Impaired Immunity in Harbour Seals (*Phoca vitulina*) Exposed to Bioaccumulated Environmental Contaminants: Review of a Long-term Feeding Study

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Mass mortalities among seals and dolphins inhabiting contaminated marine regions have led to speculation about a possible involvement of immunosuppression associated with environmental pollution. To evaluate whether contaminants at ambient environmental levels can affect immune function of seals, we carried out an immunotoxicological study under semifield conditions. Two groups of 11 harbour seals (*Phoca vitulina*) originating from a relatively uncontaminated area were fed herring from either the highly polluted Baltic Sea or the relatively uncontaminated Atlantic Ocean. Changes in immune function were monitored over a 2 1/2-year period. The seals that were fed contaminated Baltic herring developed significantly higher body burdens of potentially immunotoxic organochlorines and displayed impaired immune responses as demonstrated by suppression of natural killer cell activity and specific T-cell responses. During a 2-week fasting experiment performed at the end of the feeding study, mobilization of organochlorines from the blubber did not lead to a strong increase of contaminant levels in the blood, and no enhancement of the existing immunosuppression was observed. These results demonstrate that chronic exposure to environmental contaminants accumulated through the food chain affects immune function in harbour seals, whereas short-term fasting periods, which are normal for seals, do not seem to pose an additional risk. The seals of this study were not exposed perinatally to high levels of environmental chemicals, and body burdens of organochlorines measured near the end of the study were lower than those generally observed in free-ranging seals inhabiting many contaminated regions. Therefore, it may be expected that environmental contaminants adversely affect immune function of free-ranging seals inhabiting contaminated regions at least as seriously as observed in these studies. — Environ Health Perspect 104(Suppl 4):823–828 (1996)

Key words: harbour seals, *Phoca vitulina*, marine mammals, environmental contaminants, organochlorines, immunotoxicology, review

Introduction

In recent years, serious disease outbreaks among seals and dolphins were attributed to infection with established or newly recognized morbilliviruses [for review, see De Swart (*1*)]. The first identification of a morbillivirus as the causative agent of a mass mortality among marine mammals was in 1988, when the previously unrecognized phocine distemper virus (PDV) caused the death of 20,000 harbour seals (*Phoca vitulina*) in northwestern Europe (*2*). A similar epizootic among Baikal seals (*Phoca sibirica*) in Siberia in 1987 was later attributed to infection with canine distemper virus (CDV) (*3,4*). A virus isolated from stranded harbour porpoises (*Phocoena phocoena*) between 1988 and 1990 proved to be yet another new member of the genus *Morbillivirus*, distinct from PDV and CDV and more closely related to rinderpest virus and peste-des-petits-ruminants virus—porpoise morbillivirus (*5–7*). A similar virus, dolphin morbillivirus (DMV), was the primary cause of a mass mortality among striped dolphins (*Stenella coeruleoalba*) in the Mediterranean from 1990 to 1992 (*8,9*). These morbillivirus-related mass mortalities among aquatic mammals led to speculation about the possible involvement of environmental pollution-induced immunosuppression in the severity and extent of these outbreaks (*10*).

In the early 1970s laboratory animal studies demonstrated that the mammalian immune system can be a sensitive target for environmentally occurring toxic chemicals (*11,12*). This finding led to the identification of several groups of chemicals with immunotoxic properties. Of these, the polyhalogenated aromatic hydrocarbons (PHAH), including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs), seemed the most likely candidates for causing immunotoxic effects in the marine environment (*13*). The receptor-mediated mechanism elucidated in laboratory animal studies has suggested an additive toxicity of different PHAH congeners. Furthermore, the most immunotoxic congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), was shown to cause immune alterations at exposure levels below 1 μg/kg body weight (bw) in certain species (*14*). Top predators inhabiting contaminated marine regions carry high body burdens of lipophilic PHAH (*15,16*), and estimated yearly

