Report

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Impairment of Immune Function in Harbor Seals (*Phoca vitulina*) Feeding on Fish from Polluted Waters

Disease outbreaks with high mortality rates among seals and dolphins have recently attracted considerable public and scientific interest. Although in most cases morbillivirus infections were shown to be the primary cause of the disease outbreaks, it was speculated that pollution-induced immunosuppression had played a contributory role. Here we present results of a prospective study under semifield conditions, in which two groups of harbor seals (*Phoca vitulina*) were fed herring from marine regions with different contamination levels; the highly polluted Baltic Sea and the relatively unpolluted Atlantic Ocean. During a period of 93 weeks, parameters related to immune function were monitored and compared between the two groups. We found that natural killer-cell activity and mitogen-induced proliferative T-cell responses from the seals feeding on herring from the Baltic Sea were significantly lower. In addition, we observed higher levels of circulating polymorphonuclear granulocytes in these animals, which may indicate an increase in the occurrence of bacterial infections. This is the first demonstration of impaired immunological functions in mammals associated with chronic exposure to environmental contaminants accumulated through the marine food chain.

Harbor seals inhabiting coastal waters in an industrialized area. Environmental contaminants accumulated through the food chain suppress immune function in marine mammals, which may lead to increased susceptibility to infectious diseases. Photo: J. de Boer.

INTRODUCTION

Marine mammals inhabiting polluted coastal areas are known to accumulate high levels of environmental chemicals (1–3), which has been related to the occurrence of several abnormalities. Premature parturitions and abortion in California Sea Lions (*Zalophus californianus*), caused by infection with a calicivirus, were also suggested to be associated with higher levels of pollutants in aborting animals (4). In the highly polluted Baltic Sea the occurrence of changes in the reproductive tract, in some cases leading to sterility, as well as skeletal deformities in seals have been associated with increased levels of PCBs (5–8). In Dall's porpoises (*Phocoenoides dalli*) living in the northwestern Pacific Ocean, an inverse correlation was found between serum testosterone levels and DDE-concentrations in the blubber of these animals (9). In a semifield study, seals fed fish from the heavily polluted western part of the Dutch Wadden Sea showed a significantly reduced pup production, as compared to seals fed less-polluted fish (10).

Many of the persistent lipophilic chemicals found in marine mammals have been shown to adversely affect the functioning of the immune system of laboratory animals, which in some cases has led to an increased susceptibility to infectious diseases (11). These chemicals include polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) (12), hexachlorobenzene (HCB) (13), dieldrin (14), ß -hexachloro-cyclohexane (ß -HCH) (15), and dichlorodiphenyltrichloroethane (DDT) (16). However, little is known about possible immunotoxic effects caused by chronic exposure to undefined mixtures of xenobiotics via the food chain.

To date, it has not been possible to demonstrate that environmental chemicals cause immunosuppression in marine mammals. However, the occurrence of a number of epizootics in recent years among seals and dolphins inhabiting polluted coastal areas, including baikal seals (*Phoca sibirica*) in Lake Baikal in 1987 (17–19), striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea from 1990 onward (19,20), and harbor seals in northwestern Europe in 1988 (19, 21), has led to extensive speculation about the possible contribution of environmental pollutants to these outbreaks of infectious diseases, by causing an impairment of immune function (22–26). In addition, morbillivirus infections have been observed in seals inhabiting less-polluted areas without causing any evident mortality (27, 28).

The main problem in conducting studies designed to evaluate toxic effects of environmental chemicals on the immune system of marine mammals is related to difficulties in assessing immune function in free-ranging animals in a controlled way. We therefore designed an experiment in which captive harbor seals were fed fish contaminated through the food chain of the heavily polluted Baltic Sea and of the relatively unpolluted Atlantic Ocean, to mimic exposure levels of seals living in these areas. This made it possible to sample the same animals repeatedly while

