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DEVELOPMENT OF AN INTERLEUKIN-2 RECEPTOR EXPRESSION ASSAY AND ITS USE IN EVALUATION OF CELLULAR IMMUNE RESPONSES IN BOTTLENOSE DOLPHIN (*TURSIOPS TRUNCATUS*)

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ABSTRACT: We describe optimization of a peripheral blood mononuclear leukocyte proliferation assay and development of an interleukin-2 receptor (IL-2R) expression assay for bottlenose dolphins (*Tursiops truncatus*). Peripheral blood mononuclear leukocytes obtained from both Sea World (February 1993) and the Naval Command Control and Ocean Surveillance Center (March 1993) (San Diego, California, USA) were stimulated with the mitogens concanavalin A (ConA) and phytohemagglutinin (PHA) and evaluated for optimum proliferation and IL-2R expression. Based on these optimization assays, standard conditions were established and used to assess immune function in a population of apparently healthy, free-ranging bottlenose dolphins from Sarasota Bay, Florida (USA) in June 1993. A positive correlation was observed between proliferation assays using ConA and PHA as the stimulants. However, IL-2R expression induced by both mitogens differed significantly.

Key words: Bottlenose dolphin, *Tursiops truncatus*, lymphocyte blastogenesis assay, mitogen, interleukin-2 receptor, immune function.

INTRODUCTION

Recent mass strandings of several marine mammal species (Visser et al., 1993) have led researchers to question the overall condition of marine mammal health and to investigate the extent of which pollution, infectious disease, stress, and captivity influence the health of these animals (Hokama et al., 1990; Domingo et al., 1992). Little is known about cetacean immunity (Cavagnolo, 1979; Romano et al., 1992) or the effects environmental factors have on their immune development and function. Chemical- and stress-induced immunosuppression have been documented in studies on other well characterized animal systems, such as mice (Lundberg et al., 1992) and humans (James et al., 1993). Establishing correlations between environmental conditions and immunocompetence in marine mammals will be possible only when baseline values for healthy animals have been determined. Determining the functional capability of the immune system will additionally provide an indication of an animal's overall health.

In vitro assays capable of measuring parameters of immune cell function such as proliferation, cytotoxicity, cytokine receptor expression, and cytokine secretion, can be used to establish values for immunocompetence. Proliferation in response to mitogen stimulation has been used to evaluate immune responses in different marine mammal species, including bottlenose dolphins (*Tursiops truncatus*) (Colgrove, 1978; Lahvis et al., 1993) and harbor seals (*Phoca vitulina*) (Ross et al., 1993). Proliferation assays measure the overall ability of an immune cell population to divide in response to a non-specific stimulus. Since lymphocytes and monocytes present in this population do not all have the same proliferative capacity and an assessment of proliferation does not measure other elements of an activated immune system, it is important to develop methods to evaluate responses by particular immune cell subsets and responses other than prolifer-

ation. Assays that measure activation of lymphocytes would be the most valuable, as these cells are responsible for specific immune responses.

Quantifying expression of the interleukin-2 receptor (IL-2R) provides a method of assessing lymphocyte-specific immune responses, since this marker is expressed on activated lymphocytes (Kuby, 1994). Receptor expression data can be combined with proliferation data to yield a more comprehensive picture of the functional potential of a population of lymphocytes. Our objective was to standardize conditions for both the proliferation and IL-2R expression assays. These assays then were used to establish baseline values for healthy dolphins.

MATERIALS AND METHODS

Blood samples used in the development and optimization of immune assays were collected from two sources. Blood collected from four adult females, one adult male, and one 3-yr-old female bottlenose dolphin (age obtained from birth record) at Sea World, San Diego, California (USA) in February 1993 was used to optimize mitogen concentration and IL-2R expression over time. Blood obtained from two adult females and one adult male bottlenose dolphin at the Naval Command Control and Ocean Surveillance Center (NCCOSC), San Diego, California, in March 1993 was used to determine the optimal incubation time for proliferation assays. Two 10 ml vacutainers (Becton Dickinson Vacutainer Systems, Rutherford, New Jersey, USA) containing acid citrate dextrose (ACD) as an anti-coagulant were filled with approximately 8 ml of blood drawn from the tail fluke. The blood was shipped on, but not in direct contact with, ice and prepared for evaluation 24 to 30 hr after blood collection. When received, the blood was placed on a rotator for 30 min at 25 C prior to its use in assays. Blood was collected once from each source and all described experiments were carried out concurrently to minimize variability in experimental conditions.

