IMMUNE FUNCTION IN FREE-RANGING HARBOR SEAL (PHOCA VITULINA) MOTHERS AND THEIR PUPS DURING LACTATION

Authors: Ross, P. S., Pohajdak, B., Bowen, W. D., and Addison, R. F.

Source: Journal of Wildlife Diseases, 29(1) : 21-29

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-29.1.21

Journal of Wildlife Diseases, 29(1), 1993, pp. 21–29

IMMUNE FUNCTION IN FREE-RANGING HARBOR SEAL (*PHOCA VITULINA*) MOTHERS AND THEIR PUPS DURING LACTATION

P. S. Ross,[1] B. Pohajdak,[1] W. D. Bowen,[2] and R. F. Addison[2]

[1] Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4J1
[2] Bedford Institute of Oceanography, Dartmouth, Nova Scotia, Canada B2Y 4A6
[3] Present address: Laboratory of Immunobiology, National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, The Netherlands

ABSTRACT: Immune function in harbor seal mothers and their pups during lactation was studied on Sable Island, Nova Scotia, Canada, during the springs of 1989 and 1990. Methods included total white blood cell and differential counts, a Protein A enzyme linked immunosorbent assay (ELISA) for total immunoglobulin G (IgG) quantification, and functionality testing of lymphocytes in vitro using the T-cell mitogen concanavalin A (ConA). Lymphocyte functionality and total IgG levels were reduced in the mothers at the end of lactation, suggesting a reduction in immune function, possibly as a result of the stress of fasting, or hormonal changes associated with lactation and estrus. By contrast, lymphocyte functionality and total IgG levels in pups were low at birth and higher at the end of lactation. Pups at birth and females late in lactation may therefore be more susceptible to infection by viral and bacterial agents. This study represents the first broad examination of immune function in a free-ranging pinniped population.

Key words: Immune function, harbor seals, *Phoca vitulina*, pinnipeds, lactation, nursing, leukocytes, immunoglobulin G.

INTRODUCTION

Pinniped immunology is still in its infancy, despite widespread interest in recent as well as past mass mortalities associated with disease. The deaths of 17,000 harbor seals (*Phoca vitulina*) in Europe in 1988 sparked a fierce debate about the possible role of pollution (Dietz et al., 1989) as a factor in the susceptibility of the seals to the causal agent, phocid distemper virus (PDV) (Mahy et al., 1988; Osterhaus et al., 1990). Extensive laboratory evidence of polychlorinated biphenyl (PCB)-induced immunosuppression in mammals (Thomas and Hinsdill, 1978; Vos and Luster, 1989), coupled with the high levels of these and other pollutants in the harbor seal populations in Europe (Helle et al., 1976; Reijnders, 1980), fueled such speculation. However, the lack of baseline immunological information on harbor seals, and of methods to evaluate their immunocompetence, made it impossible to address the possible role of pollution-induced immunosuppression in the PDV epizootic.

Some work has been done to characterize the immune system of the northern fur seal, *Callorhinus ursinus* (Cavagnolo and Vedros, 1978; Cavagnolo, 1979), and the California sea lion, *Zalophus californianus* (Nash and Mach, 1971); these studies were aimed primarily at comparative descriptions of antibody structure or lymphoid histology. Antibody profiles were established for the northern fur seal by Cavagnolo and Vedros (1979), who found a slow increase in serum levels of IgG and IgA during the first 4 mo of life. Carter et al. (1990) characterized grey seal (*Halichoerus grypus*) immunoglobulin structures and quantified these for pups and adults shortly after the PDV epidemic in Europe. In other studies, hematological characteristics of pinnipeds, including white blood cell counts and differential counts of white blood cell subpopulations, have been determined (Geraci and Smith, 1975; Needham et al., 1980; Banish and Gilmartin, 1988). These and other studies of captive seals have been used to establish normal ranges for many of these blood constituents in several pinniped species (e.g., Medway and Geraci, 1988; Dierauf, 1990), providing useful diagnostic information for zoos and aquaria. However, such approaches do not provide information on

the functioning of the immune system. In addition, few studies of immunological parameters have made efforts to control for potentially influential variables, such as age, sex, condition, and sampling regime in free-ranging seals.

The purpose of our study was to establish baseline data on immune function in a free-ranging population of harbor seal mothers and their pups over the course of lactation. The nursing period in harbor seals lasts 24 days (Bowen, unpubl.). During this time, different individuals undergo similar biological events, thus allowing for comparisons of both mothers and pups over time.

METHODS

The study was undertaken during the pupping seasons (May and June) of 1989 and 1990 on Sable Island, Nova Scotia. Sable Island is a crescent-shaped, vegetated sand bar about 40 km long located 160 km east of Nova Scotia (43°55'N; 60°00'W).