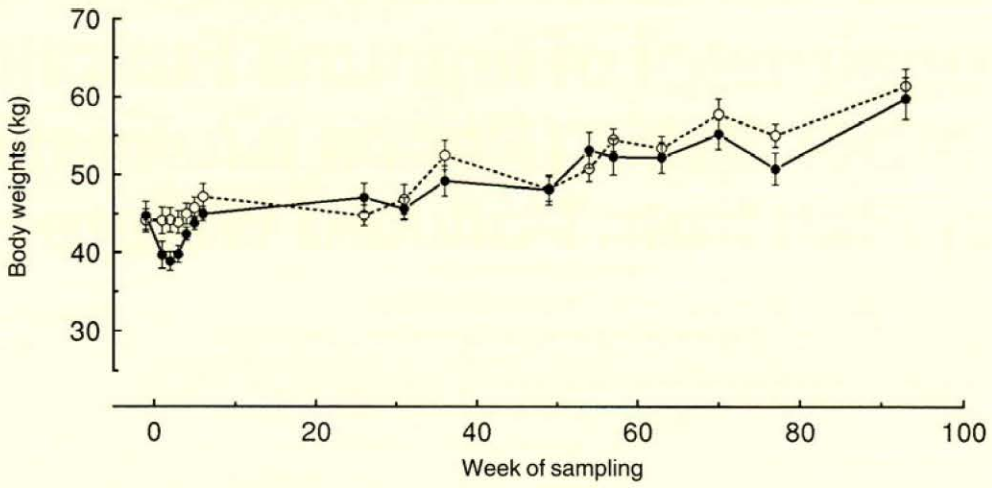


Figure 1. Mean body weights of harbor seals feeding on herring from the Baltic Sea (ϕ—ϕ) or the Atlantic Ocean (ϕ····ϕ) over the course of the feeding experiment. Each group consists of 7 females and 4 males. Vertical error bars indicate standard errors of the means. Week 0 represents the start of the feeding experiment (September 1991).

controlling for age, sex and condition, and to study longitudinal changes in parameters of immune function.

MATERIALS AND METHODS

Seals and Diets

In a semifield prospective study, two groups of juvenile harbor seals were fed fish destined for human consumption, originating from two different areas. The seals had been caught as weaned pups from the relatively unpolluted northeastern coast of Scotland (29), and were fed relatively uncontaminated herring from the Atlantic Ocean during an adaptation period of about one year. After this period, they were divided into two groups which were matched for weight and gender (seven females and four males in each group), and the diet of the first group was changed to herring caught in a polluted coastal area of the Baltic Sea (about 100 km off the southwest coast of Finland). The seals were housed at the Seal Rehabilitation and Research Center in Pieterburen (SRRC) in two similar basins with approximately 40 m^3 water and haul-out platforms of approximately 24 m^2.

Diets were similar as regards overall quality, and were both supplemented weekly with an equal, fixed amount of a mixture of vitamins per group of seals to compensate for losses during storage. The fish was stored at –25°C until use. Lipid content was lower in the herring from the Baltic Sea (on average 7.1% and 12.3%, respectively), which was compensated for by feeding the seals in Group 1 more fish than the seals in Group 2 (on average 5.6 kg and 3.7 kg per animal per day, respectively).

Toxicological Analysis of Seal Diets

Random samples were taken from each batch of fish (in both groups three different batches were used during the course of the experiment), homogenated, and organochlorine concentrations were determined on the basis of extractable fat. In addition to analyses performed as described previously (30), congener specific analyses of PCDDs, PCDFs and coplanar PCBs were carried out using previously described methods (31, 32). Daily intakes of organochlorines were estimated on a monthly basis using the average daily intake of herring by each group of seals, and the organochlorine burdens of the batches of herring fed during that month. Daily intakes of organochlorines presented in Table 1 represent the means of these monthly calculated values.

Daily intakes of aryl hydrocarbon (*Ah*)-receptor binding organochlorines in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxic equivalents (TEQ) were calculated using the international toxic equivalency factors (TEFs) for dioxins as reported by Van Zorge et al. (33), and the proposed TEFs for coplanar and mono-ortho coplanar PCBs as given by Safe (34), on the basis of the estimated daily intakes of the 17 2,3,7,8-chlorine substituted PCDDs and PCDFs, and measured congeners of PCBs (IUPAC numbers 77, 118, 126, 156, 169, 189).