All cell populations were incubated and all reagents were diluted in Dulbecco's Modified Eagle media (DMEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 5 U/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum (JRH Biosciences, Lenexa, Kansas, USA). Mitogen stocks of concanavalin A (ConA) and phytohemagglutinin (PHA) (Sigma Chemical Company, St. Louis, Missouri, USA) were reconstituted in non-supplemented DMEM to 1 mg/ml, and stock solutions of 100 μg/ml were used to make dilutions.

To isolate peripheral blood mononuclear leukocytes (PBML's) for functional studies, whole blood was centrifuged at 800 × *G* for 15 min. The buffy coat containing the white blood cells was transferred to a 15 ml conical tube and an equal volume of phosphate-buffered saline (PBS), pH 7.4, was added to the tube. Mononuclear cells were separated from the remaining leukocytes and residual erythrocytes by density gradient centrifugation. Five ml of cell suspension were underlayed with 4 ml Histopaque-1077 (Sigma Diagnostics, St. Louis, Missouri) and centrifuged at 500 × *G* for 20 min at 25 C. Alternatively, 4 ml of cell suspension were underlayed with 3 ml Isolymph (Gallard-Schlesinger Industries Inc., Carle Place, New York, USA) and centrifuged at 400 × *G* for 35 min at 25 C. The mononuclear layer was transferred to a 15 ml conical tube and washed twice with PBS. Cells were counted on a hemacytometer (Fisher Scientific, Pittsburgh, Pennsylvania, USA), and a final suspension of 2×10^6 PBML's/ml was made in supplemented DMEM.

Proliferation assays were performed in 96-well tissue culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, New Jersey). Peripheral blood mononuclear leukocytes were added to each well at a volume of 100 μl/well (2×10^5 cells/well). Based on cell number titration using 50, 100, 200, and 400×10^3 cells/well, we found that proliferative responses increased with increasing cell number (data not shown). As dolphin blood samples were limited and 200,000 cells/well gave reliable proliferation results, it was the standard cell concentration used in these proliferation assays. Cells from each animal were evaluated in triplicate for each mitogen concentration using both ConA and PHA. Mitogens were serially diluted (starting from a stock of 100 μg/ml) and added to the appropriate wells at a volume of 100 μl/well. Final mitogen concentrations ranged from 0.01 to 10.0 μg/ml. Negative control wells contained 100 μl of media only. Tissue culture plates were incubated at 37 C in 5% CO_2. After incubating 24 to 168 hr, 1 microcurie (μCi) ^{3}H-thymidine (specific activity 2 Ci/mmol, Amersham Life Science, Arlington Heights, Illinois, USA) was added at a volume of 20 μl/well. Sixteen hours later, the cells were harvested onto glass fiber filters using a PHD® Cell Harvester (Cambridge Technology, Inc., Watertown, Massachusetts, USA) and counts per minute (CPM) were determined in a Beckman LS-3801 scintillation counter (Beckman Instruments, Inc., Palo Alto,

California). Data were summarized as means of three replicates.

Isolated PBML's were analyzed for IL-2R expression after incubation with mitogens. Cells were added to a 24-well tissue culture plate (Falcon, Becton Dickinson) at 250 μl/well (5×10^5 cells/well). Concanavalin A was added to a final concentration of 1.0 μg/ml and PHA to 0.5 μg/ml. Cells were stained as per manufacturers instructions with the Fluorokine® kit (R&D Systems, Minneapolis, Minnesota, USA) and analyzed for receptor expression every 12 hr using flow cytometry. Briefly, cells were removed from the wells, washed in 1 ml RDF1 buffer (R&D Systems), centrifuged in a microcentrifuge at 300 × *G*, and resuspended in 100 μl of RDF1 buffer. Twenty-five microliters were transferred to a new tube and either 10 μl of streptavidin-phycoerythrin (PE) for a negative control or 10 μl recombinant human IL-2-PE were added. Cells then were incubated on ice for 1 hr, washed in RDF1 buffer, resuspended in PBS, and collected on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, California) at 488 nm. Ten thousand events were collected in list mode, and forward and side scatter features were used to set a live gate on the lymphocyte/monocyte population. Data were analyzed using Lysis II software (Becton Dickinson).