Field work and sample preparation

Mothers and their newborn pups were captured and restrained using capture nets made of two aluminum poles connected by nylon netting (Day 0). Blood samples were taken by venipuncture of the extradural vein. Animals were marked with a fluorescent orange paint for identification purposes, released, and again sampled at mid-lactation (Day 14) and late-lactation (Day 20).

To avoid clotting, needles and syringes were heparinized (0.5 ml sodium heparin; 250 U/ml), and heparinized vacutainers (Becton Dickinson, Rutherford, New Jersey, USA) were unstoppered immediately prior to filling. Two 10 ml vacutainers were filled with 7 ml of whole blood for each animal, stoppered, and inverted gently 15 times. A 10 ml serum separation tube (SST) (Becton Dickinson) also was filled. The SST was centrifuged at 500 × g for 20 min within 4 hr of sampling; 1 ml of serum was frozen at −20 C for later determination of IgG levels with an enzyme-linked immunosorbent assay (ELISA) assay.

Red blood cells were lysed prior to a white blood cell count using 50 µl of whole blood from the vacutainers in 1 ml of a 2% acetic acid solution. White blood cells were then counted using a Spotlite hemocytometer (American Hospital Supply Corporation, McGaw Park, Illinois, USA). A blood smear was prepared, allowed to air dry and fixed for 5 min in 95% methanol. The slide was stored at 21 C for later staining and counting.

Whole blood from the vacutainers was layered on a Histopaque-1077 (Sigma Chemical Company, St. Louis, Missouri, USA) density gradient and centrifuged for 30 min at 400 × g. The mononuclear layer was aspirated and transferred to 5 ml Corning brand cryovials. Cells were not washed because the fixed-angle centrifuge available in the field did not produce an adequate cell pellet. Cryovials were filled about one-third full, and then were filled with RPMI-1640 cell culture medium (Sigma Chemical Company) containing 20% heat inactivated fetal calf serum (FCS) and 10% dimethylsulfoxide (DMSO). Samples were frozen at a controlled rate and stored in liquid nitrogen vapor (approximately −140 C). The controlled rate of freezing was done manually as follows: 15 min in a −20 C freezer; 5 min in the open neck of a 10 l liquid nitrogen container; and in three successive steps of 10 min each, samples were lowered into the liquid nitrogen vapor. Once in the vapor, samples were maintained at −140 C or colder until analysis.

Laboratory analysis

Blood smears were stained using May-Grunwald/Giemsa (Hudson and Hay, 1980). One hundred leukocytes were classified as band cells, neutrophils, lymphocytes, monocytes, eosinophils, or basophils.

Immunoglobulin G levels were determined using a Protein A ELISA which strongly binds most mammalian IgG (Goding, 1978; Langone, 1982; Lindmark et al., 1983). Protein A is used routinely in serological testing of harbor seal serum for specific antibody levels (Osterhaus et al., 1985; Visser et al., 1989). Briefly, 2 µl of serum from each animal was placed in 200 µl coating buffer (50 mM $NaHCO_3$, pH 9.5) in the first of 12 wells in 96-well, high-binding, flat-bottomed ELISA plates. The other 11 wells contained 100 µl of coating buffer. Contents of the first well were mixed and 100 µl transferred from well to well, creating sequential 50:50 dilutions. Plates were incubated for 1 hr at 37 C, and washed twice with Tris-buffered saline-Tween solution (TBS-Tween) (Promega Corp., 1991). Two hundred µl of a gelatin solution (20 µg/ml) was added to each well to block nonspecific reagent binding and plates were incubated for 30 min at 37 C. Plates were washed twice in TBS-Tween, and 100 µl of a 1:2,000 solution of Protein A-horseradish peroxidase in TBS-Tween was added to each well. Plates were incubated at 21 C for 30 min and then washed five times in TBS-Tween. One hundred µl of a 50:50 solution of horseradish peroxidase substrate (2,2'-azino-di-[3-ethyl]-benzthiazoline-6-sulphonic acid and hydrogen peroxide) (BioRad,

Richmond, California, USA) was added to each well, and stopped with 100 μl of 2% oxalic acid after ten min. Plates were read at 405 nm using an EL 309 microplate autoreader (Bio-tek Instruments, Winooski, Vermont, USA).