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Abbreviations used: Ah, aryl hydrocarbon; bw, body weight; CDV, canine distemper virus; ConA, concanavalin A; DTH, delayed-type hypersensitivity; DDA, dimethyldioctadecylammonium bromide; DMV, dolphin morbillivirus; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; MLR, mixed lymphocyte responses; Ova, ovalbumin; PBMC, peripheral blood mononuclear cells; PDV, phocine distemper virus; PHA, phytohemagglutinin; PWM, pokeweed mitogen; PHAH, polyhalogenated aromatic hydrocarbons; PCBs, polychlorinated biphenyls; PCDDs, polychlorinated dibenzo-*p*-dioxins; PCDFs, polychlorinated dibenzofurans; RV, rabies virus vaccine; rhIL-2, recombinant human interleukin 2; TT, tetanus toxoid; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TEQ, TCDD toxic equivalents.

intakes of these compounds expressed in TCDD toxic equivalents (TEQ) often exceed the 1 μg/kg bw level (*17*). The association of bioaccumulated organochlorine contaminants in marine mammals with adverse biological effects, including reproductive failure, endocrinological alterations, developmental irregularities and increased tumor incidence, has been the subject of many reports over the last decades (*18,19*).

These considerations prompted us to initiate a series of studies in captive harbour seals kept under semifield conditions, in order to evaluate whether environmental contaminants at ambient environmental levels can affect immune function in this marine top predator. Seals consuming contaminated herring accumulated higher body burdens of potentially immunotoxic organochlorines than seals fed relatively uncontaminated herring and displayed impaired immune responses as evidenced by suppression of NK-cell activity and specific T-cell responses (*17*). The present paper provides an overview of the results of this study.

Measurement of Immune Function Parameters in Harbour Seals: Establishment of Techniques Also Used for Humans and Rodents

Technical Approach

Immunotoxicological studies are usually carried out in laboratory rodents, exposing groups of animals to a range of concentrations of a potentially immunotoxic compound and assessing immunocompetence by performing functional immunological *ex vivo/in vitro* and *in vivo* assays (*20*). Results of these studies are then extrapolated to estimate the potential immunotoxicity of these compounds in humans or wildlife (*21*). Additional information on immunotoxic effects for humans may be obtained from events of accidental exposure (*14*). In general, data obtained from laboratory animal studies provide insight into the effects of acute exposure to single compounds or relatively uncomplicated mixtures of chemicals. In contrast, limited information is available on the immunotoxic effects of chronic exposure to complex mixtures of xenobiotics as they occur in the food chain.

To carry out immunotoxicological studies in seals, we adapted a series of functional immunological assays routinely used in rodent and human immunotoxicology for use in this species. It should be noted that extensive information on the immune system and its components as available for rodents and humans is largely lacking for free-ranging animals. First, mitogen- and antigen-induced proliferative responses of peripheral blood mononuclear cells (PBMC) isolated from harbour seals were measured using ^{3}H-thymidine incorporation methods. It was shown that concanavalin A (ConA) and pokeweed mitogen (PWM) induced strong proliferative responses, while phytohemagglutinin (PHA) and lipopolysaccharide (LPS) induced lower responses (*22*). Proliferation of mitogen-stimulated PBMC in response to recombinant human interleukin 2 (rhil-2) and *ex vivo/in vitro* antibody production by PBMC were measured to discriminate between T- and B-cell responses. ConA and PHA stimulated phocine T cells, PWM stimulated both T and B cells, whereas LPS predominantly stimulated phocine B cells (*22*). Antigen-specific immune responses were measured after immunization of seals with an inactivated rabies virus vaccine (RV), tetanus toxoid (TT), keyhole limpet hemocyanin (KLH), or ovalbumin (Ova). Specific proliferative PBMC responses, *ex vivo/in vitro* antibody production, and the presence of specific antibody forming cells were demonstrated in PBMC cultures of immunized animals (*22,23*). Responses measured *ex vivo/in vitro* correlated well with specific serum antibody production *in vivo*. In addition, one-way mixed lymphocyte (MLR) responses of PBMC were measured using the harbour seal lymphosarcoma cell line PV1.P1 (ATCC CRL 6526) as irradiated stimulator cells, following methods routinely applied for humans and rodents (*23*). In addition to the measurement of *in vivo* B-cell-mediated antibody responses, a method was developed to measure delayed-type hypersensitivity (DTH) responses in seals. DTH responses correlated well with results of *ex vivo/in vitro* tests of T-lymphocyte function, implicating this cell type in the reaction (*24*). Natural killer cells are leucocytes that play an important role in the first line of defense against virus infections. The natural cytotoxic activity of harbour seal PBMC was characterized and found to be IL-2 responsive, sensitive to the NK-cell-specific antibody anti-Asialo GM1, and higher against a virus-infected target cell line, like NK cells described for other mammalian species (*25*).