Blood was drawn from six male and seven female free-ranging bottlenose dolphins, 2- to 50-yr-old, captured in June 1993 in Sarasota Bay, Florida (USA) (27°24′ to 27°31′N, 82°35′ to 82°41′W). Dolphin ages were determined in two ways. The birth years for many individuals were known from ongoing observational studies over the last 24 yr (Scott et al., 1990). The ages of the remaining animals were determined from examination of growth layer groups in the dentine of teeth (Hohn et al., 1989). Under local anesthesia, a single tooth was extracted from the lower jaw of selected dolphins. Each tooth was decalcified, sectioned, stained, and examined under magnification.

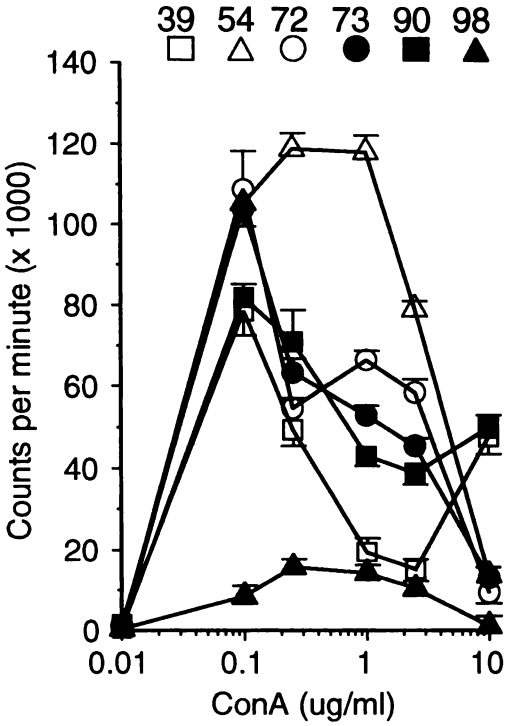
Immune responses of isolated PBML's from the free-ranging dolphin blood were measured using optimal parameters for the two functional assays (ConA and PHA at 0.1 μg/ml, 72 hr incubation for proliferation, and 36 hr incubation for IL-2R expression). Statistical analysis of all data was performed using SigmaStat Statistical Software, version 1.01 (Jandel Scientific Software, San Rafael, California). Statistically significant differences between the various responses of the wild dolphin population were determined by the Student's *t*-test when the samples were drawn from normally distributed populations as determined by the Kolmogorov-Smirnov normality test. Sample values that did not pass the Kolmogorov-Smirnov normality test were assessed for statistically significant differences by the Mann-Whitney Rank Sum Test. Statistically significant correlations between the various responses were determined by the Pearson Product Moment Correlation test. A *P* value of less than 0.05 was considered to have statistical significance for all tests performed.

RESULTS

Although a wide range of proliferative responses to mitogen concentrations was observed between the six Sea World animals, the optimum was consistently observed using 0.1 to 1.0 μg/ml for both ConA and PHA (Fig. 1). Cells incubated with media alone had values between 200 and 600 CPM (data not shown). When blood from the three NCCOSC animals was used in the experiment to determine optimal culture time, the greatest proliferation occurred when cells were stimulated with mitogens for 48 to 72 hr (Fig. 2).

Peripheral blood mononuclear leukocytes from the six Sea World animals were evaluated for IL-2R expression every 12 hr. As with the proliferation data, there was considerable animal variation for IL-2R expression, both in the number of cells expressing the receptor and in the receptor's surface density (Fig. 3). Peak expression for most animals was between 24 and 48 hr.

Based on the optimization assays, conditions chosen to evaluate a population of free-ranging dolphins were 0.1 μg/ml for ConA and PHA, 2×10^5 cells/well and 72 hr of incubation for the proliferation assay; and 5×10^5 cells/well and 36 hr of incubation for the IL-2R expression assays. Thirteen individuals were evaluated. For ConA-stimulated PBML's, the mean (±SD) CPM value for proliferation was 27,616 ± 33,010, and the mean (±SD) percentage for IL-2R expressing cells was 50% ± 11%. For PHA-stimulated PBML's, the mean (±SD) CPM value for proliferation was 35,338 ± 35,624, and the mean (±SD) percentage for IL-2R expressing cells was 68% ± 12%. There was a positive correlation between ConA- and PHA-induced prolif-