Cryopreserved lymphocyte samples were tested for their proliferative ability in the presence of the T-cell mitogen concanavalin A (ConA) (Sigma Chemical Company). Cells were thawed by bathing cryovials in a 37 C water bath, which was followed by a step-wise dilution of samples using 20% FCS-RPMI 1640 solution in 15 ml conical tubes (Callery et al., 1980). Phenol red-free RPMI 1640 was used so as to avoid interference with absorbance readings at the end of the assay (Mosmann, 1983). Cells were washed twice by centrifugation at 400 × g for 10 min. Each time, the supernatant was removed, fresh phosphate buffered saline (PBS) added, and the cell pellet re-suspended. A volume of 0.5 ml 20% FCS-RPMI 1640 was added to the final pellet, which was re-suspended, and cells counted using a hemocytometer. Cell solutions were adjusted to 1×10^6 cells/ml. One hundred μl of these solutions was placed in each of 12 wells (100,000 cells/well) of a prepared 96-well cell culture plate for each animal (one row represented one animal) containing triplicate controls, and triplicate wells of 100 μl ConA at a final concentration in the wells of 2.5 μg/ml, 5 μg/ml, and 10 μg/ml. Plates were incubated at 37 C in a 5% CO_2 atmosphere for 96 hr, 24 hr longer than is usual. This allowed the cells an additional 24 hr to adjust to the assay conditions following their cryopreservation and thawing (Fujiwara et al., 1986).

Plates were removed from incubation, centrifuged at 200 × g for 5 min, and 100 μl supernatant removed. The colorimetric assay developed by Mosmann (1983) was used for cellular and metabolic quantification. Twenty μl of a tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-1,5-diphenyl tetrazolium bromide-PBS solution (MTT-PBS) was placed in each of the wells, the plate was incubated again for 4 hr, and the reaction stopped using 150 μl 0.04 N HCl-isopropanol. Plates were read at 560 nm using an EL 309 microplate autoreader. Data were corrected by subtracting blanks, and a stimulation index was calculated by dividing the stimulated absorbance values by the control well absorbance values for each individual.

All statistical analyses were undertaken using SYSTAT software (Wilkinson, 1989).

RESULTS

Total white blood cell counts did not differ significantly over time in mothers or in pups (Fig. 1). Mean (±SD) combined

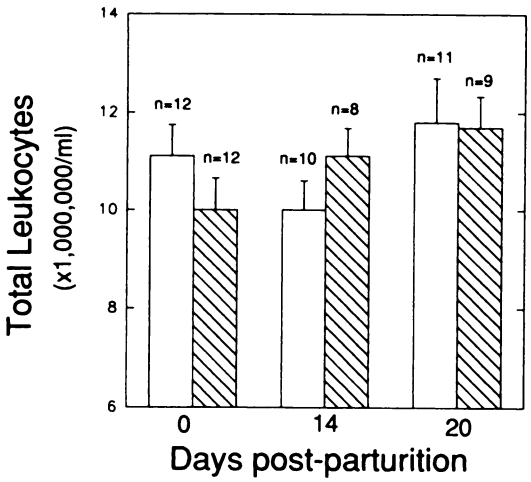

FIGURE 1. Total leukocyte counts in harbor seal mothers (not shaded) and their pups (shaded) at parturition (Day 0), mid-lactation (Day 14), and late lactation (Day 20). Values represent mean ±SE of animals sampled.

counts from the three sampling points were 11.1 (±2.5) for the mothers; and 10.9 (±2.1) for the pups. Using a *t*-test, there were no significant differences between mothers and pups for any of the sampling points. Leukocyte subpopulations also remained relatively static through lactation for both mothers and pups, though a large amount of variation was apparent among individuals (Table 1). This was particularly true for neutrophils and lymphocytes in both mothers and pups late in lactation. But using a *t*-test, mothers had significantly ($P \leq 0.001$) higher numbers of eosinophils and basophils than did pups at both Day 0 and Day 20, suggesting varying degrees of an inflammatory response.

Mean absorbance values for the serial serum dilutions were used as indicators of protein A-binding IgG trends in both mothers and pups (Fig. 2). Using univariate repeated measures analysis (univariate *F*-test), there was a significant ($P < 0.001$) increase in IgG levels in pups over the course of lactation, with this change occurring between Day 0 and Day 14. There was no significant change in serum IgG in the pups between Day 14 and Day 20, suggesting a rapid rise within the first 2 wk following birth. Relative IgG levels in

TABLE 1. Differential leukocyte counts for harbor seal mothers and pups at two points during lactation. Values represent mean ± SD, and figures in parentheses represent the full range of sample counts.