Haematological and Immunological Parameters

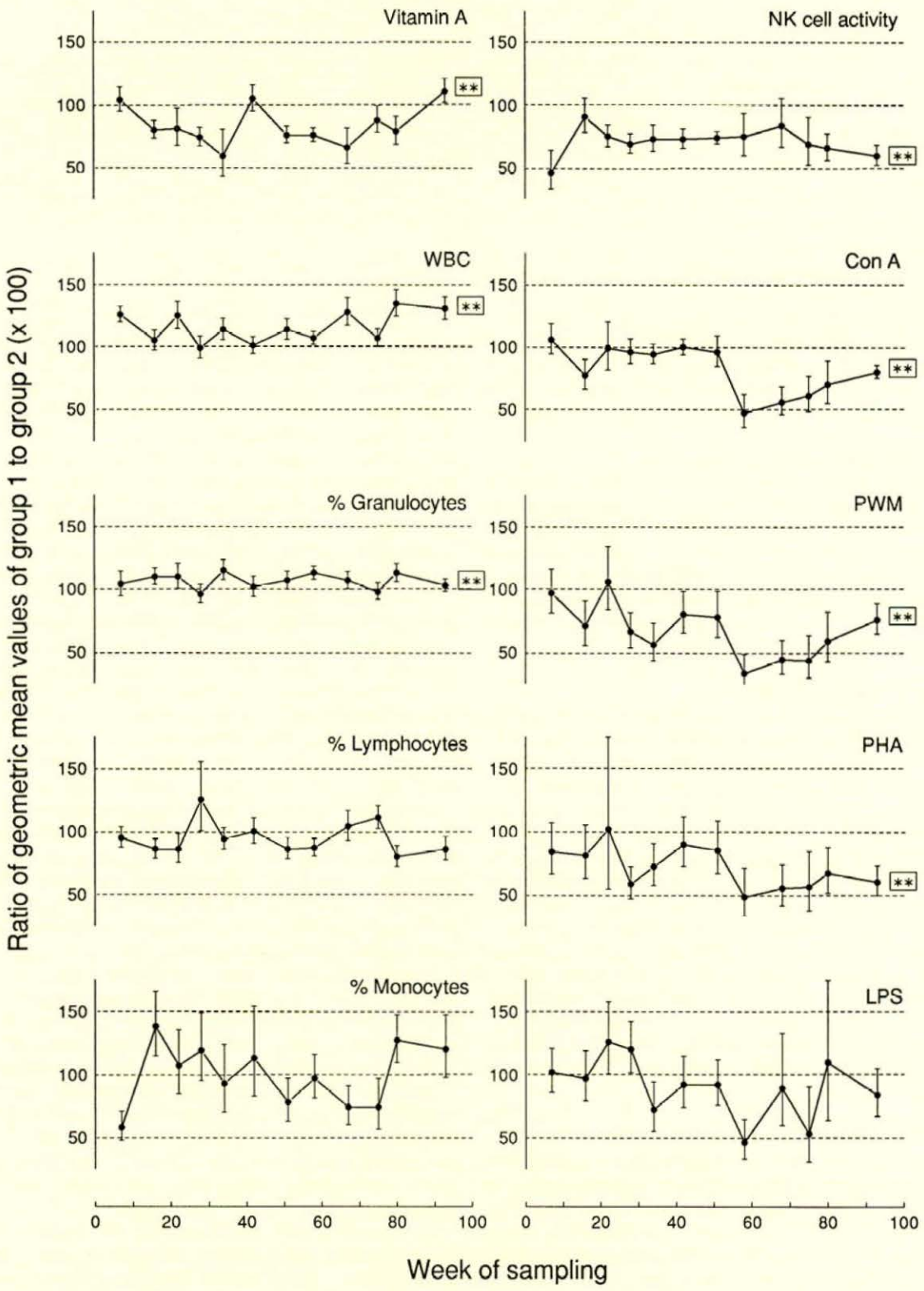
Every six to nine weeks following the start of the feeding experiment, blood samples were taken from the epidural vein for measurement of haematological and immunological parameters. Vitamin-A levels were determined in serum by HPLC analysis, after extraction of retinoids by hydrolysis (35). Vitamin-A concentrations measured in this way showed a good correlation (r^2=0.89) with retinol concentrations measured in plasma (Dr. A. Brouwer, pers. comm.) using methods previously described (36). White blood cell (WBC) counts were determined in whole blood using EDTA as an anti-coagulant, with an automated haematology analyzer (Sysmex E-5000) with differentiation of leukocyte subsets. Samples were kept shielded from direct daylight at 4°C until analysis within five hours after blood sampling.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized epidural venous blood as previously described (37), within eight hours of sampling. Isolated PBMC were stored overnight on ice in culture medium containing 20% fetal bovine serum before immunological assays were carried out. Natural killer (NK) cell activity was determined in a chromium release assay with YAC-1 cells as targets (38). 10^6 YAC-1 cells were labelled with 100 μ Ci ^{51}Cr and incubated in triplicate for 6 hours with seal PBMC at an effector:target ratio of 100:1. Mitogen-induced proliferative responses were measured as described previously (37). Triplicate cultures of PBMC were stimulated with optimal concentrations of the mitogens concanavalin A (Con A), pokeweed mitogen (PWM), phytohaemagglutinin-M (PHA) and lipopolysaccharide from *Salmonella typhimurium* (LPS) (5 μg ml^{-1}, 2.5 μg ml^{-1}, 20 μg ml^{-1} and 100 μg ml^{-1}, respectively) (37). Proliferation was quantified by measuring the incorporation of ^3H-labelled thymidine on day 4 for Con A, PWM and PHA, and on day 5 for LPS. Means of control cultures were subtracted from means of stimulated cultures prior to statistical analyses.