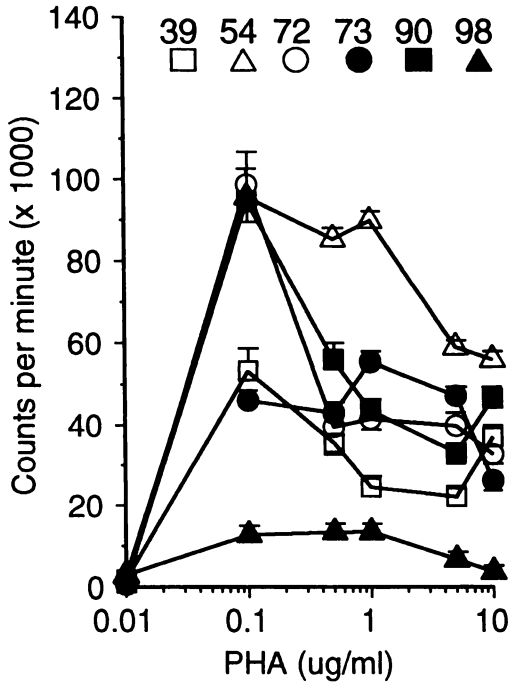
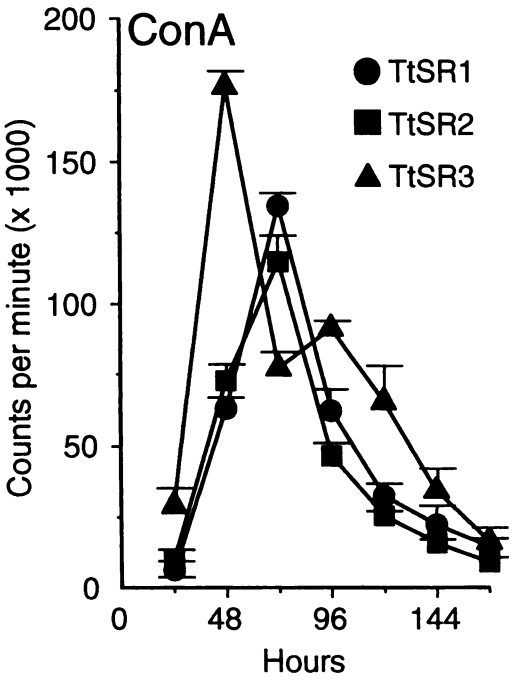


FIGURE 1. Response of bottlenose dolphin peripheral blood mononuclear leukocytes to increasing concentrations of concanavalin A (ConA) or phytohemagglutinin (PHA). Numbers at top refer to individual dolphins. Bars represent standard deviations based on three replicates.



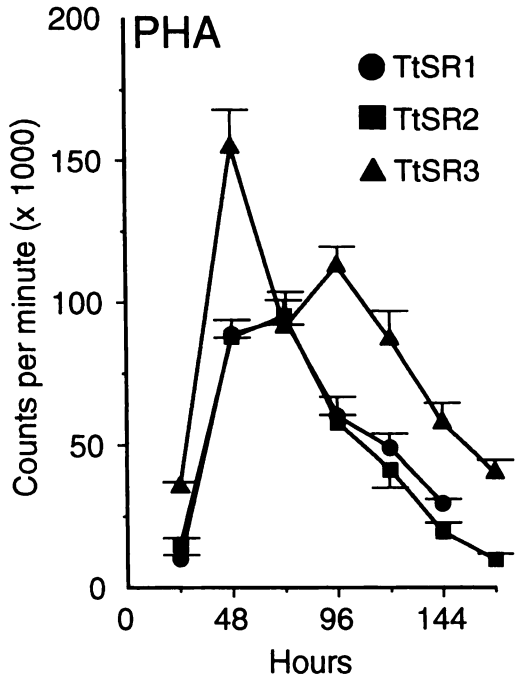


FIGURE 2. Response of bottlenose dolphin peripheral blood mononuclear leukocytes over time with 1.0 μg/ml concanavalin A (ConA) or 0.5 μg/ml phytohemagglutinin (PHA). Numbers at top refer to individual dolphins. Bars represent standard deviations based on three replicates.