	Mothers		Pups	
	Day 0	Day 20	Day 0	Day 20
Band cells	3.5 ± 4.0 (0–12)	4.5 ± 4.2 (0–12)	3.6 ± 4.1 (0–13)	5.4 ± 4.5 (1–14)
Neutrophils	62.7 ± 5.8 (54–70)	59.9 ± 14.6 (40–81)	68.6 ± 8.3 (53–86)	60.1 ± 15.7 (31–77)
Lymphocytes	24.7 ± 7.1 (15–39)	27.6 ± 10.1 (15–40)	24.6 ± 6.1 (13–35)	30.7 ± 11.5 (19–53)
Monocytes	3.2 ± 4.8 (0–14)	3.6 ± 4.1 (0–9)	2.3 ± 3.9 (0–12)	3.3 ± 4.3 (0–11)
Eosinophils	5.8 ± 2.1 (3–14)	4.3 ± 2.5 (1–9)	0.9 ± 0.9 (0–3)	0.4 ± 0.8 (0–2)
Basophils	0.3 ± 0.6 (0–10)	0.1 ± 0.3 (0–9)	0.0 ± 0.0 (0–0)	0.0 ± 0.0 (0–0)

mothers showed a significant decrease over the course of lactation (univariate repeated measures analysis; $P = 0.015$), though the magnitude of the change was not large. Using univariate F-tests, these differences were limited to between Day 0 to Day 14 ($P = 0.027$), and did not occur between Day 14 and 20.

A concentration of 5 μg/ml of the T-cell mitogen ConA elicited a maximal proliferative response when incubated with seal leukocytes (Fig. 3). This was consistent with the recent observation of De Swart et al. (in press), who have confirmed the specificity of ConA in the stimulation of T-lymphocytes in the harbor seal. Using this assay, no significant temporal differences were evident for mothers or pups, but stimulation indices were significantly higher in pups on both Day 0 and Day 14 than in mothers on Day 14 (t-test; $P = 0.012$ and $P = 0.007$, respectively) (Fig. 4). Therefore, pup lymphocytes at both points in lactation gave a stronger proliferative response to a polyclonal antigen than did the mothers' late in lactation.

DISCUSSION

Our field approach to assessing immune function in harbor seals could be used in the study of other wildlife species for which specialized immunologic reagents are not available. This approach provides an overview of both humoral and cellular components of the immune system. Conditions under which field samples must be taken are not always optimal. However, by keeping needles, syringes and vacutainers at room temperature (approximately 21 C), and removing the needle from the syringe and the top of the vacutainer, we obtained high quality samples. Samples free from even small-scale clotting were essential to optimal lymphocyte recovery and subsequent proliferation assays.

Total white blood cell counts in mothers and pups during lactation in this study were somewhat higher than those reported by Medway and Geraci (1988) for harbor seals (7 to 9 × 10^6 ml), with the latter likely reflecting results from "normal" individuals. Although we found no significant differences over the lactation period, our results paralleled the white blood cell counts in female Australian sea lions (*Neophoca cinerea*) which declined from 11.7 to 9.8 × 10^6/ml from early to late lactation (Needham et al., 1980). Three- to 6-mo-old Hawaiian monk seals (*Monachus schauinslandi*) had a normal range of 7.2 to 10.4 × 10^6/ml (Banish and Gilmartin, 1988). The significantly higher propor-

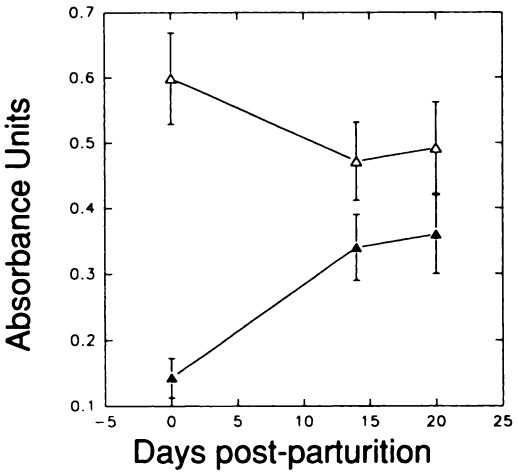

FIGURE 2. Total relative IgG in harbor seal mothers (open triangles) and pups (closed triangles) over the course of lactation, as measured by a protein A based ELISA. Bars represent mean ±SE for animals sampled.