Experimental Approach

Several approaches have been used in attempts to correlate body burdens of environmental contaminants with either immunological dysfunction or mortality due to infectious agents in marine mammals. The most straightforward approach appears to be the collection of information from free-ranging animals. Lahvis et al. (*26*) reported an inverse correlation between blood levels of PHAH and cellular immune function in free-ranging bottlenose dolphins (*Tursiops truncatus*), but this study was based on a very limited sample size (n=5). Results of studies aimed at correlating contaminant levels with disease or mortality have proved inconclusive in most cases, since the experimental design was flawed by unavoidable and uncontrollable factors (*27–29*). Most of these confounding factors may be controlled when carrying out immunotoxicological studies with animals held in captivity and by mimicking the natural situation and exposure levels of free-ranging animals as closely as possible.

Along these lines, Harder et al. (*30*) exposed captive harbour seals to contaminants by feeding them PCB-spiked fish for a short period and subsequently monitored their resistance to challenge infection with PDV. No differences were found in mortality rates or other virological parameters between PCB-exposed and -unexposed animals. However, the conclusions were based on a small sample size and limited to effects associated with PCB body burdens that were much lower and exposure times that were much shorter than those observed in free-ranging animals inhabiting contaminated areas.

In our experiments, we fed fish originating from marine regions with different contaminant levels to two groups of young harbour seals. The fish of both diets was originally destined for human consumption. This experimental design allowed us to mimic the natural situation as closely as possible by exposing the animals for a prolonged period of time to different levels of contaminants occurring in the aquatic food chain. It should however be noted that the duration of exposure was shorter than under natural circumstances, since perinatal exposure was not feasible.

An Immunotoxicological Feeding Study in Seals under Semifield Conditions

Toxicological Aspects

During a 2 1/2-year period, two groups of seven female and four male harbour seals each were fed herring from either the heavily polluted Baltic Sea or the relatively uncontaminated Atlantic Ocean, and

longitudinal changes in immune function were assessed (*17*). The animals had been caught as weaned pups off the relatively uncontaminated northeast coast of Scotland, and were fed relatively uncontaminated herring for an adaptation period of one year prior to this feeding study. Analyses of chemical residue were performed on the fish fed throughout the experiment, and on seal blood and blubber samples taken during the final stages of the feeding study. Estimated daily intakes of potentially immunotoxic organochlorine contaminants were three to more than ten times higher in the seals fed Baltic herring (*17*). Persistent compounds such as *p,p'*-DDT and total PCBs were biomagnified from herring to seal blood and blubber, whereas lipid-based levels of aryl hydrocarbon (Ah) receptor-binding organochlorines (sum TEQ) were not higher in seals than in the fish which they were fed (*31*). The latter finding confirms that seals have the capacity to metabolize or excrete these compounds (*32,33*). This was also illustrated by the fact that estimated body burdens of *p,p'*-DDT were in the same order of magnitude as the estimated cumulative intakes of this compound (almost 50% in both groups), whereas estimated body burdens of sum TEQ totaled 7 and 2.2% of the estimated cumulative intakes by seals fed Atlantic or Baltic herring, respectively (Figure 1). However, blubber and blood levels of these chemicals were still three times higher in the seals fed Baltic herring (Table 1). In general, higher percentages of the total contaminant intakes accumulated in the Atlantic group as compared to the Baltic group, which may have reflected higher induction of liver enzymes and subsequently more efficient biotransformation in the Baltic group (*31*). Total PCB levels in the blubber of seals fed Baltic herring were between 15 and 20 mg/kg bw (Table 1), which is relatively low compared to seals inhabiting polluted waters like the Baltic Sea or the Dutch Wadden Sea (*16*).