Statistical Analysis

Accumulated longitudinal data were analyzed with ANOVA split plot analysis with time, sex and diet as factors (39), after log-transformation to cater for the effect of heteroscedasticity. Significant differences over time determined using this method are indicated in Figure 2 by asterisks ($p < 0.01$). Error bars in fi-

Figure 2. Differences in means of serum vitamin-A levels, haematological and immunological values between harbor seals feeding on herring from the Baltic Sea (Group 1) or the Atlantic Ocean (Group 2). Values are shown as ratios of geometric mean values of Group 1 (n=11) to Group 2 (n=11). Vertical error bars indicate the 66% confidence intervals of this ratio, as determined from the anti-log transformation of the differences between the two groups on the log-scale plus or minus the standard errors of these differences. Week 0 represents the start of the feeding experiment (September 1991). Asterisks indicate a significant difference between the two groups ($P < 0.01$) as determined by split plot analysis of variance. Results of the same assays carried out 21 weeks before the start of the experiment led to the following ratios (± standard errors): vitamin A 125 ± 22, WBC count 110 ± 16, % granulocytes 91 ± 7, % lymphocytes 121 ± 23, % monocytes 85 ± 32, NK cell activity 146 ± 68, Con A 145 ± 34, PWM 126 ± 36, PHA 157 ± 46 and LPS 135 ± 26.



gures represent standard errors of means (Fig. 1) or ratios (Fig. 2) at each individual sampling point.

RESULTS

Body Weights and Daily Intakes of Organochlorines

The mean body weights of the seals in both groups increased from 44 kg (range 36–52) to 61 kg (range 49–78) during the experimental period of 93 weeks. Body weights of the seals in Group 1 dropped immediately after the seals were switched from Atlantic to the Baltic Sea herring, since the animals initially refused to eat. However, their body weights caught up with those of the second group within the next five weeks (Fig. 1).

As shown in Table 1, estimated daily intake of organochlorines was three to more than ten times higher in the seals of the first group. Estimated daily intakes of aryl hydrocarbon (*Ah*)-receptor-binding organochlorines in TCDD toxic equivalents were 288 ng TEQ per day per seal in Group 1 and 29 ng TEQ per day per seal in Group 2. Since the animals were fed in groups and not individually, only estimates of the daily intakes of organochlorines per seal could be determined.

Table 1. Estimated daily intakes of organochlorines by seals feeding on herring from the Baltic Sea (Group 1) or the Atlantic Ocean (Group 2) in μg day^{-1} and in ng TEQ day^{-1}.

Compounds	Estimated daily intakes			
	Group 1		Group 2	
	μg day^{-1}	ng TEQ day^{-1}	μg day^{-1}	ng TEQ day^{-1}
PCBs[1]	1460	203	260	23
PCDDs[2]	0.07	10	0.02	1
PCDFs[2]	0.4	75	0.03	5
HCB	42	n.a.	6	n.a.
Dieldrin	491	n.a.	54	n.a.
β-HCH	17	n.a.	<5	n.a.
sDDT	497	n.a.	102	n.a.