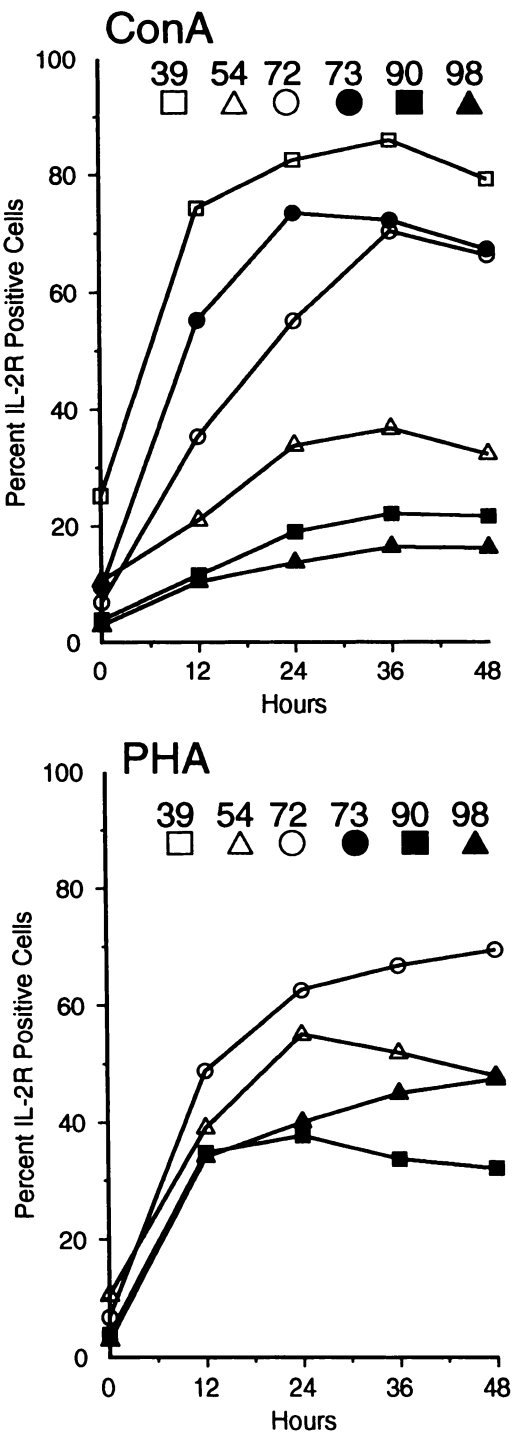


FIGURE 3. Expression of the interleukin-2 receptor (IL-2R) over time by bottlenose dolphin peripheral blood mononuclear leukocytes in response to concanavalin A (ConA) at 1.0 μg/ml or phytohemagglutinin (PHA) at 0.5 μg/ml. Results are expressed as the percent of cells positive for IL-2R. Numbers at top refer to individual dolphins. Animals 39 and 73 were not stimulated with phytohemagglutinin due to low cell numbers.

erative responses ($r = 0.85$, $P < 0.001$). This was not the case for the mitogen-induced IL-2R expression assay ($r = 0.51$ $P = 0.11$). The difference between the mean values of the ConA- and PHA-induced IL-2R expression was statistically significant ($P = 0.002$).

No statistically significant relationship existed between the proliferation and IL-2R expression assays for either mitogen (ConA, $r = 0.334$, $P = 0.315$; PHA, $r = 0.42$, $P = 0.18$). Based on Student's t-test, no significant differences were associated with age or sex. Except for the ConA-induced proliferation data, all other results passed the Kolmogorov-Smirnov normality test; thus, the wild dolphin samples probably were representative of a normally-distributed group.

DISCUSSION

We describe two assays developed specifically for dolphin blood mononuclear cells that measure both early and late events in immune cell activation. This also is the first description of a technique to measure IL-2R expression by dolphin lymphocytes. Interleukin-2 receptor expression involves membrane proteins and secondary messengers and occurs early in lymphocyte activation (Kuby, 1994). This was supported by the 24 to 48 hr optimum time range with significant increases detected as early as 6 hr after incubation with mitogens. Proliferation, however, occurs after IL-2R upregulation and secretion of the autocrine cytokine interleukin-2 (Kuby, 1994). For this reason more time (48 to 96 hr) was required to reach optimum levels of proliferation.