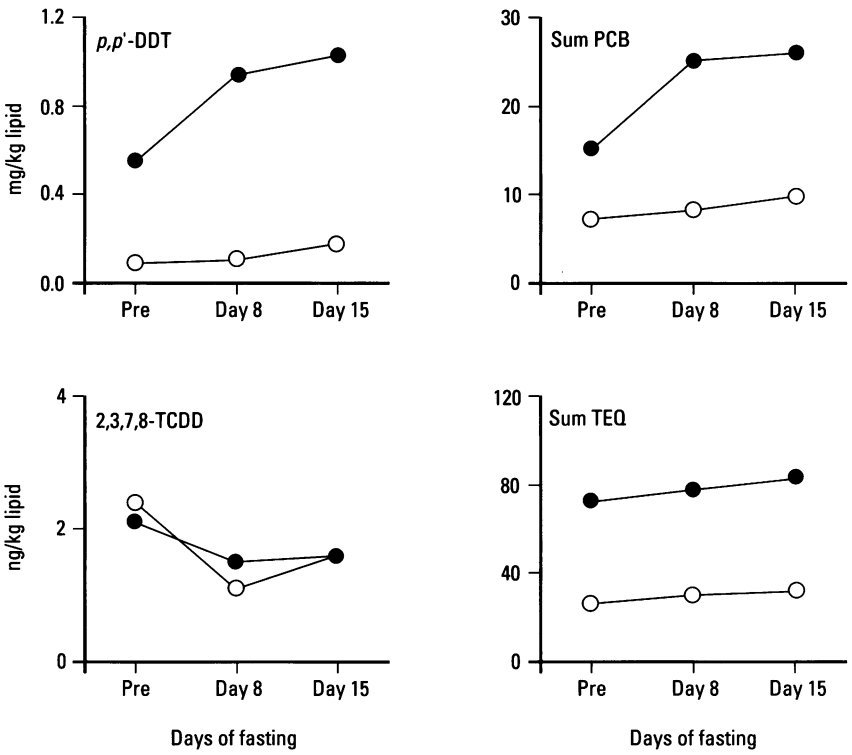

Figure 1. Levels of *p,p'*-DDT, sum PCBs, 2,3,7,8-TCDD, and sum TEQ, measured on basis of extractable lipid, in pooled blood samples from seals of the Atlantic group (open symbols) or the Baltic group (closed symbols), six weeks before (pre), and at days 8 and 15 of the fasting experiment. Data De Swart et al. (*31*).

Table 1. Organochlorine contaminants in herring and in seal blubber or pooled blood samples taken after 2 and 2 1/2 years on the different diets, respectively.

	Herring[a]		Seal blubber[b]		Seal blood[c]	
	Atlantic	Baltic	Atlantic	Baltic	Atlantic	Baltic
p,p'-DDT[d]	31 ± 3	272 ± 35	306 ± 2448	2448 ± 368	89	552
Sum PCBs[d]	875 ± 158	4398 ± 715	6884 ± 493	16488 ± 1023	7109	15063
Sum TEQ[e]	42 ± 4	426 ± 83	62 ± 4	209 ± 12	26	72

[a]Means ± SE of three batches of herring. [b]Means ± SE of 11 seals (7 females, 4 males). [c]Concentration in pooled blood sample of 11 seals. [d]Levels in microgram per kilogram lipid. [e]Levels in nanogram per kilogram of lipid. Data from De Swart et al. (*31*).

Immunological Aspects

During the course of the 2 1/2-year feeding study, blood samples were taken at regular intervals from all seals for PBMC isolation and subsequent assessment of *ex vivo/in vitro* immunological parameters. NK-cell activity of PBMC obtained from seals fed Baltic herring was consistently and significantly reduced to a level approximately 25% lower than that observed in seals fed Atlantic herring (*25*). Interestingly, an apparent seasonal pattern emerged in the responses of the seals in both groups, with NK-cell activity in winter being approximately half of that observed during the summer months.