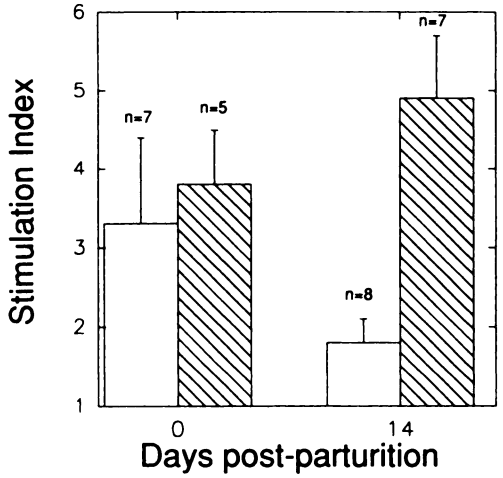

FIGURE 4. Lymphocyte proliferation in the presence of 5 μg/ml ConA using the MTT assay for harbor seal mothers (not shaded) and pups (shaded) at Day 0 and Day 14. Bars represent mean ±SE for 1989 cryopreserved samples from Sable Island.

tions of eosinophils and basophils in the mothers than in the pups is probably due to the presence of parasites in the mothers, since these leukocyte subsets are associated with parasitic infestation and accompanying inflammation (Needham et al. 1980; Bass, 1984). Leukocyte numbers and proportions are affected by numerous hormones (Thomson and Geraci, 1986), but seemed to have remarkable stability through lactation and nursing in our study, despite dramatic physiological changes in our study animals.

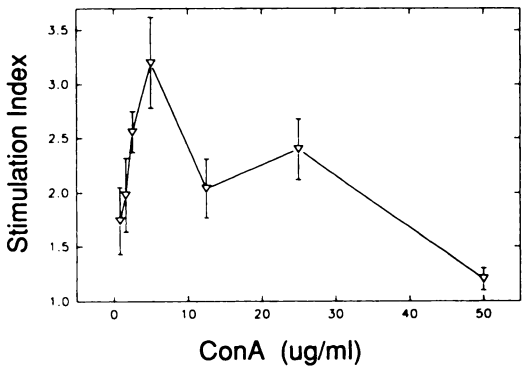

FIGURE 3. The optimal dose-response range for harbor seal lymphocytes in the presence of the T-cell mitogen concanavilin A (ConA). Maximal proliferation, as measured by the colorimetric MTT assay, occurred at 5 μg/ml. Data points represent mean ±SE of captive harbor seals tested (n = 7).

While IgG levels reflect general antibody production by B-lymphocytes, newborns often receive temporary protection from maternally-derived IgG. Although it is impossible to distinguish between endogenously-produced and maternally-transferred IgG in the harbor seal pups, we believe that the low serum levels in the newborn and the high levels at 14 days postpartum reflect a low rate of transplacental transfer but a high rate of transfer through colostrum and milk. Carter et al. (1990) found relatively high levels of IgG in the colostrum of gray seals, but did not see a rapid rise in IgG in the serum of pups during the course of lactation. Although the maternal transfer of IgG either via placenta or colostrum and milk provides some protection for the newborn pup, this will be limited by the short time spent with the mother.

The decrease in total IgG levels in the mothers was somewhat difficult to interpret. Though significant, the decline was not large. It may reflect a change in the production or metabolism of immuno-

globulins, either at parturition or later in lactation.

Proliferative responses to ConA provide a measure of T-lymphocyte activity. It generally is held that the cryopreservation of lymphocytes does not alter functionality, providing that cells are frozen at a controlled rate and that a step-wise dilution in 20% fetal calf serum is undertaken during thawing (Oldham et al., 1976; Callery et al., 1980). We found that culturing cells for 96 hr rather than the usual 72-hr period resulted in more consistent stimulation assays.

We found no significant differences in the stimulation indices in either the mothers or pups over the course of lactation. However, this may simply reflect the small sample of mothers ($n = 7$) and pups ($n = 5$) available to test the hypothesis. Based on the significantly higher proliferative responses of pup lymphocytes at mid-lactation as compared to their mothers, we believe that T-lymphocyte function is well developed in pups, despite their young age. However, the proliferation results only reflect the general function of their T-lymphocytes, and a lack of immunological memory likely limits the response against specific antigens. This has been found in humans, where polyclonal responses to mitogens are strong in newborns, but antigenic memory is limited from lack of prior exposure (Hayward, 1981).

There is little doubt that the mothers undergo a great deal of physiological stress during lactation and fasting. If the observed changes in immune function in the females represent a real decline from normal values, a number of stressors or combination of factors may be involved. Both malnutrition (Chandra, 1980; Manderino and Watson, 1988) and stress resulting from dietary changes (Watson, 1988) affect immune function in mammals. Even though a fasting period and observed loss of nearly 40% of body weight (Bowen, unpubl.) would appear to be a significant nutritional stressor for the mothers, pinnipeds have almost certainly adapted to this pattern of parental care (Oftedal et al., 1987). Reproductive hormones, including progesterone and oestradiol-17β, rise during the two weeks following parturition in harbor seals, reflecting the approaching oestrus (Reijnders, 1990). Both of these hormones have been associated with immunosuppression at physiological levels in female mammals (Schuurs and Verheul, 1990).

