition of this T lymphocyte activation antigen following cell stimulation (Fig. 4A). A greater percentage of lymphocytes were activated by Con A, relative to PWM, though the relative activation antigen density profiles at 36 hr were very similar (Fig. 4). The fact that lymphocyte activation induced by PWM did not correlate with Con A induction would support the contention that these two mitogens stimulate different lymphocyte subpopulations. This observation was made possible by the short-term nature of IL-2r expression which permits a relatively precise enumeration of the number of T lymphocytes capable of being activated; this is in contrast to the 72-hr blastogenesis assay in which the percentage of cells in the initial sample that were activated cannot be determined. This latter feature of

blastogenesis is due to an inability to determine if a small number of lymphocytes underwent extensive expansion or a large number of lymphocytes underwent limited expansion.

In our initial studies directed at titrating the various blastogenesis assay parameters demonstrated, we noted that no single set of conditions was optimal for all animals tested. However, these data were used as a basis for selecting blastogenesis conditions (mitogen concentration, incubation time, and cell number) for establishment of baseline values from free-ranging harbor seals. Two deviations from the initial optimization data were employed due to laboratory ease of setting up the assay; while PWM responses peaked at  $\geq 120$  hr of incubation time, a time of 72 hr was selected as this appeared optimal for Con A and PHA. A cell number of  $2 \times 10^5/\text{ml}$  was employed (rather than  $4 \times 10^5/\text{ml}$ ) due to limited blood volume typically available. Those conditions selected for blastogenesis also were selected for analysis of lymphocyte activation (IL-2r expression) using samples from free-ranging harbor seals, with the exception that the incubation period was 36 hr. This short incubation period permitted analysis of the actual number of T lymphocytes in the original sample that were mitogen-activated. Extensive animal variability (Table 1) was observed relative to blastogenesis responses; this was most obvious when using PWM or PHA as the stimulus. Animal variability in the percentage of cells expressing IL-2r was less than the variability observed in blastogenesis and was especially evident when using Con A as the stimulus. This was not surprising as minor differences in the number of cells initially activated (IL-2r expression) were not amplified by proliferation (blastogenesis) over a 72 hour period.

Based on these data generated from free-ranging animals, the samples were derived from a group of animals with a normal distribution (Kolmogorov-Smirnov normality test) with the exception of PHA-



induced proliferation. While we would speculate that those animals with the lowest immune function responses would be those most susceptible to health-related problems, an animal tagging and monitoring program would be required to substantiate this hypothesis.

In summary, in vitro immunologic assays such as those described here can be good indicators of cellular immunocompetence in marine mammals. A range of immune responses determined for healthy harbor seals should enable the identification of lymphocyte abnormalities that can be used diagnostically. Both assays used concurrently may provide a more comprehensive and definitive representation of immunocompetence. The IL-2r expression assay will offer the additional advantage of identifying T lymphocyte activation at the subpopulation level as monoclonal antibodies specific for such subsets become available.

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