Proliferative responses of PBMC obtained from the Baltic group of seals after stimulation with the T-cell mitogens ConA and PHA and the T/B cell mitogen PWM were also significantly reduced compared to responses in the Atlantic group. Impairment of these responses was particularly evident during the second part of the experiment, and mean responses during this period showed an inverse correlation with TEQ levels in blubber biopsies taken 2 years after the onset of the experiment (*23*). MLR-induced PBMC proliferation, reflecting a nonspecific immunological response involving a complex sequence of events involving antigen processing and presentation, was significantly lower in seals fed Baltic herring (*23*). Mean MLR responses from the second half of the experiment were also inversely correlated with total TEQ levels in blubber biopsies. When seals were immunized with RV (before the start of the feeding study) and TT (half-way through the feeding study), specific proliferative responses of PBMC from the Baltic group were also significantly reduced. Again, these impaired cellular responses were most pronounced

toward the end of the experiment, with RV-induced responses showing an inverse correlation with total TEQ levels in blubber biopsies. Evidence for the *in vivo* relevance of these impaired T-cell responses came from measuring DTH responses to Ova in the seals. The skin reaction to this antigen, characterized by the appearance of mononuclear cells peaking at 24 hr after intradermal administration, was significantly lower in the Baltic group. These DTH responses correlated with *ex vivo/in vitro* ConA- and PHA-induced lymphoproliferative responses, but not with PWM- or LPS-induced responses, implicating T cells as effectors.

In contrast to the impairment of T-cell responses, lymphoproliferative responses induced by the B-cell mitogen LPS remained largely unaffected in the Baltic group and did not correlate with blubber TEQ levels (*23*). In line with this observation, *ex vivo/in vitro* mitogen-induced total antibody production proved to be unaffected in the Baltic group. Furthermore, primary antigen-specific serum antibody responses to immunization with RV, TT, and poliovirus antigen also were not lower in the Baltic group. Serum antibody responses to Ova, which antigen was used to elicit the DTH responses in the seals, were significantly lower in the Baltic group. We speculated that this difference was related to the use of the adjuvant dimethyldioctadecylammonium bromide (DDA) in the latter study, which has a major effect on the induction of T-helper-cell responses. Consequently, the difference observed in these serum antibody titers may be predominantly related to an impaired T-cell response in the Baltic group (*23*).

An important aspect of immunotoxicological studies is to ascertain that effects measured in specific immunological assays are caused by a direct influence of the chemicals under investigation and do not result from indirect causes including nutritional status, impaired protein synthesis, or stress. A full set of routine diagnostic parameters was therefore evaluated to control for such potential indirect effects in our experiments. Hematology and clinical chemistry parameters were monitored longitudinally, as possible indicators of immunotoxic stress as well as indicators of general health state (*34*). The collective data demonstrate an insensitivity of clinical chemistry parameters to the effects of the chronic contaminant exposure, but suggest the induction of clear alterations in hematology profiles. The most striking finding was an increase in neutrophil counts in the Baltic group. This increase became more pronounced toward the end of the experiment (*34*). We speculated that this might indicate an increased occurrence of subclinical bacterial infections in these animals due to the observed impairment of immune function. However, it is also possible that an effect at the myeloid stem cell level is responsible for this observation: in experimental animals, lack of T-cell activity is sometimes compensated for by an increased activity of myeloid stem cells, leading in turn to a higher production of both monocytes and granulocytes. A summary of the immunological effects observed in the seals fed Baltic herring is shown in Table 2.

Effects of Short-term Fasting on Immune Function in Seals with High Body Burdens of Organochlorines