Lowered immune responses in mammals during pregnancy and after parturition appear to be relatively common (Lloyd, 1983). Wells et al. (1977) found significantly lower lymphocyte proliferative responses to phytohemagglutinin (PHA) in dairy cows immediately postparturition, as compared to non-pregnant cows. Purswell et al. (1989) found a significant reduction in natural killer cell activity during the reproductive cycle of swine, with the lowest activity being observed 2 wk post-parturition. Guidry et al. (1976) found significantly lower phagocytic activity by neutrophils in cows 2 wk post-parturition compared to the activity at parturition itself, although it was not clear which value was more representative of normal activity.

The immunological immaturity of harbor seal newborns and the possible suppression of immune function in mothers during lactation may increase their susceptibility to disease. Thus, the pupping season for harbor seals may represent a sensitive stage in the life history of this and perhaps other pinniped species. Disease has played a significant role in the dynamics of pinniped populations, as shown by the recent mass mortalitites in Europe (Dietz et al., 1989), Lake Baikal (Grachev et al., 1989), and North America (Geraci et al., 1982). While detailed age distributions of affected individuals are not always known, 90% of the seals that died in the 1979-80 epidemic in North America were <3-yr-old (Geraci et al., 1982). In studies of "healthy" breeding pinnipeds, elevated rates of infection by numerous pathogens are found among pups (Baker and Baker,

1988; Baker, 1989; Steiger et al., 1989). Although considerably more information is needed on the factors affecting immune function in pinnipeds, we conclude that studies of free-ranging populations are possible, despite the obvious difficulties of working under isolated field conditions, and the lack of specific immunologic reagents.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Warwick Kimmins and John Young of the Marine Mammal Laboratory, Dalhousie University, for access to a group of captive harbor seals; and to the staff at the Dalhousie Animal Care Centre for their advice regarding the care of the study seals on Sable Island during sampling. Research was supported by a Natural Sciences and Engineering Research Council (NSERC) Postgraduate Scholarship to PSR, Department of Fisheries and Oceans and NSERC grants to WDB, and a NSERC-DFO subvention grant to I. A. McLaren. O. T. Oftedal, D. Boness, S. J. Iverson, and D. Benoit assisted in the collection of field samples.

LITERATURE CITED

BAKER, J. R. 1989. Natural causes of death in non-suckling grey seals (*Halichoerus grypus*). The Veterinary Record 125: 500–503.

———, AND R. BAKER. 1988. Effects of environment on grey seal (*Halichoerus grypus*) pup mortality. Studies on the Isle of May. Journal of Zoology, London: 216: 529–537.

BANISH, L. D., AND W. G. GILMARTIN. 1988. Hematology and serum chemistry of the young Hawaiian monk seal (*Monachus schauinsland*). Journal of Wildlife Diseases 24: 225–230.

BASS, D. A. 1984. Eosinophils. *In* Fundamentals of clinical haematology, 2nd ed., J. L. Spivak (ed.). Harper & Row, Publishers, Philadelphia, Pennsylvania, pp. 135–152.

CALLERY, C. D., M. GOLIGHTLY, N. SIDELL, AND S. GOLUB. 1980. Lymphocyte surface markers and cytotoxicity following cryopreservation. Journal of Immunological Methods 35: 213–223.

CARTER, S. D., D. E. HUGHES, AND J. R. BAKER. 1990. Characterization and measurement of immunoglobulins in the grey seal (*Halichoerus grypus*). Journal of Comparative Pathology 102: 13–23.

CAVAGNOLO, R. Z. 1979. The immunology of marine mammals. Developmental and Comparative Immunology 3: 245–257.

———, AND N. A. VEDROS. 1978. Identification and characterization of three immunoglobulin subclasses in the northern fur seal *Callorhinus ursinus*. Developmental and Comparative Immunology 2: 689–697.

———, AND ———. 1979. Serum and colostrum immunoglobulin levels in the northern fur seal *Callorhinus ursinus*. Developmental and Comparative Immunology 3: 139–146.

CHANDRA, R. K. 1980. Immunology of nutritional disorders. Edward Arnold Company, London, United Kingdom, 110 pp.

DE SWART, R. L., L. M. G. KLUTEN, C. J. HUIZING, E. J. VEDDER, P. J. H. REIJNDERS, I. K. G. VISSER, F. G. C. M. UYTDEHAAG, AND A. D. M. E. OSTERHAUS. In press. Mitogen and antigen induced B and T cell responses of peripheral blood mononuclear cells from the harbour seal (*Phoca vitulina*). Veterinary Immunology and Immunopathology.