Immune cell proliferation is the culmination of numerous cellular events, including lymphocyte-specific IL-2R expression, and is responsible for the expan-

sion of immune cells in response to antigenic stimuli. However, measuring the ability of a population of immune cells to proliferate in response to a stimulus does not provide specific information about the number and types of immune cells that are proliferating. Thus, immune cell proliferation is a population measurement from which immune competence can be inferred, while IL-2R expression is a quantitative and specific indicator of lymphocyte activation. In support of this, we found that, in contrast to the positive correlation between proliferative responses using ConA and PHA, no correlation was observed between ConA- and PHA-induced IL-2R expression. Thus, because of its ability to specifically quantify activated lymphocytes, we were able to use the IL-2R expression assay to substantiate that ConA and PHA stimulate different lymphocyte subpopulations (Kuby, 1994).

Immune proliferative responses of dolphin blood mononuclear cells to mitogens have been reported by Colgrove (1978) and Lahvis et al. (1993). Both authors reported overall significantly lower values for proliferation using optimal concentrations of mitogens that were higher (1.25 to 12 μg/ml for ConA and 0.25 to 2 μg/ml for PHA) and required longer incubation times (144 and 156 hr for ConA and PHA, respectively) to reach maximum levels. There may be several reasons for these apparent discrepancies. Probably the two most critical differences were in the preservative used for shipping and the preparation of blood mononuclear cells. Relative to the differences in preservatives, in the other two reports, blood was collected in vacutainer tubes containing heparin or disodium ethylenediamine tetraacetate (EDTA). Prior to our study described here, we compared the viability and proliferative capacity of PBML's collected in tubes containing heparin, EDTA and ACD. We found consistently higher values for viability and proliferative capacity of dolphin blood mononuclear cells from samples collected in ACD vacutainers for blood prepared for evaluation 24 to 30 hr after collection (data not shown).

Relative to the differences in preparation of PBML's, we previously determined that mitogen-stimulated cultures containing a higher number of neutrophils consistently yielded lower proliferative and IL-2R expression values than cultures that had neutrophils removed (data not shown). Colgrove (1978) did not enrich for blood mononuclear cells, and Lahvis et al. (1993) used ficoll density centrifugation. In preparation for our study, different density mediums were compared, including ficoll, for their ability to purify blood mononuclear cells of contaminating neutrophils. We determined that Isolymph and Histopaque-1077, both used in this report, produced the most purified populations of mononuclear cells from dolphin blood (data not shown). Even though these differences between our experimental procedures and those of Colgrove (1978) and Lahvis et al. (1993) likely contributed to the differences observed in proliferative responses, cellular proliferation is a relative measure and may be susceptible to many influences. For this reason other assays, such as the IL-2R assay described in this report, are necessary to provide more definitive and complementary measures of immune cell function.

Because proliferation and IL-2R expression are important factors associated with immune responses, the potential relationship between the data obtained from free-ranging dolphins for both assays was determined. No statistically significant correlation was found between these assays with either mitogen stimulus. This supports the notion that immune cell activation is not inextricably linked to proliferation. Although increased proliferation usually was associated with increased expression of IL-2R, the exceptions support the use of both assays to evaluate immune performance. In addition, under conditions of immunosuppression, both assays used in conjunction would be helpful in pinpointing the site of inhibition.

Based on the standard deviations observed, the free-ranging population exhibited a wide variation in individual animal responses. This was to be expected from a normally distributed population of free-ranging animals. Perhaps low responders were at a greater health risk, but this can only be confirmed by repeated sampling and analysis of the same animals over time. The importance of using both assays was illustrated by dolphin 94 who had very low responses for mitogen-induced proliferation (ConA, 3,918 CPM; PHA, 5,772 CPM) but had relatively high values for IL-2R expression (ConA, 57%; PHA, 78%). Had only the proliferation assay been performed, this animal would have been considered immunologically impaired relative to the other animals. Therefore, although this animal's PBML's had a diminished ability to proliferate in response to both mitogens, his lymphocytes clearly were capable of being activated and made functional.

In summary, immunity to any given pathogen is dependent on the host's ability to organize and focus a diverse array of immune cells and soluble products against the pathogen. Utilizing two assays that measure both early and late events in the immune response can provide a more definitive assessment of immune competence. A decrease in immune proliferation is evidence for an inability to amplify the immune response but does not address the activation state of the cells present which may be producing soluble factors that can mediate many events leading to the elimination of pathogen. Alternatively, immune cell activation, as determined by increase in expression of IL-2R, is a measure of the number of cells responding to a pathogen and not their ability to amplify that response. Therefore, the two assays described in this manuscript are complementary and when used together can be used to find differences in the ability of dolphin leukocytes to be activated or to proliferate in response to antigenic or mitogenic stimuli.

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