Fasting periods are a normal phenomenon in the natural life history of seals and may occur in relation to their reproductive cycle or the moulting season. Although true seals (family *Phocidae*) are physiologically adapted to long fasting periods, the risk posed by the release of environmental chemicals from their lipid reserves during these episodes may be of concern, since potentially immunotoxic chemicals may be mobilized and induce acute toxic effects. To test this hypothesis, we subjected the seals of our study to an experimental 2-week fasting directly after the end of the feeding study. The animals of both groups lost an average 11.1 kg bw, representing 16.5% of their body weights. Metabolization of blubber lipids led to an approximate 2-fold increase in blood levels of persistent PHAH, but did not influence blood levels of Ah receptor-binding organochlorines (Figure 1). This is not inconsistent with the observation that few differences in immunological parameters were observed between the Baltic and Atlantic groups (*30*). A drop of about 35% in circulating lymphocytes and a slight increase in NK-cell activity were observed in both groups, whereas mitogen- and antigen-induced lymphoproliferative responses of the Baltic group remained within previously observed ranges. Unexpectedly, lymphoproliferative responses of the Atlantic group were reduced after the 15-day fasting, which could not be explained. Taken together, our results suggest that short-term fasting does not pose a major additional immunotoxic threat to seals with high organochlorine body burdens. Since thyroid hormone responses to the stress of fasting were lower in the Baltic group, it may be speculated that marine mammals with high body burdens of environmental chemicals are less capable of coping with stressful situations than animals with lower burdens, such as those inhabiting relatively uncontaminated areas (*30*).

Conclusions

The experiments described here have underlined the usefulness of a semi-field approach to immunotoxicological studies of complex mixtures of environmentally accumulated contaminants. Although major efforts are needed to adapt functional immunological assays for use in the species under investigation, once established these assays allow the changes in immune function to be monitored over time in relation to contaminant mixtures as they occur in the natural environment. The numbers of assays used and the immune parameters studied in an integrated manner may justify the extrapolation of experimental data to

Table 2. Summary of differences in immunological parameters between the two groups of seals.

Parameter	Assay	Effect	Reference
NK cell	^{51}Cr release assay	↓[a]	(*17,25*)
T lymphocyte	Mitogen-induced proliferation	↓	(*17,23*)
	Antigen-induced proliferation	↓	(*23*)
	Mixed lymphocyte reaction	↓	(*23*)
	Delayed-type hypersensitivity skin test	↓	(*24*)
B lymphocyte	Mitogen-induced proliferation	–[b]	(*17,23*)
	Specific serum antibody responses	–/↓	(*23,24*)
	Ex vivo/in vitro immunoglobulin production	–	(*23*)
Hematology	Lymphocyte counts in peripheral blood	–	(*17,34*)
	Neutrophil counts in peripheral blood	↑[c]	(*17,34*)

[a]Significantly lower responses in the seals fed Baltic herring compared to the seals fed Atlantic herring. [b]No significant differences over time between the two groups of seals. [c]Significantly higher responses in the seals fed Baltic herring compared to the seals fed Atlantic herring.

conclusions on the impact of contaminant exposure on immunity and disease.

Using this approach we detected differences in functional immunological parameters between two groups of seals fed fish destined for human consumption, which originated from areas with different levels of environmental pollution. Results of *ex vivo/in vitro* and *in vivo* assays strongly reinforced each other, which added to the significance of the results. The most striking findings were impairment of NK-cell and T-cell function induced by exposure to environmentally accumulated xenobiotics. Previously, chronic exposure to PHAH has been suggested to specifically affect cellular immunity rather than humoral immunity, which is consistent with our observations. The NK- and T-cell responses measured showed an inverse correlation with TEQ levels in blubber biopsies, which suggests that the impairment was largely related to exposure to Ah receptor-binding PHAH. This suggestion was supported by the results of the fasting experiment, in which no aggravation of immunosuppression was observed during fasting, whereas blood levels of many non-Ah receptor-binding PHAH increased but TEQ levels remained largely unaffected. However, we cannot rule out a possible immunosuppressive action of non-Ah receptor-binding PHAH or any other group of environmental contaminants. For ethical reasons and because of legal restrictions, it was not possible to challenge the seals with PDV or another infectious agent to assess differences in host resistance between the two groups of seals. However, since the functionally impaired NK and T cells mentioned above play a major role in immunity to virus infections, and contaminant levels in marine mammal species affected by the recent viral epizootics were in many cases substantially higher than levels in the seals of our study, we speculate that exposure to immunotoxic chemicals acted as a co-factor in these mass mortalities. This may have facilitated the emergence of the recent epizootics by aggravating the severity and extent of the infection, leading to increased numbers of affected animals and higher fatality rates.

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