DIERAUF, L. A. (editor). 1990. CRC handbook of marine mammal medicine: Health, disease, and rehabilitation. CRC Press, Boca Raton, Florida, 735 pp.

DIETZ, R., M.-P. HEIDE-JORGENSEN, AND T. HARKONEN. 1989. Mass deaths of harbor seals (*Phoca vitulina*) in Europe. Ambio 18: 258–264.

FUJIWARA, S., M. AKIYAMA, M. YAMAKIDO, T. SEYAMA, K. KOBUKE, M. HAKODA, S. KYOIZUMI, AND S. L. JONES. 1986. Cryopreservation of human lymphocytes for assessment of lymphocyte subsets and natural killer cytotoxicity. Journal of Immunological Methods 90: 265–275.

GERACI, J. R., AND T. G. SMITH. 1975. Functional hematology of ringed seals (*Phoca hispida*) in the Canadian Arctic. Journal of the Fisheries Research Board of Canada 32: 2559–2564.

———, D. J. ST. AUBIN, I. K. BARKER, R. G. WEBSTER, V. S. HINSHAW, W. J. BEAN, H. L. RUHNKE, J. H. PRESCOTT, G. EARLY, A. S. BAKER, S. MADOFF, AND R. T. SCHOOLEY. 1982. Mass mortality of harbor seals: Pneumonia associated with Influenza A virus. Science 215: 1129–1131.

GODING, J. W. 1978. Use of staphylococcal Protein A as an immunological reagent. Journal of Immunological Methods 20: 241–253.

GRACHEV, M. A., V. P. KUMAREV, L. V. MAMAEV, V. L. ZORIN, L. V. BARANOVA, N. N. DENIKINA, S. I. BELIKOV, E. A. PETROV, V. S. KOLESNIK, R. S. KOLESNIK, V. M. DOROFEEV, A. M. BEIM, V. N. KUDELIN, F. G. NAGIEVA, AND V. N. SIDOROV. 1989. Distemper virus in Baikal seals. Nature 338: 209.

GUIDRY, A. J., M. J. PAAPE, AND R. E. PEARSON. 1976. Effects of parturition and lactation on blood and milk cell concentrations, corticosteroids, and neutrophil phagocytosis in the cow. American Journal of Veterinary Research 37: 1195–1200.

HAYWARD, A. R. 1981. Development of lymphocyte responses and interactions in the human fetus and newborn. Immunological Reviews 57: 39–59.

HELLE, E., M. OLSSON, AND S. JENSEN. 1976. DDT and PCB levels and reproduction in ringed seal from the Bothnian Bay. Ambio 5: 188–189.

HUDSON, L., AND F. C. HAY. 1980. Practical immunology, 2nd ed. Blackwell Scientific Publications, Oxford, United Kingdom, 359 pp.

LANGONE, J. J. 1982. Protein A of *Staphylococcus aureus* and related immunoglobulin receptors produced by streptococci and pneumonococci. Advances in Immunology 32: 157–252.

LINDMARK, R., K. THOREN-TOLLING, AND J. SJOQUIST. 1983. Binding of immunoglobulins to Protein A and immunoglobulin levels in mammalian sera. Journal of Immunological Methods 62: 1–13.

LLOYD, S. 1983. Effect of pregnancy and lactation upon infection. Veterinary Immunology and Immunopathology 4: 153–176.

MAHY, B. W. J., T. BARRETT, S. EVANS, E. C. ANDERSON, AND C. J. BOSTOCK. 1988. Characterization of a seal morbillivirus. Nature 336: 115.

MANDERINO, G., AND R. R. WATSON. 1988. Nutritional stresses and thymic hormone functions. *In* Clinical and experimental nutrition, Vol. 1: Nutrition, disease resistance, and immune function, R. R. Watson (ed.). Marcel Dekker, Inc., New York, New York, pp. 285–298.

MEDWAY, W., AND J. R. GERACI. 1988. Clinical pathology of marine mammals. *In* Zoo and wild animal medicine, 2nd ed., M. E. Fowler (ed.). W. B. Saunders Company, Philadelphia, Pennsylvania, pp. 791–797.

MOSMANN, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65: 55–63.

NASH, D. R., AND J.-P. MACH. 1971. Immunoglobulin classes in aquatic mammals. Journal of Immunology 107: 1424–1430.

NEEDHAM, D. J., C. F. CARGILL, AND D. SHERRIFF. 1980. Haematology of the Australian sea lion, *Neophoca cinerea*. Journal of Wildlife Diseases 16: 103–107.