[1] Estimated daily intakes of PCBs in μg day^{-1} are based on total PCB concentrations in lipids, determined as described by Boon et al. (30). Estimated daily intakes of PCBs in ng TEQ day^{-1} were calculated on the basis of congener specific concentrations of coplanar PCBs (IUPAC numbers 77, 126, 169) determined as described by Van der Velde et al. (32), and mono-ortho substituted PCBs with IUPAC numbers 118, 156 and 189, determined as described by Boon et al. (30). [2] estimated daily intakes of PCDDs and PCDFs both in μg day^{-1} and in ng TEQ day^{-1} are based on 17 2,3,7,8-chlorine substituted congeners only, determined as described by Liem et al. (31). n.a. = not applicable.

Comparison of Haematological Data

Vitamin-A levels proved to be significantly lower in serum of the seals of the first group ($P < 0.01$, Fig. 2), confirming results of a previous experiment with a similar setup in which reproductive disorders had been observed (10, 36). WBC counts were significantly higher in seals of the first group ($P < 0.01$), which resulted from significantly higher numbers of granulocytes ($P < 0.01$). No significant differences were found in the numbers of circulating lymphocytes or monocytes (Fig. 2).

Comparison of Immunological Data

For comparison of immune function in the seals of both groups, peripheral blood mononuclear cells (PBMC) were isolated (37), and a series of *in vitro* functional immunological assays was carried out. Natural killer (NK) cell activity, as determined by a chromium release assay with the YAC-1 tumour cell line as target, was significantly lower in PBMC from the seals of the first group ($P < 0.01$; Fig. 2). Lymphocyte function was evaluated by measuring proliferative responses of PBMC to stimulation with the mitogens Con A, PWM, PHA and LPS. Proliferative responses to Con A, PWM and PHA were significantly lower in the seals of the first group ($P < 0.01$; Fig. 2). No significant differences were found in responses to LPS stimulation. Sex-related differences in the reduction of lymphocyte proliferation were observed, with the responses of the females being more reduced than those of the males (Con A, PWM and PHA, $P<0.05$). No sex-related differences were observed in the reduction in NK cell activity.

DISCUSSION

The data presented show a functional impairment of cells of both the innate and the adaptive immune system of harbor seals after chronic exposure to environmental contaminants at concentrations occurring in their natural habitat. Measurement of serum vitamin-A levels was used as a control for the exposure levels of organochlorines. Reduction of serum retinol concentrations is generally observed in mammals following exposure to organochlorines, as a consequence of an interaction of these chemicals with the serum carrier protein for retinol (40, 41). Furthermore, in a previous study in which harbor seals were fed fish containing different levels of contaminants, vitamin A and thyroid hormone levels were shown to be significantly reduced in seals feeding on polluted fish (36).

The observed reduction in NK cell activity may have direct consequences for the host resistance of these animals, as these cells are known to act as a first line of defence against viral infections (42). The reduced proliferative lymphocyte responses after stimulation with Con A, PWM and PHA suggest an impaired T cell function in these animals, as we have previously shown that these mitogens stimulate phocine T cells (37). T cells, especially cytotoxic T-lymphocytes (CTLs), are known to be of crucial importance in the clearance of virus infections (43), which has also been documented for morbillivirus infections (44). These results are in line with findings in laboratory animals, in which impaired NK cell activity has been demonstrated after exposure to PCBs and HCB (12, 38), and reduced proliferative responses of lymphocytes to mitogens have been observed after exposure to PCBs, PCDDs, PCDFs, Dieldrin, and ß -HCH (12, 14, 15).

The observed increase in levels of granulocytes may be related to these impaired immunological functions, as elevated levels of these cells may reflect an increase in the occurrence of bacterial infections (45). The results of a sampling carried out 21 weeks before the start of the experiment (see legend of Fig. 2) make a genetic bias in immunological responsiveness of the seals in one of the groups unlikely.

It remains difficult to determine whether environmental pollution did indeed play a major role in the recent morbillivirus-induced mass mortalities among marine mammals, as morbillivirus infections can be accompanied by high morbidity and mortality rates in previously unexposed populations (46). However, as both NK cells and T cells play an important role in the immune response against virus infections it is not unlikely that a functionally impaired NK and T cell response led to increased susceptibility to morbillivirus infections in marine mammals, and thus contributed to the severity and extent of the recent epizootics.

These results add malfunction of the immune system to previously identified biological effects of the contaminants that accumulate in the food chain, showing again that their present levels are a tangible threat to mammals inhabiting the marine ecosystem.

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