OFTEDAL, O. T., D. J. BONESS, AND R. A. TEDMAN. 1987. The behavior, physiology, and anatomy of lactation in the Pinnipedia. *In* Current mammalogy, Vol. 1, H. H. Genoways (ed.). Plenum Publishing, New York, New York, pp. 175–245.

OLDHAM, R. K., J. H. DEAN, G. B. CANNON, J. R. ORTALDO, G. DUNSTON, F. APPLEBAUM, J. L. MCCOY, J. DJEU, AND R. B. HERBERMAN. 1976. Cryopreservation of human lymphocyte function as measured by *in vitro* assays. International Journal of Cancer 18: 145–155.

OSTERHAUS, A. D. M. E., H. YANG, H. E. M. SPIJKERS, J. GROEN, J. S. TEPPEMA, AND G. VAN STEENIS. 1985. The isolation and partial characterization of a highly pathogenic herpesvirus from the harbor seal (*Phoca vitulina*). Archives of Virology 80: 239–251.

———, J. GROEN, H. E. M. SPIJKERS, H. W. J. BROEDERS, F. G. C. M. UYTDEHAAG, P. DE VRIES, J. S. TEPPEMA, I. K. G. VISSER, M. W. G. VAN DE BILDT, AND E. J. VEDDER. 1990. Mass mortality in seals caused by a newly discovered morbillivirus. Veterinary Microbiology 23: 343–350.

PROMEGA CORP. 1991. Promega protocols and applications guide, 2nd ed. Promega Corp., Madison, Wisconsin, 422 pp.

PURSWELL, B. J., D. L. DAWE, AND J. BROWN. 1989. Lymphocyte reactivity to mitogens and natural killer cell activity in crossbred swine during the reproductive cycle. Veterinary Immunology and Immunopathology 22: 29–37.

REIJNDERS, P. J. H. 1980. Organochlorine and heavy metal residues in harbour seals from the Wadden Sea and their possible effects on reproduction. Netherlands Journal of Sea Research 14: 30–65.

———. 1990. Progesterone and oestradiol-17B concentration profiles throughout the reproductive cycle in harbour seals (*Phoca vitulina*). Journal of Reproduction and Fertility 90: 403–409.

SCHUURS, A. H. W. M., AND H. A. M. VERHEUL. 1990. Effects of gender and sex steroids on the immune response. Journal of Steroid Biochemistry 35: 157–172.

STEIGER, G. H., J. CALAMBOKIDIS, J. C. CUBBAGE, D. E. SKILLING, A. W. SMITH, AND D. H. GRIBBLE. 1989. Mortality of harbor seal pups at different sites in the inland waters of Washington. Journal of Wildlife Diseases 25: 319–328.

THOMAS, P. T., AND R. D. HINSDILL. 1978. Effect of polychlorinated biphenyls on the immune responses of rhesus monkeys and mice. Toxicology and Applied Pharmacology 44: 41–51.

THOMSON, C. A., AND J. R. GERACI. 1986. Cortisol, aldosterone, and leucocytes in the stress response of bottlenose dolphins, *Tursiops truncatus*. Canadian Journal of Fisheries and Aquatic Sciences 43: 1010–1016.

VISSER, I. K. G., M. W. G. VAN DE BILDT, H. N. BRUGGE, P. J. H. REIJNDERS, E. J. VEDDER, J. KUIPER, P. DE VRIES, J. GROEN, H. C. WALVOORT, F. G. C. M. UYTDEHAAG, AND A. D. M. E. OSTERHAUS. 1989. Vaccination of harbour seals (*Phoca vitulina*) against phocid distemper with two different inactivated canine distemper virus (CDV) vaccines. Vaccine 7: 521–526.

VOS, J. G., AND M. I. LUSTER. 1989. Immune alterations. *In* Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products, 2nd ed, R. D. Kimbrough and A. A. Jensen (eds.). Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 295–322.

WATSON, R. R. 1988. Stress caused by dietary changes: Corticosteroid production, a partial explanation for immunosuppression in the malnourished. *In* Clinical and experimental nutrition, Vol. 1: Nutrition, disease, and immune

function, R. R. Watson (ed.). Marcel Dekker, Inc., New York, New York, pp. 273–283.

WELLS, P. W., C. BURRELLS, AND W. B. MARTIN. 1977. Reduced mitogenic responses in cultures of lymphocytes from newly calved cows. Clinical and Experimental Immunology 29: 159–161.

WILKINSON, L. 1989. SYSTAT: The system for statistics. SYSTAT Inc., Evanston, Illinois, 822 pp.

Received for publication 26 July 1991.